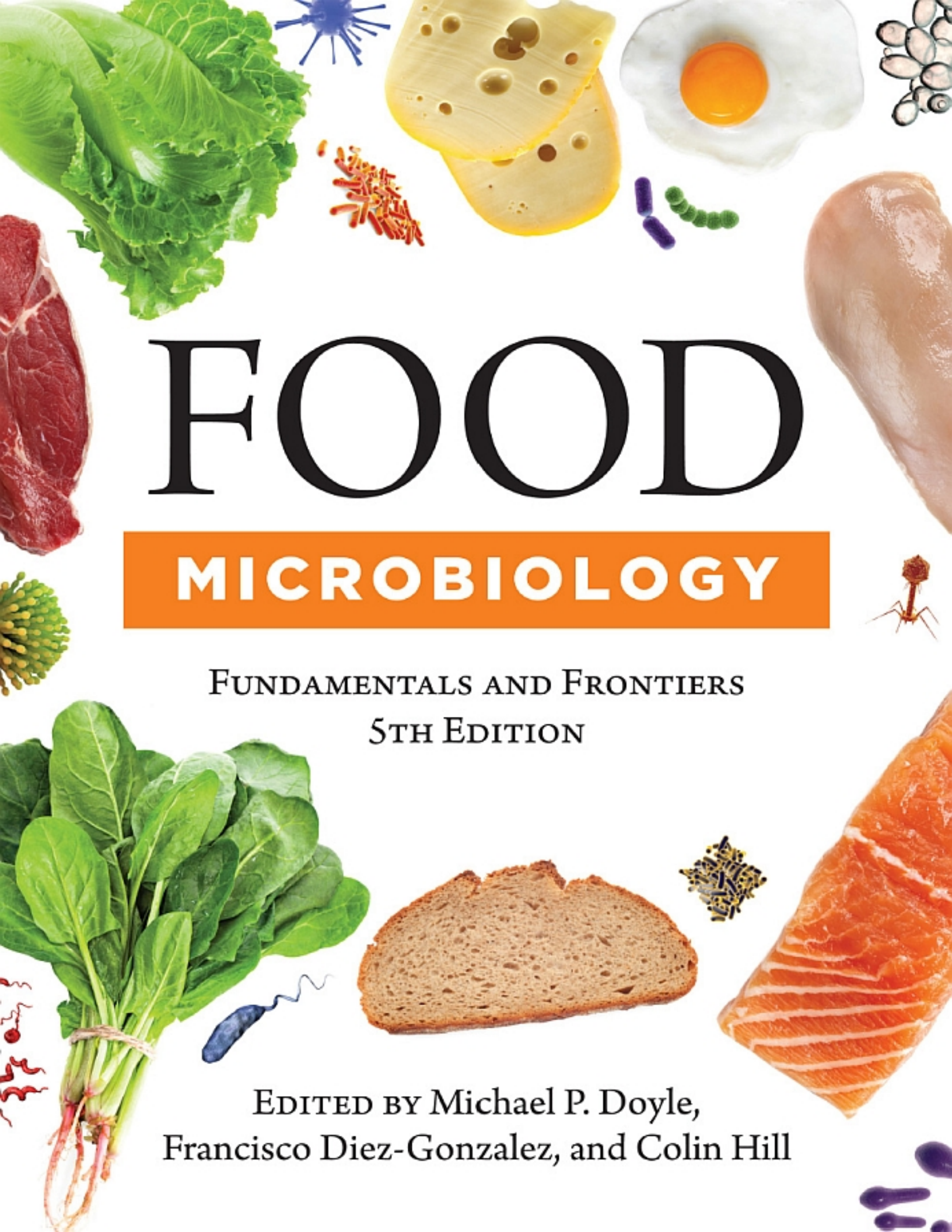


FOOD

MICROBIOLOGY

FUNDAMENTALS AND FRONTIERS
5TH EDITION

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Preface

Microbiologists are using an ever rapidly expanding tool chest of new and extraordinary molecular methods to address many of the challenges associated with the microbial world of foods. Methods involving "-omics," whole-genome sequencing, CRISPR-Cas9, and the microbiome, to name a few, are revolutionizing the field of food microbiology by helping us better understand how microbes behave in foods. Such methods will also facilitate the development of innovative approaches (i) to reduce and control contamination of foods by harmful and spoilage microbes, (ii) to better detect outbreaks of microbe-associated foodborne illnesses, (iii) to deliver microbially produced foods of higher nutritional and functional quality, (iv) to detect and characterize undesirable microbes in foods more quickly and accurately, (v) to enhance the overall microbiological safety of foods, and much more.

The 5th edition of *Food Microbiology: Fundamentals and Frontiers* (*FMFF*) addresses these and many more topics focused on the microbes associated with foods. Those chapters that have long been fundamental to the field of food microbiology have been updated with new research findings and related information, and new chapters on recent topics in food microbiology have been included. Since its introduction in 1997, the purpose of *FMFF* has been to serve as an advanced reference that explores the breadth and depth of food microbiology. Hence, for most chapters, there is only a minimal review of the basic principles and techniques of food microbiology, as this book is written at a level that presumes a general background in microbiology and biochemistry that is needed to understand the principles of food microbiology at a basic scientific level.

The book is composed of seven primary sections that address major areas of the field based on the roles that microorganisms play in the production, preservation, safety, and quality of foods. The first

section, “Factors of Special Significance to Food Microbiology,” addresses the growth, survival, and inactivation characteristics of foodborne microbes, the significance of spore-forming microbes in foods, proper use of microbiological criteria and indicator microorganisms, and the importance of stress response mechanisms in microbes that enable them to persist in foods under adverse conditions. The second section, “Microbial Spoilage and Public Health Concerns,” focuses on the quality and safety issues associated with three primary food groups, i.e., dairy, meats, and produce. These commodity-oriented chapters address the types of microbial spoilage associated with foods and innovative approaches for prevention and control. In addition, the types of harmful microbes that have been associated with these commodities and their adverse consequences on public health are described.

The book’s third and fourth sections, “Foodborne Pathogenic Bacteria” and “Nonbacterial Pathogens and Toxins,” cover the major harmful microbial agents associated with foods. Each chapter addresses the diseases caused by each agent, its epidemiology and etiology, the virulence determinants of pathogens, and control measures to prevent foodborne illnesses. Considerable molecular information regarding virulence and toxicity has been added to the pathogenic bacteria section, as has new information on control measures to reduce contamination along the food chain.

The fifth section, “Preservatives and Preservation Methods,” provides state-of-the-science information on the three approaches to preserve foods, namely physical, chemical, and biological methods. With special interest by consumers and the food industry in reducing the use of manufactured chemicals in foods, the chemical chapter includes coverage of natural antimicrobials that may be applicable to foods. In addition, a chapter on bacteriophages, which is another “natural” approach to controlling foodborne pathogens, has been added to this section. Each chapter addresses traditional and emerging preservation techniques including their mechanisms of microbial inactivation or suppression.

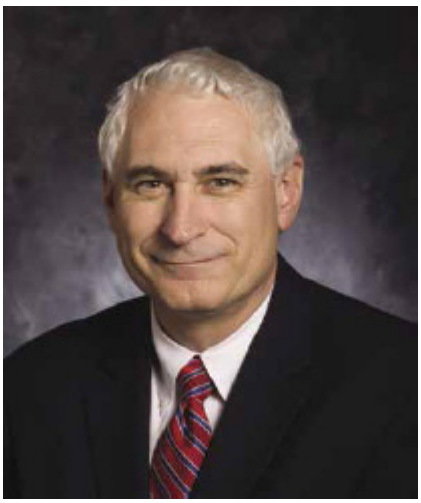
The sixth section, “Fermentations and Beneficial Microbes,” addresses microbes and microbial substrates and end products that promote human health. Studies of the human intestinal microbiome have revealed exciting information regarding the significance of diet to the gut microbiota and the effects of these microbes on health. This is opening new avenues to defining approaches for more healthy eating. In addition, major advances have been made to perfect the use and control of microbes used as starter cultures to produce fermented foods such as cheese and sausages. This section covers these topics and more.

The final section, “Current Issues and Advances in Food Microbiology,” covers a variety of “front and center” topics of special interest to food microbiologists. These include antimicrobial resistance and its relationship to gut microbes and human health; the application of genomics and metagenomics to food microbiology; the influence of food production environments on food microbiomes; the use of whole-genome sequencing in molecular source tracking of foodborne illness outbreaks and molecular subtyping of foodborne pathogens; advances in the application of predictive microbiology and risk assessment models for food safety; modern food management systems in the food industry that are used to mitigate contamination by undesirable microbes; the microbiological constraints for the use of reclaimed and reconditioned water in food production and processing operations; and the relevance of food microbiology issues to current trends in food production and imported foods. These chapters provide scientific and technical insights that may not otherwise be readily available in one convenient source.

We thank our coauthors for their diligence and commitment to developing timely and informative chapters to broaden our knowledge of the rapidly expanding field of food microbiology. It is their contributions that are the foundation for this book. We also thank our reviewers for their critical evaluations which enabled us to fine-tune each chapter.

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About the Editors



Michael P. Doyle is a retired Regent's Professor of Food Microbiology and founding Director of the Center for Food Safety at the University of Georgia. He was a Wisconsin Distinguished Professor of Food Microbiology at the Food Research Institute, University of Wisconsin–Madison. Dr. Doyle has published more than 600 scientific papers and has given more than 900 invited presentations at national and international scientific meetings on food microbiology and food safety topics. He is a fellow of the American Academy of Microbiology, the American Association for the Advancement of Science, the Institute of Food Technologists, the International Association for Food Protection, and the National Academy of Inventors, and is a member of the National Academy of Medicine.



Francisco Diez-Gonzalez is the Director of the Center for Food Safety at the University of Georgia. He earned his MS and PhD in Food Science from Cornell University. In 1999, he joined the Department of Food Science and Nutrition at the University of Minnesota, where he was professor for 17 years and served as department head from 2014 to 2016. He has taught courses on food safety and food microbiology, has authored more than 90 peer-reviewed articles, and serves on the editorial board of several journals. His research interests center on the control and detection of foodborne bacteria in different food commodities.



Colin Hill, PhD DSc, is Professor of Microbial Food Safety at University College Cork, Ireland. He is also a Principal Investigator at APC Microbiome Ireland in Cork, a research center devoted to the study of the role of the gut microbiota in health and disease. In 2009 he was elected to the Royal Irish Academy and in 2010 he

received the Metchnikoff Prize in Microbiology and was elected to the American Academy of Microbiology. He has published more than 540 papers and holds 18 patents. His main interests are in the role of the gut microbiome in protecting against microbial infections.

I

Factors of Special Significance to Food Microbiology

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1
Behavior of Microorganisms in Food: Growth, Survival, and Death

Monitoring microbial population density in any environment (including food) over time generally reveals one of three states or behavioral modes: growth, survival, or death (Fig. 1.1). If the monitoring period is extended, more than one state may be observed. Growth involves replicating the microbial genome, which is often followed by replication of other cell constituents and culminates in cell division, i.e., multiplication. Growth is measured empirically by the increase in biomass, as is the case for molds, or by cell count, as is the case for bacteria and yeasts; other means of monitoring growth are discussed below. A microorganism in food may enter a state of arrested growth (described here as survival), and the density of its viable population remains constant or fluctuates minimally (e.g., a ±0.25 log change in cell count) during the experimental monitoring period. Therefore, the "surviving" microorganism temporarily loses its ability to multiply, which can be regained if environmental conditions (e.g., medium composition) change during the period of observation. In contrast, death occurs when a cell loses its ability to multiply regardless of how favorable the growth conditions are. Experimentally, death becomes evident when the microorganism fails to reproduce upon subculturing.

GROWTH BEHAVIOR IN FOOD

Beneficial microorganisms can grow during food fermentation; hence, monitoring their growth can provide valuable information about the progress of the fermentation process. Microbial

populations may increase during the storage of food, causing its spoilage or rendering it unsafe for human consumption. Hence, monitoring growth of undesirable microorganisms also is important for ensuring food quality and safety. Growth can occur when an inoculum of a microbe is introduced into a new medium (e.g., food), and both the medium composition and environmental factors influence its growth. The presence of available water and essential nutrients (e.g., carbon and nitrogen sources, growth factors, and micronutrients) and the lack of hostile components (e.g., antimicrobials) or unfavorable pH are important characteristics of food that influence microbial growth. Factors providing a growth-supporting environment include suitable temperatures and gaseous environments. Different microorganisms grow over different temperature ranges. Psychrophiles, psychrotrophs, mesophiles, thermophiles, and extremophiles are categories of microorganisms that grow at temperatures ranging from subfreezing to higher than that of boiling water. Food microbiologists are mostly concerned about psychrotrophs, mesophiles, and thermophiles. Gaseous requirements for growth depend on the specific microorganism. Oxygen is toxic to strict anaerobes (e.g., some *Clostridium* spp.), but it is essential for the growth of aerobes, such as *Bacillus* spp. and fungi. In addition to growth media and environmental conditions, exposure time is also an important factor influencing microbial growth. A bacterium divides and doubles its population under a set of conditions, during a defined time interval called generation time or doubling time. Generation times are affected by different conditions; hence, the time required to complete a growth cycle can vary.

Growth recognition requires the measurement of microbial population density repeatedly on a time scale. Demonstrating the changes in microbial population density over time is used to determine if growth occurred. There should be a clear distinction between growth in food, as just described, and growth on a microbiological counting medium. The two events are interrelated, and both involve genome multiplication and cell division; however,

the latter is done merely to determine the population density in food at a given time point. Additionally, multiplication of a cell into a colony on an agar medium is described qualitatively as growth, whereas the increase in the population of a microorganism or microbiota in food over time quantifies that growth.

Measuring Microbial Populations in Food

The plate count method is one of the oldest and most commonly used methods to determine the viable microbial population in a food sample (1). The method may involve placing the food directly in a plate (petri dish) containing a suitable growth medium with agar (direct plating) or preparing dilutions of the food before selected dilutions are plated (dilution plating). The inoculated agar plates are incubated, and the resulting microbial colonies are counted. Colony counts on plates are used to determine the population count in food, which may be expressed as colony-forming units (CFU) per unit volume (e.g., milliliters) or weight (e.g., grams) of food. Considering that the population density under optimum growth conditions can increase by several orders of magnitude, the population is often presented in scientific notation (e.g., 2.7×10^6 CFU/g) or the logarithm to base 10 ($\log_{10}$ or simply log) of that number. In the example just described, the microbial population will be 6.4 log CFU/g. When the population, expressed as a log value, is plotted against time, it is relatively easy to determine if the microbial behavior in food is growth, survival, or death (Fig. 1.1). The plate count method is most suitable for counting sizable populations of single-celled, easyto-culture microorganisms. The method fails to produce satisfactory results when the foodborne microorganism is present in a small population (e.g., most pathogenic bacteria), has a tendency to produce cell clumps (e.g., staphylococci), or requires host cells to grow (e.g., parasites). The method also is inherently labor-intensive, costly, and slow. It requires 24 hours or more before colonies on plates are countable and the microbial population is measurable.

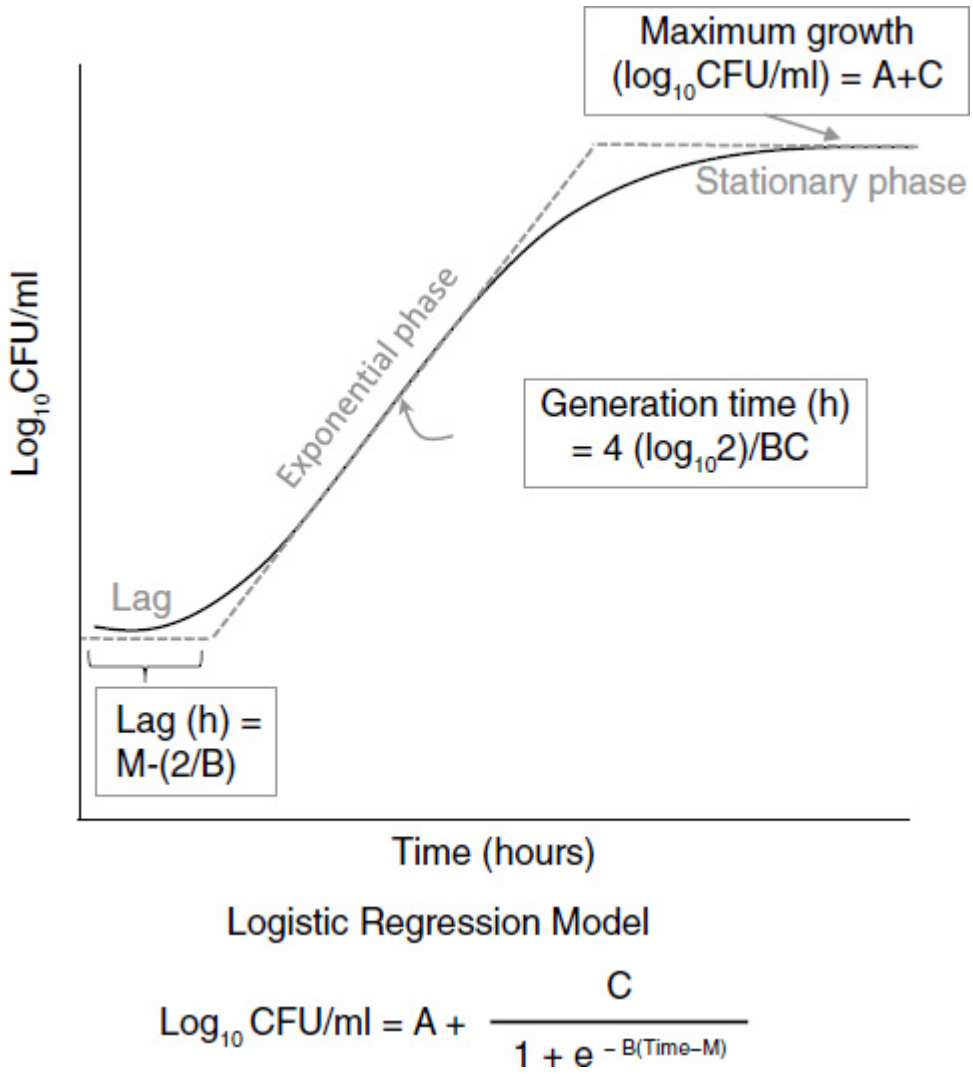


Figure 1.1 Conceptual behavior of microorganisms in various environments. The scale of the *y* axis symbolizes populations of single-celled foodborne microorganisms.

The most-probable-number technique was introduced to overcome some of the deficiencies of the plate count method, particularly the inability to enumerate small microbial populations. The technique involves making dilutions of food in a number of tubes containing a suitable broth and incubating the tubes of diluted food to enable growth of the small microbial population. After incubation, the numbers of positive and negative tubes (i.e., with detectable and undetectable growth, respectively) are compared to numbers in standard tables and used to estimate the population size. Results

are reported as the most probable number per unit of food. The method has been used for enumerating pathogen populations (e.g., *Listeria monocytogenes*) in food (1, 2).

Spiral plating relies on colony counting but reduces labor, cost, and time compared with the plate count method (3). A spiral plater uses a robotic arm to dispense a sample aliquot in an Archimedean spiral onto the surface of a rotating agar plate. The volume of the sample decreases from the center to the periphery of the plate so that a concentration range is established on a single plate. Counting of colonies is achieved using a specialized counting grid. The use of fewer dilution bottles or tubes and less agar medium and the ability to process a large number of samples in one batch are the main advantages. Some of the drawbacks of this method are the high limit of detection and the great variation in counts observed when small populations are counted, particularly in samples of solid foods.

Speedy measurement of microbial populations in food has been attempted using different methods. The direct microscopic count method involves using a specially designed glass slide having a small chamber and a microruler. A small measured volume of the sample, entrapped in the chamber, is examined with a microscope, cells are counted within a ruled area (e.g., 1 mm^2), and the estimated population per unit volume of the sample is calculated. A commonly used slide is the Petroff-Hausser counting chamber, which is suitable for counting prokaryotes in food. The method can be conducted easily, quickly, and inexpensively, but live and dead cells cannot be differentiated unless a vital-staining technique is included in the procedure. The method is well suited for rapid cell counting in liquid foods such as milk but less well suited for cell counting in solid foods.

Flow cytometry uses an optical system which enables rapid and highly sensitive estimation of cell numbers in a given suspension. Cells are stained with fluorescent dyes to allow the analysis of functional cell properties. Flow-cytometric methods have been used

to determine total bacterial counts in food (4). The high cost of flow cytometry equipment and the management of instrumental data are some limitations of this method.

A biosensor-based approach for real-time counting of microbial populations in food has been investigated. Biosensors are analytical devices that convert a biological response into electrical signals. Many of these devices consist of two main components: (i) a biorecognition element or a bioreceptor, which recognizes the analyte, and (ii) a transducer, which converts recognition events into measurable electric signals. Optical biosensors were tested successfully to quantify *Escherichia coli* O157:H7 in ground beef (5) and *L. monocytogenes* and *Salmonella enterica* serovar Enteritidis in frankfurters (6, 7). Electrochemical sensors were tested to monitor *E. coli* O157:H7 in milk (8) and to quantify *Campylobacter jejuni* in chicken carcasses (9). These assays have a short sample-to-answer time (10). Efforts continue to develop reliable, sensitive, rapid, accurate, selective, and cost-effective biosensor-based techniques. Such a technique could offer a commercial advantage to the food manufacturing and processing sectors by facilitating the rapid detection of foodborne pathogens and the release of uncontaminated foodstuff in hours, rather than the several days required for culture-based methods.

The methods described above were developed with the assumption that bacteria represent the majority of the food microbiota. Although some of these methods have been adapted for counting molds and yeasts, monitoring the growth of these eukaryotic organisms, particularly molds, in food may produce unsatisfactory results. When molds grow on food, they form a multicellular structure (mycelium) that is not quantifiable in terms of cell count or CFU. When mold hyphae are chopped during homogenization of a food sample, resulting pieces (propagules) may produce colonies whose number depends on the degree of chopping/homogenization. Growth of mold also results in the production of a large number of spores (e.g., conidiospores and sporangiospores), each of which is potentially capable of producing a colony on agar medium.

Therefore, it is likely that the count monitored over time is underestimated before spores are formed and overestimated as the spores are released from mycelia.

Phases of Microbial Growth in Food

Similar to a batch culture, microbial growth in an inoculated food can be described by the classical growth curve. When the log population count is plotted against time, four distinct phases can be discerned: lag phase, exponential phase, stationary phase, and death. Microorganisms dividing by binary fission (i.e., bacteria) in a liquid food (e.g., milk) produce growth curves with four recognizable phases. Careful consideration of the growth of other microorganisms in various food matrices can reveal the phases of a classic growth curve. The following discussion addresses the first three phases of growth; the death phase is addressed later.

Lag Phase and Shelf Life Extension

When a microorganism is introduced into a fresh environment, a period of growth cessation (lag phase) is observed. During this lag in growth, it is presumed that the microorganism is adapting to the new environment through retooling and synthesizing new components prior to resumption of cell division. For example, expression of a new set of enzymes may be needed if the microorganism is introduced into a medium different from the one in which it was growing previously. Once adaptation is successful, DNA replication resumes and cell division ensues. The length of the lag phase varies with the nature of the medium and the stress condition of the microorganism. If the inoculum is old (e.g., the stock culture was preserved at −80°C) or is transferred into a chemically different medium, the lag phase will be long. In contrast, the lag phase will be short or even absent when a vigorously growing young culture is transferred into a fresh medium having the same composition.

Bacterial growth in a laboratory setting starts with inoculation of a microorganism into a microbiological medium. Growth in most foods starts with an accidental contaminant gaining access to the food. When a microorganism enters a food, the nutritional and inhibitory characteristics of this new environment dictate the length of lag imposed on the microorganism. Availability of water and nutrients and favorability of the temperature, pH, oxygen level, and other environment factors are important determinants of duration of the lag phase. The presence of antimicrobial agents in food could suppress cell multiplication, thus extending the lag period. Food processors design processes to extend the lag period of food microbial contaminants; in essence, this approach extends the shelf life of the newly prepared food. Processing food (e.g., pasteurization) may render modifications in food composition and its environment, whereby even resident microorganisms that survived the process may undergo a lag to adapt to the new conditions.

Exponential Phase

A microorganism transitions from the lag to the exponential growth phase when cells start to multiply at measurable rates. Under favorable conditions, a microorganism multiplies at an increasing pace until a state of balanced growth is reached. During this state, the microorganism doubles its population at equal time intervals, called the generation time or doubling time (t_d). Therefore, the growth at this balanced-growth stage can be described by the following equation:

$$N_t = N_0 \times 2^n$$

where N_0 is the initial population at this stage, N_t is the population at time t, and n is the number of generations in time t. This equation can be used to derive the doubling time, which can be calculated as follows:

$$t_d = \frac{0.301t}{\log N_t - \log N_0}$$

In addition to the generation time, the specific growth rate is another way of expressing growth during the exponential phase. This is based on the assumption that the rate at which the cell population increases at a given time (t) depends on the population size (N) at that time.

$$\frac{dN}{dt} = \mu N$$

where μ is the specific growth rate, which is species and environment dependent. Integration of the above equation gives the following exponential function:

$$N_t = N_0\, e^{\mu t}$$

These equations can be used to derive μ as follows:

$$\mu = \frac{d\ln\, N}{dt}$$

Therefore, μ equals the slope of the line representing the exponential phase when the natural logarithm of population density (ln CFU per milliliter) is plotted against incubation time (typically in hours). The following is a more conventional way to calculate μ using Commonly plotted growth curves, i.e., $\log_{10}$ CFU per milliliter versus incubation hours.

$$\mu = \frac{\log N_t - \log N_0}{0.434\ t}$$

Comparing the above equations, for t_d and μ, results in the following relationship between these two exponentialgrowth-phase parameters:

$$\mu = \frac{0.693}{t_d}$$

At the exponential phase, the cell population is relatively uniform in terms of physiological and biochemical characteristics; therefore, microbial cultures in the exponential phase are used often in microbiological studies.

Exponential microbial growth in food

Knowledge of growth kinetics during the exponential phase is indispensable to food microbiologists. Understanding growth kinetics also helps processors quantify the behavior of beneficial bacteria and alleviate the harm of spoilage and pathogenic microorganisms. Fermentation, spoilage, and toxin accumulation in food are the result of vigorous microbial growth activities. During food fermentation, growth of beneficial microorganisms results in a wide range of changes in food components that lead to nutritional enrichment, improved safety, and promotion of health. Rapid growth of a starter culture during fermentation often inhibits the growth of foodborne pathogens. A microorganism may exhibit metabolic activities that differ with the phase of growth. For example, the proteome and transcriptome of *Lactobacillus rhamnosus* GG change significantly during the transition from the exponential to the stationary phase. This transition is accompanied with a shift from glucose fermentation to galactose utilization and from homolactic to mixed-acid fermentation end products (11). In a strain of wine yeast, the number of proteins involved in ethanol production and sulfur metabolism during alcoholic fermentation is different during the exponential and stationary phases (12).

Food spoilage is an outcome of microbial growth and metabolism. These activities can result in changes in the pH of food, release of off odors, generation of undesirable flavors, formation of gas and slime, and product discoloration (13). Accumulation of toxins in food also is the outcome of active growth of toxigenic microorganisms. For example, production of staphylococcal enterotoxins occurs during the exponential and stationary growth phases of *Staphylococcus aureus* (14). Toxigenic molds also grow on

stored food, including fruits, with the production of mycotoxins during the mold's growth cycle (15).

Stationary Phase

The exponential growth phase ends with a stage of growth deceleration due to the depletion of critical nutritional components or the development of unfavorable environmental conditions, such as low pH, low redox potential, or the release of inhibitory metabolites. During the transition to the stationary phase, the pace of growth decreases to a negligible rate and population density reaches an asymptote. Despite the lack of noticeable multiplication during the stationary phase, the cell population may remain metabolically active. Prokaryotes have evolved several strategies to survive a starvation similar to that encountered during the stationary phase. Under this condition, cells frequently produce starvation proteins (chaperones) which increase peptidoglycan cross-linking, protect DNA, and prevent protein denaturation (16). These chaperones make starved cells difficult to kill and resistant to further starvation or other stresses. Moreover, starvation-resistant cells may become more virulent (17), an observation which has major implications for the safety of foods. The following are stationary phase-associated phenomena of great significance in food microbiology.

Quorum sensing

Bacterial behavior during the stationary phase is linked to the quorum-sensing phenomenon. At the onset of this phase, bacteria reach a high cell density and experience physiological stress due to nutrient limitation and other unfavorable conditions. Quorum sensing is a process by which bacteria cope with stationary-phase stress and shift their central metabolism toward production of components that benefit the population as a whole (18). For example, quorum sensing modulates the cellular metabolism of *Burkholderia* spp. to produce oxalate, which enables their survival during the stationary phase. In *Burkholderia glumae*, quorum

sensing serves as a metabolic brake to halt multiplication but maintain the cells' primary metabolism.

The term "quorum sensing" was first suggested to describe how bacteria communicate with each other through signaling molecules released into the environment during growth. Several cellular processes are triggered when the density of bacterial cells and the concentration of the signaling molecules reach a threshold. The signaling molecules required for communication can be divided into four categories: (i) *N*-acyl homoserine lactones (AHLs), which are generally known as autoinducer 1 molecules and are used by Gram-negative bacteria for facintraspecies communication (19); (ii) furanosyl borate diester, known as autoinducer 2, which serves as a universal signal and is used for interspecies and intraspecies communication by Gram-positive and Gram-negative bacteria (20); (iii) autoinducer 3, which is used by enterohemorrhagic *E. coli* for expression of virulence genes and cross talk with mammalian host cell signaling (21); and (iv) autoinducing peptides, which are produced by Gram-positive bacteria (22). However, other molecules similar to those of quorum-sensing systems have been described in the published literature. Quorum sensing occurs when there is a high cell density. Microbial spoilage of food also is a high-celldensity event. Hence, researchers have investigated the potential role of quorum sensing in spoilage. Additionally, quorum sensing has been investigated in the context of how it regulates food spoilage (23), how production of signaling molecules by pathogens is impacted by food system conditions (24), and how quorum sensing affects expression of survival and virulence genes of foodborne pathogenic bacteria (25). Understanding quorum sensing may help in disrupting cellto-cell communication and thereby preventing food spoilage. This may be achieved by targeting the synthesis of cell-signaling molecules or blocking these signaling systems. An alternative approach, referred to as quorum quenching or quorum sensing inhibition, involves using quorum-sensing molecules to generate false sensing and confuse bacteria, leading to the arrest of their growth or metabolic activity (26). Plants have

received considerable interest for their ability to produce compounds capable of interfering with quorum sensing in bacteria. Plant extracts may contain AHLdegrading enzymes which are capable of disrupting quorum sensing and are potentially useful in extending food's shelf life.

Biofilm formation

Bacteria in the planktonic state in an aqueous environment may form biofilms by residing on surfaces and undergoing sessile growth. Once a biofilm is formed on a surface, bacteria become more difficult to remove, and their susceptibility to antimicrobial agents or adverse environmental changes decreases relative to that of planktonic cells. It is not surprising that the formation of biofilms in food processing environments is difficult to control. Biofilm formation includes four stages: (i) initial attachment, which may occur to moist food or pipeline surfaces; (ii) microcolony formation; (iii) biofilm maturation; and (iv) dispersion. A cyclic increase and decrease in the population of a contaminant may occur in the processing environment, such as that which may be observed in effluents collected during the cleaning of pipes. During this cycle, the decrease in population may correspond to the initial stages of biofilm formation and the increase may coincide with the dispersion phase.

Biofilm formation is influenced by environmental factors (e.g., pH and temperature), surface characteristics (electrostatic forces, roughness, and hydrophobicity), bacterial factors (e.g., flagella and pili), and genetic factors (e.g., the polysaccharide intercellular adhesion gene). Biofilm formation may be regulated by quorum sensing, which activates several genes that are necessary for expression of extracellular polymeric substances. Control of bacterial biofilms in food processing facilities is vital to mitigate the occurrence of pathogens in food. Disinfection, cleaning, enzymatic treatment, and application of bacteriophages have been reported as effective treatments in controlling biofilms (27). Microbial metabolites, such as surfactin from *Bacillus subtilis*, prevented

biofilm formation by *Salmonella* Typhimurium (28). In addition to chemical and biological control strategies, new physical treatments, such as laser decontamination and antimicrobial coating of surfaces, have been proposed.

Predictive Modeling of Microbial Growth

When growth of a microorganism is monitored experimentally, plotting the lag, exponential, and stationary phases against incubation time typically produces a classic sigmoid curve (Fig. 1.2). Mathematical modeling of such a curve helps researchers and processors predict the growth behavior of the microbe in food under various processing and storage conditions. This predictability has important food safety and quality implications. A predictive growth model developed for a specific microorganism in a given food should be reevaluated if the food composition or storage environment is changed.

There are generally two types of models used in predictive microbiology: stochastic (probabilistic) and deterministic models. The latter type is often used for modeling growth curves, and it includes empirical analytical expressions and rate equations (29, 30). In order to build a deterministic model, growth has to be measured first; then growth data (e.g., measurements of CFU per milliliter for 24 hours) are fitted mathematically with the selected model using nonlinear regression statistical software, and the goodness of the model's fit is determined statistically. Data fitting involves determining model constants, i.e., the model's mathematical parameters. These parameters vary with the species, medium characteristics (e.g., pH and aw), and environmental Factors (e.g., temperature). Gompertz and logistic models are some of the most commonly used models for microbial growth. The following are equations representing these models.

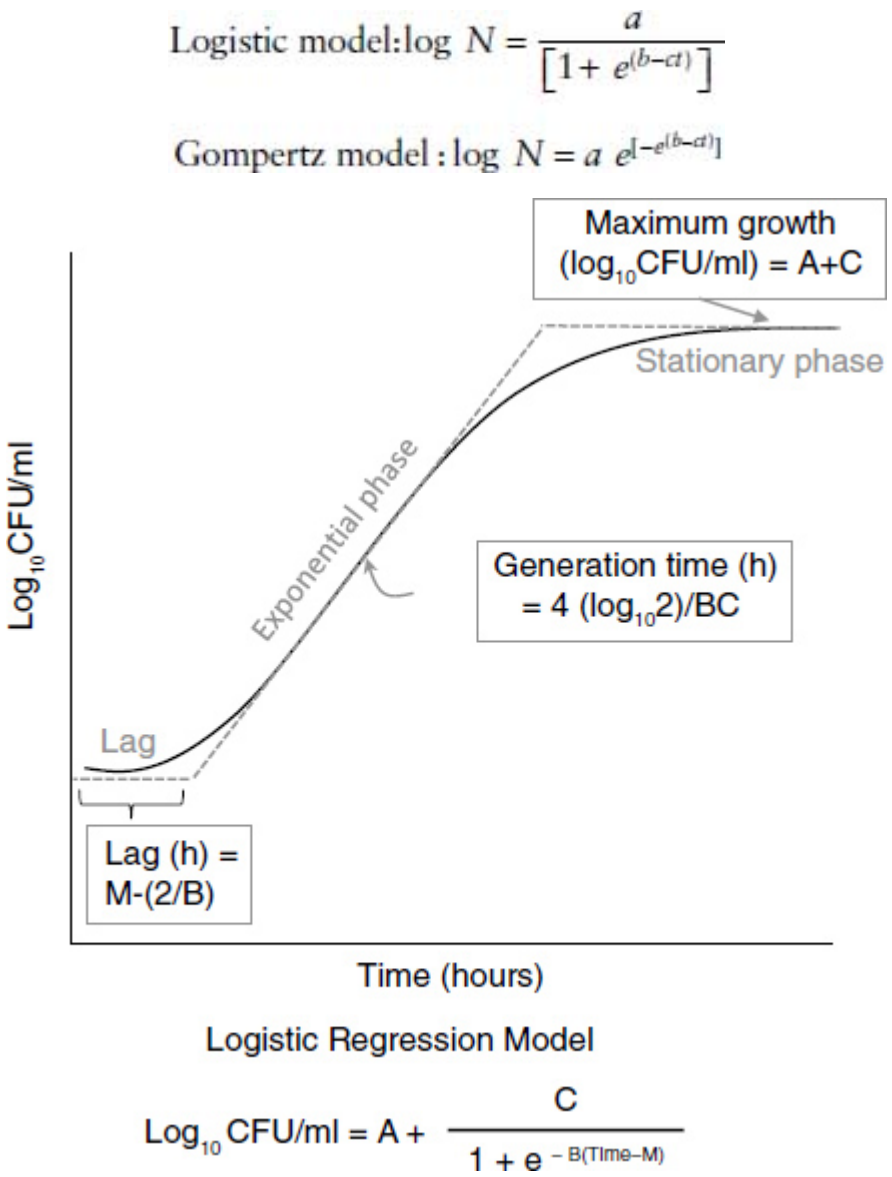


Figure 1.2 Typical bacterial growth curve, fitted with a logistic model.

where t is incubation time, N is population density, and a, b, and c are model parameters. Biologically meaningful parameters, such as lag phase, specific growth rate, and generation time, are calculated from the mathematical parameters. An implementation of the logistic model is illustrated graphically in Fig. 1.2 (31).

Microbial Metabolism in Food

Microbial growth per se is not the direct cause of food fermentation or spoilage; enzymes and metabolites released from the growing biomass are responsible for these changes. Covering the large number of metabolic pathways and the overwhelming number of metabolites produced by foodborne microorganisms is beyond the scope of this introductory chapter. Therefore, metabolism is addressed at the fundamental level only.

Microbial metabolism can be arranged into two categories based on how microorganisms obtain carbon for the synthesis of their biomass: (i) autotrophic (carbon is obtained by fixing CO_2) and (ii) heterotrophic (carbon is obtained from metabolized organic compounds). Utilization of carbon, regardless of its source, requires energy. Based on the source of energy that microbes use, both autotrophic and heterotrophic microorganisms can be photosynthetic (using light energy) or chemotrophic (energy derived from oxidation of chemical compounds). The chemicals providing energy could be from an organic (organotrophic) or inorganic (lithotrophic) source. Most microorganisms of importance to food are chemo-organoheterotrophs. These microorganisms use organic compounds as both carbon and energy sources. They also may derive their organic nutrients from dead matter or living hosts; these are termed saprophytes and parasites, respectively.

Aerobic Respiration

For many foodborne microorganisms, carbohydrate metabolism is the primary source of energy that drives vital cell reactions and the source of building blocks needed for cell growth. Different microorganisms metabolize carbohydrates (typically glucose) differently depending on the functionality or dysfunctionality of their metabolic pathways. Utilization of glucose by microorganisms capable of cellular respiration is accomplished through glycolysis, which is also called the Embden-Meyerhof-Parnas pathway, and the citric acid cycle, which is known as the Krebs cycle (Fig. 1.3). The

outcome of these metabolic activities is formation of energy-rich ATP and a reducing power in the form of NADH and $FADH_2$. These reduced molecules are used to produce more ATP at the electron transport chain, which is embedded in the cytoplasmic membrane of prokaryotic cells or the inner membrane of mitochondria in the case of eukaryotic cells. The reduced products (particularly NADH) are oxidized, becoming positively charged and releasing electrons to the electron transport chain.

$$NADH \rightarrow NAD^+ + H^+ + 2e^-$$

This chain is composed of a series of membrane-embedded protein complexes as well as mobile carriers (e.g., cytochrome *c*). Along the chain, the released electrons undergo a series of redox reactions, losing energy that is used to pump protons from the cell cytosol side (or mitochondrial matrix) to the outside of the membrane. The energy gradient generated across the membrane is used by ATP synthetase, which is also embedded in the membrane, to phosphorylate ADP (in the presence of inorganic phosphate) to ATP in a process called oxidative phosphorylation. At the end of the chain, electrons are passed to oxygen, the final electron acceptor, producing water.

$$2e^- + \tfrac{1}{2}O_2 \rightarrow H_2O$$

The metabolism of one glucose molecule by aerobic cellular respiration (glycolysis + citric acid cycle + electron transport) yields 30 to 32 ATP molecules, but some references report larger numbers. Most of these ATPs are the result of oxidative phosphorylation. The aerobic respiration pathways are fairly highly conserved in both prokaryotic and eukaryotic cells. In bacteria, glycolysis and the citric acid cycle occur in the cell cytosol and the electron transport takes place at the cytoplasmic membrane. In fungi and other eukaryotes, glycolysis occurs in the cell cytosol, and the citric acid cycle takes place in the mitochondrial matrix, with electron transport occurring in the inner membrane of mitochondria. Some bacteria use

alternative pathways for glycolysis and the citric acid cycle. *Pseudomonas fluorescens*, for example, metabolizes sugar using the keto-deoxy-phosphogluconate pathway, instead of the Embden-Meyerhof-Parnas pathway, which is partially defective in this species (32).

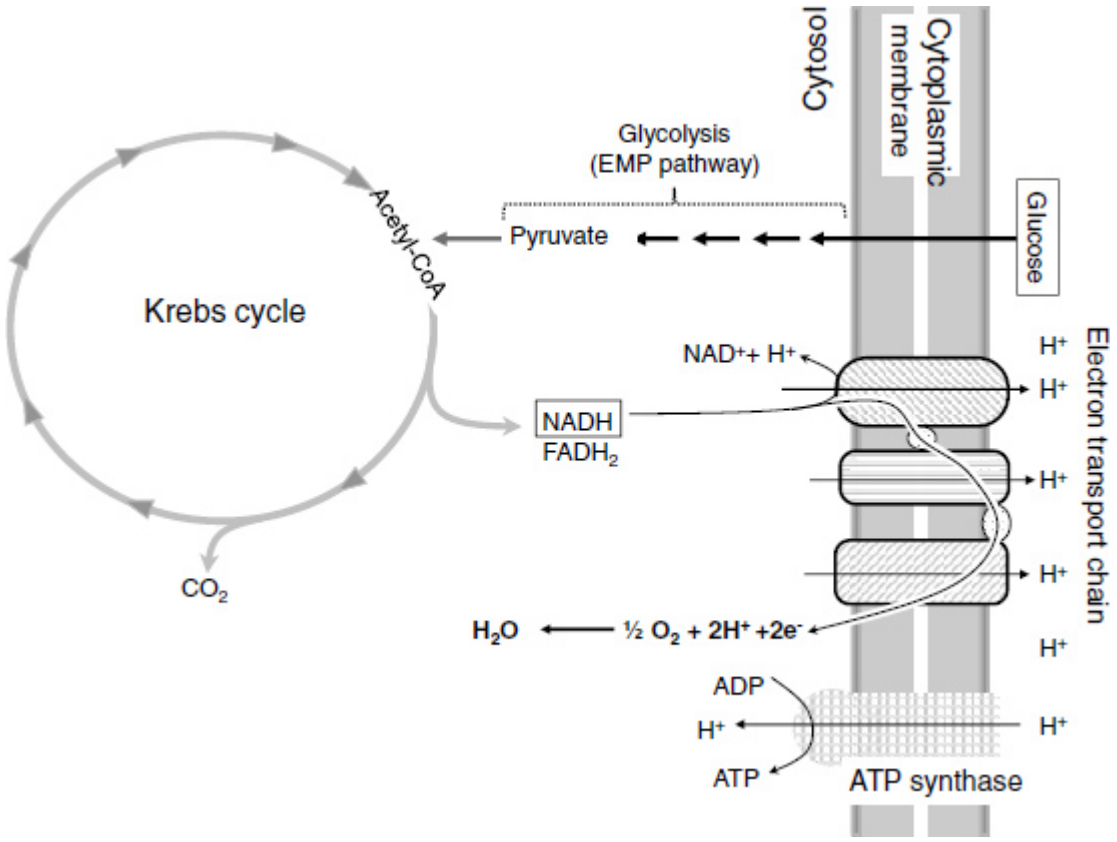


Figure 1.3 Glucose metabolism through aerobic respiration. In the absence of oxygen, the presence of an alternative oxygen acceptor results in anaerobic respiration. EMP, Embden-Meyerhof-Parnas.

Aerobic microorganisms such as *Bacillus* spp. and most molds are rapid growers and often cause food spoilage. Beneficial molds, however, are useful in making moldripened cheeses and other fermented products. These molds are grown on the cheese surface (e.g., Camembert cheese) or inside the cheese matrix (e.g., blue and Roquefort cheeses) (33). In the latter case, cheese blocks are spiked with needle-like rods to allow the air to contact the mold inside the block.

Anaerobic Respiration

The respiratory pathways just described can occur in the absence of molecular oxygen when alternative electron acceptors are reduced at the end of the electron transport chain. Alternative electron acceptors include nitrate, sulfate, fumarate, tetrathionate, and dimethyl sulfoxide. These alternatives have less oxidizing power than oxygen; thus, less energy is produced than in aerobic respiration. Utilization of nitrate during anaerobic respiration can be a multistep denitrification process that involves more than one cellular enzyme. The enzymes needed for complete denitrification, in conjunction with electron transport, are nitrate reductase, nitrite reductase, nitric oxide reductase, and nitrous oxide reductase (Fig. 1.4). Some foodborne bacteria (e.g., members of the *Enterobacteriaceae*) are capable of completing the first step only, i.e., the reduction of nitrate to nitrite in the presence of cellular nitrate reductase (34). *Staphylococcus carnosus*, a starter culture for naturally fermented sausages, also is capable of converting nitrate to nitrite. Considering that nitrites inhibit the growth of *Clostridium botulinum* in food, this denitrification step can be significant for the safety of food. Additionally, the products of denitrification can affect the color of raw red meat and some processed meats.

Intriguingly, anaerobic respiratory metabolism can be linked to microbial pathogenesis and virulence. *Salmonella* pathogenicity islands which carry virulence genes can harbor genes encoding specific metabolic pathways that are important for the successful infection of a host. Inflammation of the intestine caused by *Salmonella* Typhimurium infection results in the release of reactive oxygen species, which oxidize intestinal thiosulfate into tetrathionate. Considering that *Salmonella* Typhimurium is capable of utilizing tetrathionate as a final electron acceptor (anaerobic respiration), this permits *Salmonella* to multiply and colonize the intestinal tract, giving the pathogen a competitive advantage over the predominantly fermentative gut microbiota (35).

Fermentation and Anaerobic Bacteria

Bacteria having a dysfunctional citric acid cycle are anaerobes and produce energy through fermentation. These bacteria carry out glycolysis to produce pyruvate which is converted to other organic end products (Fig. 1.5). These products include ethanol, acetate, lactate, and butyrate. Gases such as CO_2 and H_2 may be produced as fermentation end products. Fermentative bacteria generate ATP through substrate-level phosphorylation, where phosphatecontaining high-energy organic compounds convert ADP to ATP. There are fermentative bacteria that can tolerate oxygen (e.g., many lactic acid bacteria), whereas others (e.g., some *Clostridium* spp.) have an extremely low tolerance to oxygen (i.e., strict anaerobes).

Lactic acid and propionic acid bacteria are fermentative microorganisms with great importance in food processing. Fermented dairy products, meats, and vegetables are made using lactic acid bacteria, and Swiss-type cheeses are made using propionic acid bacteria. Recent genomic analyses of lactic acid bacteria revealed novel metabolic pathways that led to the discovery of unusual phenotypic characteristics. Arginine catabolism by these bacteria was found to contribute to stationary-phase survival and acid resistance. Engineering fermentative bacteria by introducing new cellular processes at central metabolic pathways enabled the production of novel metabolites. For instance, engineering *Lactococcus lactis* subsp*cremoris* NZ9000 with a four-enzyme cascade enabled the production of guanosine diphosphate l-fucose (36). l-Fucose is a biologically important monosaccharide. Metabolic engineering of lactic acid bacteria may reduce their nutritional requirements and enable them to ferment low-cost polysaccharides.

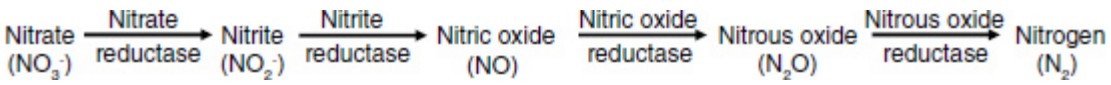


Figure 1.4 Microbial reduction of nitrate and other inorganic nitrogen during anaerobic respiration.

Facultative anaerobes

Some food microbiota are capable of metabolizing glucose using multiple pathways. In other words, these microorganisms can switch between aerobic respiration and anaerobic pathways (anaerobic respiration or fermentation) depending on oxygen availability. Members of the *Enterobacteriaceae* are facultative anaerobes; thus, they can complete aerobic respiration in the presence of oxygen (using glycolysis, the citric acid cycle, and the electron transport chain), and in the absence of oxygen, they metabolize glucose by anaerobic respiration using alternative oxygen acceptors. In the absence of any electron acceptors, *Enterobacteriaceae* are fermentative, producing a mixture of acetate, formate, lactate, and succinate. These metabolites are products of multiple pathways; hence, this mode of glucose metabolism is known as mixed-acid fermentation (Fig. 1.5). This versatility gives facultative anaerobes a competitive advantage in environments having a variety of microbiota. The persistence of coliforms in the intestine, despite the complexity of gut microbiota, illustrates their high metabolic adaptability to various environments. Some yeasts (e.g., baker's yeast) carry out aerobic respiration and anaerobic fermentation.

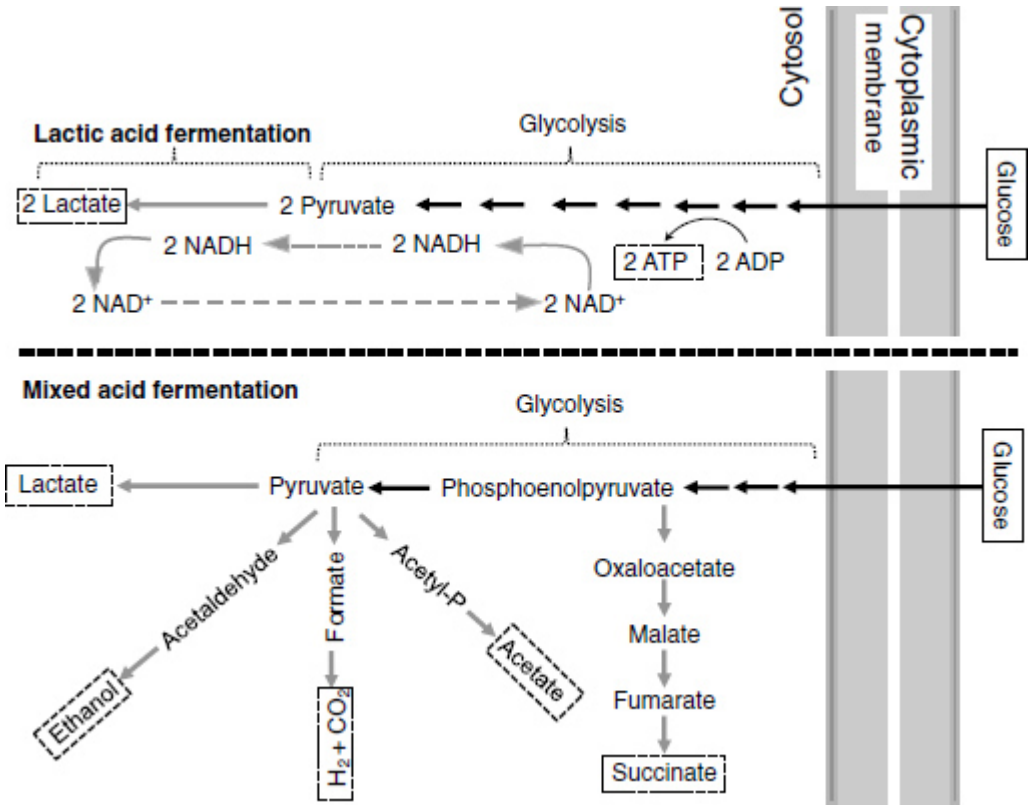


Figure 1.5 Anaerobic metabolism of glucose through fermentation. (Top) Lactate fermentation. (Bottom) Mixed-acid fermentation. Final fermentation products are in dotted boxes.

Consequences of Microbial Growth and Metabolic Activity in Food

Microbial growth in food can lead to desirable or undesirable effects. Fermentation, biocontrol, spoilage, and increased disease risk are the main outcomes of this growth.

Food fermentations are the bases for an important sector of the food industry. There should be a clear distinction between "metabolic" fermentation, which is discussed above, and industrially relevant food fermentations, which are addressed in this section. Industrially relevant fermentations use desired microorganisms to convert raw or minimally processed products into fermented foods regardless of the metabolic pathways contributing to this conversion. The source of the inoculum used in food fermentations

could be (i) microorganisms naturally present in raw food, (ii) microbe-containing food products from a previous fermentation (a process known as back slopping), or (iii) an in-house-prepared or commercial starter culture. Small inocula generally must grow to produce the desired effect, whereas large inocula may accomplish the fermentation with limited growth due to the metabolic activity of the microbial biomass. There are many desirable bacterial groups, including lactic acid bacteria, which have been used in food fermentation.

Spoilage is a result of an unintended microbial growth and its associated metabolic activities. Release of metabolites and enzymes (with or without celllysis) is the direct cause of the spoilage. Spoilage microorganisms could be part of the natural microbiota of food or may have gained access to food during handling or processing. Indications of food spoilage include changes in odor, color, or texture; accumulation of gas; slime formation; and accumulation of exudates. Certain conditions can promote the sequence of events that lead to food spoilage. These are (i) food having characteristics that support microbial growth; (ii) presence of microbial contaminants compatible with food characteristics; (iii) growth of the contaminant to high population density (commonly greater than 106 CFU/ml or CFU/g), which occurs when storage conditions favor microbial growth (e.g., temperature abuse); and (iv) food being held for a sufficient length of time under the growth-favoring conditions. Many microorganisms that are naturally present or unintentionally added to food are capable of causing its spoilage.

Increased risk of disease transmission is an important but undesirable consequence of growth of microorganisms in food. Based on the mode of causing illness, microbial foodborne diseases can be grouped into intoxications, infections, and toxicoinfections (37). Intoxication is an illness resulting from ingestion of a toxin produced during growth of the toxigenic microorganism in food. Once the toxin is produced in an active form in a food, there is no need for the microbial cells to be viable for illness to occur.

Foodborne intoxications include botulism, staphylococcal gastroenteritis, and *Bacillus cereus* gastroenteritis. Growth of molds (e.g., some *Aspergillus* and *Fusarium* species) in food may result in the accumulation of mycotoxins which cause human and animal intoxications. Infections, in contrast, require ingestion of live microorganisms which may or may not have grown in the food. Some infectious pathogens (e.g., *L. monocytogenes* and *E. coli* O157:H7) have low infectious doses, so their growth in food is not a prerequisite for illnesses to occur; however, growth of these pathogens in food increases the risk of disease transmission. Toxicoinfection results from ingestion of a live pathogenic microorganism with contaminated food, growth of the pathogen in the gastrointestinal tract, and production of a toxin in the intestine. A classic example of toxicoinfections is cholera, which is caused by *Vibrio cholerae*.

Pseudogrowth in Food

An increase in microbial populations in food cannot always be translated into microbial growth. For example, the population of *L. monocytogenes*, measured as CFU per gram, increased almost 10-fold during the manufacture of Colby cheese (38). This increase, however, was most likely due to entrapment of the pathogen in the curd while syneresis (i.e., whey expulsion) was occurring. Similar pseudogrowth may be observed in other events. Concentration of milk, whey, or juices during ultrafiltration most likely results in an increase in counts (CFU per milliliter or gram) that has nothing to do with the growth of food microbiota.

SURVIVAL BEHAVIOR IN FOOD

When conditions in food are neither conducive to growth nor hostile enough to cause death, microbial populations may remain relatively unchanged for a period of time (Fig. 1.1). This state is described in this chapter as “survival.” Conceptually, when a food known to support microbial growth is treated with a bacteriostatic

agent (i.e., a growth inhibitor), the treatment prevents contaminants from multiplying, thereby leading to a state of survival. Published literature contains many studies revealing the survival behavior as described here; however, some researchers use the word to describe subpopulations that remain viable after lethal treatments (i.e., treatment persisters). Despite the inconsistent use of this term in the published literature, microbial survival, as used in this chapter, is intended to represent a phenotypic state where neither growth nor death is noticeable. When the physiological and molecular bases for survival are further investigated, uniformity in the usage of the term is expected. Considering the difficulty of demonstrating survival, as intended here, in a complex matrix such as food, the term is used even when some changes in microbial population are observed during lengthy storage of a product.

Pathogen survival in food was demonstrated in a 1986 study on the behavior of *L. monocytogenes* in Cheddar cheese (39). Although most strains of the pathogen had died gradually during the lengthy storage of cheese, the population of *L. monocytogenes* V7 persisted relatively unchanged, or minimally decreased, in some batches held at 6°C for more than 200 days. Dry conditions are conducive to lengthy microbial survival, particularly when the contaminated product is stored at temperatures that are lower than ambient. Populations of *Salmonella* Enteritidis phage type 30 on the surfaces of raw almonds did not change considerably (~1-log reduction) during storage at 4°C or −20°C for over 550 days (40). Similarly, populations of *L. monocytogenes* and *E. coli* O157:H7 remained unchanged when inoculated almonds and pistachios were stored for 12 months at 4°C or −19°C (41). In the same study, nuts were inoculated with *E. coli* O157:H7 and *L. monocytogenes* and stored at 24°C. Populations of these two pathogens declined 0.60 and 0.71 log CFU/g/month on almonds and 0.35 and 0.86 log CFU/g/month on pistachios, respectively. *Salmonella* Enteritidis PT 30 was capable of long-term survival on the surface of in-shell walnuts (42). The population of the pathogen declined at 4°C by 0.1 log CFU/nut/month, whereas under ambient conditions, the decline

ranged from 0.55 to 2.5 log CFU/nut/month. In a study on fresh produce, *E. coli* O157:H7 persisted on lettuce and parsley after seedlings were planted in soils amended with contaminated composts or contaminated irrigation water (43).

One may presume that microorganisms in the survival mode are growing and dying in small increments, for short periods, so that the overall outcome is a population size that is relatively constant over time. In other situations, the survival state may reflect a quasi-equilibrium between cell division and death. Alternatively, cells of the surviving population may have stopped their metabolic activity, leading to growth arrest. In the latter case, it is unlikely that microorganisms maintain this survival state for a long time; they may adapt to these conditions and resume growth, or they may suffer physiological injury leading to gradual death. Physiological changes leading to the state of survival have intrigued many researchers, and phenomena contributing to this state have been investigated. Some of these phenomena are stress adaptation, persistence, dormancy, and the viable-but-nonculturable (VBNC) state; these are discussed below.

Stress Adaptation

When microorganisms sense unfavorable changes in their surroundings, they may respond by altering gene expression in a way that increases their tolerance to the perceived stresses. This stress adaptation phenomenon has greater implications than just improving the survival ability of the microorganism. Stress adaptation could alter virulence characteristics of bacterial pathogens and enhance their ability to infect a host. It is plausible that adaptation occurs more readily when microorganisms experience sublethal rather than lethal stresses (44). Mild treatments during food processing are becoming popular due to the growing demand for minimally processed foods. Microorganisms that survive these processes may respond by adapting, thus compromising the safety of food.

Various cell structures, such as proteins, DNA, RNA, cell walls, and cell membranes, undergo injury and damage as a result of exposure to stress (45). Microorganisms may respond by expressing genes for repairing such damage and aiding their survival. Stress responses Include production of proteins that maintain cellular homeostasis, repair damage, or remove the stress agent. The pre-exposure of pathogens to sublethal levels of a given stress, leading to tolerance to harsher levels of the same or different types of stress, is the basis for stressadaptive or cross-adaptive responses, respectively.

The response of bacteria to stress can be general or specific. Since many stresses have similar effects on vital cellular structures, bacteria have evolved general stress responses controlled by sigma factors. Binding of these factors to RNA polymerase core enzyme directs transcription of a set of genes to support cell survival. In Gram-negative bacteria (*Salmonella*, *Vibrio*, and *E. coli*), RpoS is a main sigma factor, whereas SigB is an alternative sigma factor with similar functions in Gram-positive bacteria (*S. aureus*, *L. monocytogenes*, and *B. subtilis*). These regulators are the key players in coordinating response to stresses such as high osmolarity, starvation, higher-than-optimum temperature for growth, and exposure to bacteriocins, antibiotics, and low pH (46). Specific stress responses involve the production of proteins that protect bacteria against specific suboptimal conditions in their environments. Examples of these proteins include heat shock proteins, cold shock proteins, acid shock proteins, systems responsible for potassium uptake and accumulation of compatible solutes, catalase, and superoxide dismutase (47–49). In foodborne pathogens, stress adaptation and virulence may be interrelated. For example, a mutation in *opuC*, a gene encoding the osmolyte transport system, reduced the ability of *L. monocytogenes* to colonize and cause infection in mice (50). Similar studies revealed a link between salt tolerance and virulence in *S. enterica* (51).

Adaptation to stress can be derived from mutations that select for phenotypes that enable microorganisms to survive under selective environments. Development of resistance to high hydrostatic

pressure (HHP) among strains of *E. coli* has been attributed to the probable acquisition of specific genetic determinants that maintained HHP resistance for 80 generations (52). After a single exposure to an HHP treatment of 400 MPa for 20 min, a piezotolerant strain of *L. monocytogenes* was isolated. This isolate had increased cell size, lacked flagella, exhibited morphological alterations, and had increased tolerance to acid, heat, and hydrogen peroxide treatments (53). This multi-stress-resistant phenotype was attributed to a deletion in the glycine-encoding repeat region of *ctsR*, a gene that encodes the class 3 stress gene repressor. Genetic analysis of 24 HHP-resistant variants of *L. monocytogenes* LO28 revealed seven variants with mutations in *ctsR* and two variants with deletions in the upstream region of the gene (54). However, HHP-resistant variants of *S. aureus* had no mutations in *ctsR*, suggesting that there are different mechanisms for acquisition of HHP resistance by different species (55).

Persistence

Persistence refers to the ability of a microorganism to remain viable for a period of prolonged time in a certain habitat. Although persistence can be an outcome of microbial growth, colonization, biofilm formation, or resistance to decontamination processes, it is discussed in this chapter under the survival mode of microbial behavior. Persistence of pathogens results in repeated contamination of foods, increased violation of the safety of foods, and elevated public health risks. At preharvest, persistence includes colonization of an animal by a particular pathogen that results in shedding of the pathogen for weeks or months. Bacterial pathogens also may persist in farm environments such as feed or stables. Persistence at processing facilities is manifested by repeated isolation of the same strain of a pathogen (e.g., *L. monocytogenes*) for months or even years from the same sites.

Several studies have revealed evidence for the persistence of foodborne pathogens in the food production environment. For cattle that persistently shed Shiga toxin-producing *E. coli* in their feces,

there was a greater prevalence of the pathogen in the farm environment than for cattle that occasionally shed the pathogen (56). Cattle that shed more than 10^4 CFU of *E. coli* O157:H7/g of fecal matter are described as supershedders (57). *Salmonella* can persist for years in feed-processing environments. The persistence of *Salmonella* has been explained by several mechanisms, including the ability to form biofilms, which enhances its resistance to disinfection and desiccation (58). The correlation between biofilm formation and persistence has been investigated extensively, and the biofilm-forming ability of *Salmonella* has been proposed to play a key role in its resistance in dry environments and persistence in food factory environments (59). *L. monocytogenes* can persist and survive disinfection and cleaning procedures, thereby causing crosscontamination of food products. Persistence of this pathogen is presumed to be attributable to desiccation and disinfection resistance, biofilm formation, and differential gene expression for utilization of carbon sources that confer a competitive advantage (60, 61). *Cronobacter sakazakii* can survive and persist in low-wateractivity matrices, such as powdered infant formula, for up to 2 years and in infant cereals for approximately 24 weeks (62). The mechanism for the persistence of *Cronobacter* is not fully understood; however, some researchers have related this behavior to the successful accumulation of ions (e.g., calcium) or compatible solutes (e.g., trehalose) which increase intracellular osmolarity (63). *Campylobacter* spp. contaminating work surfaces and equipment during the slaughter of infected poultry flocks can survive and contaminate *Campylobacter*-negative flocks. The persistence of *Campylobacter* may be associated with its resistance to disinfectants and antibiotics, biofilm formation, and interaction with protozoan cells (64, 65)

Dormancy

Bacterial sporulation, which is common in *Clostridium* and *Bacillus* species, represents a dormancy state that is considered the ultimate survival mechanism in nature. Spores retain viability due to reduced

metabolic activity and increased resistance to extreme physical and chemical conditions. Spore dormancy is thought to be associated with the dehydration of the spore core and reduced molecular movement. To end the state of dormancy, a spore undergoes a series of biochemical reactions that involve activation, germination, and outgrowth; these are described collectively as spore-to-cell transformation. Spores that need an extended length of time before this transformation are described as superdormant. If superdormant spores are present in processed food, they cannot be recovered by conventional detection or enumeration methods. When such food is stored, the superdormant spore may germinate, causing spoilage or a disease hazard.

Many researchers have evaluated different strategies to inactivate bacterial spores. A combination of pressure and heat applied sequentially or simultaneously proved effective for inactivating *Clostridium sporogenes* spores (66). Synergistic inactivation of spores of *C. botulinum* by heat and high pressure was shown to be strain dependent (67). Other researchers combined pressure, heat, low pH, and the addition of nisin to inactivate spores of *B. subtilis* and *C. sporogenes* (68). A germination-induced inactivation approach has been developed. This involves inducing spore germination using suitable germinants followed by exposure of the outgrowth to an antimicrobial chemical, physical, or biological agent. An example of this approach was the application of HHP, mild heat, and selected amino acids (added as germinants), which was found to be an effective sporicidal combination treatment (69). A combination of osmotic activation and treatment with nisin was proposed as a strategy to inactivate *Clostridioides* (formerly *Clostridium*) *difficile* spores (70). The application of the germinant asparagine followed by HHP treatment (586 MPa) at 73°C for 10 min killed *C. perfringens* spores in meat products (71).

VBNC State

Bacteria are considered VBNC when they cannot grow in conventional culture media but they maintain their viability.

Dormancy is similar to the VBNC state, but VBNC cells possess measurable metabolic activity that is not detectable in dormant cells. Being nonculturable, VBNC cells cannot be detected by conventional methods, which poses a great challenge for the detection of pathogens in this state. Recently, 85 species of bacteria, among which 67 are pathogenic, have been recognized to enter the VBNC state. Some foodborne pathogens have the ability to enter the VBNC state and maintain their virulence, but they cannot initiate disease until resuscitated (72). For example, VBNC cells of *L. monocytogenes* regained pathogenicity after resuscitation by incubation in embryonated eggs (73). Some researchers, however, reported that pathogens that enter VBNC state become nonpathogenic. For example, VBNC *Salmonella* Typhimurium could not infect mice in an *in vivo* experiment (74). In contrast, VBNC cells of enteropathogenic *E. coli* have been observed to retain their enteropathogenicity (75).

There is increasing evidence that correlates VBNC pathogens with the occurrence of foodborne-illness outbreaks. It was proposed that an outbreak of *E. coli* O157:H7 infection in Japan was caused by the VBNC state of the pathogen in salted salmon (76). Moreover, *Salmonella* Oranienburg entered the VBNC state in response to NaCl stress and caused an outbreak in dried processed squid (77). Failure to isolate the *E. coli* O104:H4 outbreak strain from food in Germany was attributed to the nonculturable state of this strain (78).

VBNC foodborne pathogens pose a safety risk because they escape conventional detection methods and recover their pathogenicity under certain conditions to present a human health threat. Within the supply chain, food is exposed to conditions that may be conducive to the entry of pathogens into the VBNC state. Foodborne pathogens have been recognized to enter the VBNC state in grape juice (79), vegetables (80), and milk products (81). The VBNC state of *E. coli* O157:H7 has been induced by UV disinfection on lettuce and spinach plants (82). Pulsed-electric-field treatment may have induced cells of *L. monocytogenes* and *B. cereus* to enter the VBNC

state (83). The mechanisms for induction into and resuscitation from the VBNC state need to be fully investigated.

CELL DEATH BEHAVIOR IN FOOD

Microorganisms may die spontaneously during food storage or be killed intentionally by processes designed to inactivate spoilage and pathogenic microbiota. Spontaneous death of pathogens in hard cheese, for example, is expected to occur with 60 days after manufacture (84) Food processors inactivate undesirable microbiota in food using physical factors such as heat, gamma radiation, and ultrahigh pressure; chemical factors such as antimicrobial gases and food preservatives; or biocontrol measures such as applying antagonistic beneficial microbial cultures. Details of these methods can be found in subsequent chapters. The following discussion addresses microbial death in food as a behavior or phenomenon that applies to a broad set of conditions.

Unmediated Microbial Death

Microorganisms may die in food during storage without the application of external lethal treatments. This behavior is described here as unmediated death. For example, a fraction of the population of lactic acid bacteria die and autolyze during the ripening of Cheddar cheese (85). Studies on *L. monocytogenes* revealed that the pathogen died more rapidly during storage in Parmesan cheese than in other cheeses (86). This rapid death in Parmesan cheese could be attributed to its hostile environment, such as its low water activity and the liberation of antimicrobial fatty acids during cheese ripening. Cell injury during processing (e.g., curd cooking during the preparation of Parmesan) combined with storage conditions that are unfavorable for cell repair may lead to such behavior. When *E. coli* was inoculated in apple juice and treated with a pulsed electric field, cell death continued to be observed during refrigerated storage of the juice (87). Death of *Salmonella* organisms was observed during the storage of dried cocoa beans for 30 days at room temperature

(88). The death, instead of survival, of *Salmonella* was attributed to the low water activity (0.68) of the product. A rapid death rate of *C. jejuni* was observed in Chinese-style sausage subjected to 50°C air blast drying, and a nondetectable level of the pathogen was reached in non-vacuum-packed sausage after 21 to 28 days of storage (89).

Competition between pathogens and other microbiota may explain the unmediated death of these pathogens in the production environment or food. Death of *L. monocytogenes* in a cheese microcosm was attributed to the microbial communities of Gram-positive and Gram-negative bacteria and yeasts present in Livarot cheese (90). The death behavior of *Salmonella* and *Campylobacter* in the ceca of poultry is regulated by the size and complexity of the microbial community inhabiting the alimentary gut (91, 92). The exclusion of invading pathogens from the intestinal tract may result from the competition for limited nutrients and adhesion sites, production of antimicrobial metabolites, and the occurrence of environmental conditions that are hostile to the pathogen. Unmediated cell death may also be explained by the programmed cell death (PCD) phenomenon, which is discussed below.

Programmed Cell Death

Certain stresses can lead to PCD in microbial communities. Death and lysis of these cells can provide nutrients that are usable by the surviving subpopulation. Several mechanisms of stress-induced PCD have been reported, and some are outlined here. One such mechanism in *E. coli* involves a toxin-antitoxin system encoded by a pair of genes, *mazF* and *mazE*, which constitute the *mazEF* module. These genes encode a stable toxin (MazF) and an unstable antitoxin (MazE) which prevents the lethal action of the toxin. Stress factors that inhibit transcription and translation of MazE allow its degradation, causing cell death by the action of MazF (93). High temperatures, DNA damage, and oxidative stresses were found to induce *mazEF*-mediated cell death. It is commonly believed that PCD under stress permits the survival of a small population that will act as a nucleus for the renewed population whenever the

conditions become conducive to growth. Hence, *mazEF*-mediated cell death is considered an altruistic mechanism of bacterial survival and is population dependent. Addressing PCD in foodborne pathogens can have significant implications for food safety, because this phenomenon could be used beneficially in food preservation.

Death Kinetics

Deliberate treatment of a microbial population with an antimicrobial agent may result in cell death to a degree that depends on the strength of the applied lethal factor and the duration of application. Similarly, when such a treatment is applied to raw food, it causes product decontamination to a level that depends on the delivered dose of the lethal factor. Heat, radiation, ultrahigh pressure, and antimicrobial gases are examples of lethal factors applied in food processing. Determining the rate of cell death (i.e., inactivation or death kinetics) in response to a lethal treatment is important for quantifying the effectiveness of that treatment.

Death kinetics can be measured accurately for a microorganism having a homogeneous population, exposed uniformly to a lethal treatment, and monitored carefully over a defined time period. Such a population often produces a death curve similar to that shown in Fig. 1.6. When the death behavior of bacteria is examined, the following phases may be observed: (i) viability depletion, (ii) logarithmic death, and (iii) persistence. These phases are more noticeable with mild, rather than aggressive, lethal treatments; the latter often cause a death behavior that is more linear than curved. The viability depletion phase is seen as a lag in death immediately after application of the antimicrobial agent at its full strength. This initial resistance to inactivation may be attributed to cells' innate ability to sustain the damage caused by the lethal factor. The reserve resistance response is eventually depleted, and cells then enter the logarithmic death phase.

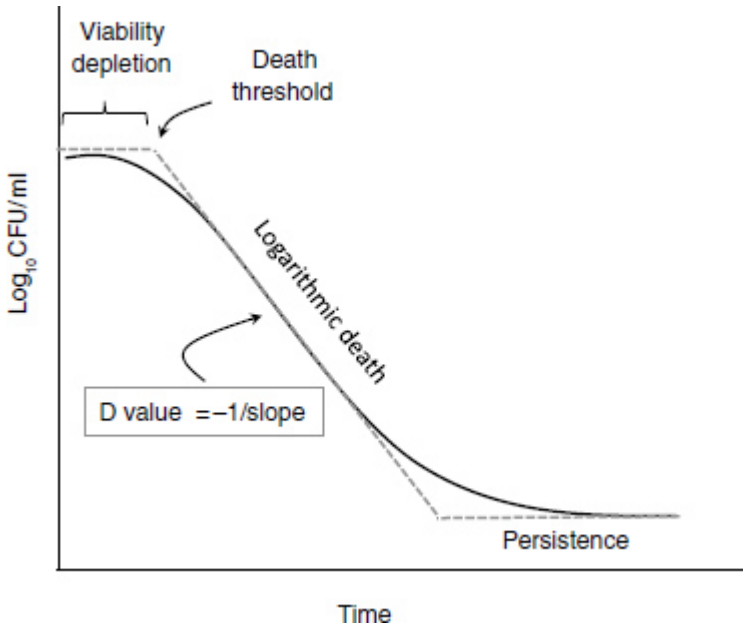


Figure 1.6 Phases of a microbial inactivation curve.

During the logarithmic death phase, the homogeneous microbial population undergoes first-order inactivation kinetics, which can be described by the following equation,

$$N_t = N_0\, e^{-kt}$$

where N_0 is the initial population, *Nt* is the population at time *t*, and *k* is the rate constant. For a population exposed to a lethal factor in a given medium, the *k* value depends on the species, the lethal factor applied, and the strength of that lethal factor. Instead of *k*, food processors use decimal reduction time (*D* value), which is a more meaningful cell inactivation parameter.

$$D = \frac{-dt}{d\log N}$$

In practical terms, the *D* value equals the negative reciprocal of the slope calculated from the linear portion of the death curve (Fig. 1.6). The linearity of death occurring during this phase (observed on a logarithmic death plot) is probably the outcome of uniform disruption of a viabilitylimiting step by the applied lethal factor.

At the end of the logarithmic phase, the rate of population death decreases, leading to the persistence phase. The size of the persistent population is often small. If the initial population is 107 CFU/ml and the persistent population is 102 CFU/ml, the latter represents only 1/105 of the original population. Many hypotheses have been suggested to explain the persistence of this subpopulation. Adaptation of members of the population to the applied lethal factor has been proposed; however, this adaptation is unlikely to develop during heating or the application of other physical lethal factors. Protection of the surviving population by lysates from inactivated cells seems plausible, but little information to support this hypothesis has been published. Genetic and phenotypic diversity within the initial population was also proposed. When a lethal factor is applied, a resistant phenotype is selected for, resulting in tailing of the curve. Some consider the persistence an outcome of flaws in techniques used to enumerate the cell population (see the discussion of these techniques above). For example, if cells of bacteria clump together, cell clumps appear to be dying at a lower rate than the rest of the population, which is made up mainly of individual cells. This observation is expected when heat is applied as a lethal factor and the viable population is counted as CFU per milliliter of food. Chemical lethal factors also may act more swiftly on planktonic cells than on cells embedded within clumps.

Persistence of a pathogen during testing and food processing minimizes the predictability of the process, which can lead to serious safety implications. If the parameters of the lethal process (e.g., temperature and time) were selected based on the *D* value for a targeted pathogen exhibiting a sigmoidal death curve, persistence of this pathogen leads to an underestimation of the processing conditions needed to make the food safe for human consumption. Understanding death kinetics of microorganisms in food, and the limitations on measuring reliable death parameters, should help processors to properly implement current and emerging

preservation technologies and to effectively mitigate disease transmission risks.

CONCLUDING REMARKS

Despite its importance, microbial behavior in food is often addressed casually in the published literature. Understanding the different patterns of this behavior is essential for promoting microbial growth when needed, inhibiting growth-associated spoilage and toxin production, and eliminating foodborne pathogens through processing. Food microbiologists, processors, and regulators need to consider the quantitative and mechanistic aspects of microbial growth, survival, and death. Behavior of microorganisms in food is intimately linked to important biological phenomena, such as quorum sensing, biofilm formation, stress adaptation, persistence, dormancy, and the VBNC state. Understanding programmed, unmediated, and induced microbial death and how these modes are interrelated could lead to new strategies in eliminating pathogenic and spoilage microorganisms in food.

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2
Spores and Their Significance

Members of the Gram-positive *Bacillales* and *Clostridiales* and some closely related genera respond to slowed growth or starvation by initiating the process of sporulation, and the resultant spores can cause practical problems in food microbiology as well as human disease. The molecular biology of sporulation and spore resistance and germination in *Bacillus subtilis* has been extensively studied for many years, and there is detailed knowledge of these processes and many of the regulatory mechanisms involved (1–12). With the recent availability of many genome sequences as well as methods for genetic manipulation of clostridia of industrial and medical importance, a detailed molecular understanding of sporulation and spore germination in clostridia is also being developed (4, 9, 13–17). This chapter describes the fundamental bases of sporulation and spore germination and resistance and the problems that spores present to the food industry.

SPOREFORMER PHYLOGENY AND SPORULATION AND GERMINATION OVERVEWS

The primary sporeformers of significance in foods are *Bacillus*, *Clostridium*, *Anoxybacillus*, *Desulfotomaculum*, *Geobacillus*, *Paenibacillus*, and *Sporolactobacillus*. These members of the *Bacillales* and *Clostridiales* have undergone extensive phylogenetic analysis, largely based on genome sequencing (17–19), which provides insight into their evolution and physiological

characteristics. Overall, the sporeformers have DNA with a low G_+C content and possess a Gram-positive cell wall structure. Other systematic, species, and physiological characteristics of these organisms have been described in detail (20). It appears likely that the ability to sporulate was acquired early in evolution, and a core of ~60 genes is associated with sporulation. Interestingly, food-related bacteria classified in the *Firmicutes* include *Listeria*, *Staphylococcus*, *Streptococcus*, and *Lactobacillus*, and these genera have lost the ability to sporulate at variable times in evolution (17). Despite the ability of sporeformers to remain in a dormant state for long periods of time, their rate of evolution appears to be the same as that of non-spore-forming bacteria (21).

Sporulation in *B. subtilis* has been most extensively studied and forms the basis of most descriptions in this chapter. The first obvious morphological event in sporulation is an unequal cell division. This creates the smaller prespore or forespore compartment and the larger mother cell compartment. As sporulation proceeds, the mother cell engulfs the forespore, resulting in a single forespore (an endospore) within a mother cell, and eventually the mother cell lyses. Note that while generation of one spore in a sporulating cell is common, there are a number of examples of sporulating cells that form multiple spores in a single cell (18). Throughout sporulation, gene expression is ordered both temporally and spatially, as some genes are expressed only in the mother cell or the forespore (2, 4–7, 9, 14, 22). The pattern of gene expression during sporulation is controlled by the ordered synthesis and/or activation of five new sigma (specificity) factors for RNA polymerase and many DNA-binding proteins. As sporulation proceeds, there are striking morphological and biochemical changes in the developing spore. It becomes encased in two and sometimes three novel layers: a large layer of peptidoglycan (PG) termed the spore cortex, whose structure differs from that of growing-cell PG, a number of spore coat layers, and in some species an exosporium. Both the coats and exosporium contain proteins unique to spores (23, 24). The spore's central region, or core, accumulates a huge

depot (~10% of spore dry weight) of pyridine-2,6-dicarboxylic acid (dipicolinic acid [DPA]) (Fig. 2.1), as well as a large number of divalent cations, including Ca^{2+}chelated with DPA (CaDPA), and the core loses much water (25). In addition, the developing forespore synthesizes large amounts of novel small acid-soluble proteins (SASP), some of which coat the spore chromosome and protect the DNA from damage (8, 12, 14). As a result of these and other changes, the spore becomes metabolically dormant and resistant to harsh conditions, including heat, radiation, and chemicals.

Despite the spore's dormancy, if given the appropriate stimulus (often a sugar or amino acid), the spore can return to life via germination (4, 9–11). Within minutes of exposure to a germinant, spores lose their unique characteristics, including loss of CaDPA by excretion, loss of the cortex and SASP by degradation, much core water uptake and swelling, and loss of resistance. Completion of germination allows progression into outgrowth, when normal metabolism of endogenous and exogenous compounds begins and macromolecular synthesis is initiated. Eventually the outgrowing spore is converted back into a growing cell. Detailed study of sporulation, spores, and spore germination and outgrowth has been motivated by a number of factors, one of which is the attraction of this developmental system. Another motivating factor is the major role played by sporeformers in food spoilage and foodborne diseases; related to this factor is the recognition of the potential for the use of spores of *Bacillus anthracis* as an agent of bioterrorism. This review focuses on molecular mechanisms of sporulation, spore resistance, and germination, most of which have been examined in *B. subtilis*, the model sporeformer for mechanistic studies on sporulation, spore germination, and spore resistance. However, there is increasing knowledge of many aspects of the sporulation and spore germination in many *Clostridium* species, most notably, *Clostridium perfringens*, *Clostridium sporogenes*, and *Clostridioides difficile* (formerly *Clostridium difficile*) (4, 9–11, 13–15). The completed genome sequences of many hundreds of Gram-positive spore-forming species are now available, and comparison of

these sequences indicates that there is a tremendous degree of conservation among genes whose products are involved in sporulation, spore resistance, and spore germination, most notably a core set of ~60 conserved genes (17–19). This conservation extends to many similar mechanisms, including regulatory mechanisms, involved in sporulation, spore resistance, and spore germination in these various species, although there are important differences in both sporulation and germination in members of the *Bacillales* and *Clostridiales* (4–7, 9–11, 13–18).

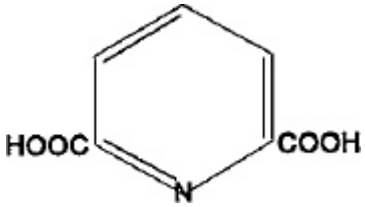


Figure 2.1 Structure of DPA. Note that at physiological pH, both carboxyl groups are ionized.

SPORULATION

Distribution of Sporeformers

The sporulating bacteria discussed in this chapter form heat-resistant endospores that contain DPA and are refractile or phase bright under phase-contrast microscopy. Most studies on sporulation, spores, and spore germination utilize species of either the aerobic bacilli or the anaerobic clostridia. However, members of many closely related genera form similar spores (17, 18).

Induction of Sporulation

Induction of sporulation in the laboratory is generally achieved by nutrient limitation, either by exhaustion of nutrients during growth or by shifting cells from a rich to a poor medium. Addition of an inhibitor (decoyinine) of guanine nucleotide biosynthesis also leads to sporulation. Although these methods cause most cells in a culture to sporulate, this may not be how sporulation is induced in

nature. Even cells in a growing culture produce a small but finite number of spores, with the percentage of spores increasing as the culture's growth rate decreases (26).

Massive sporulation of a cell population generally takes place only when cells enter stationary phase. However, sporulation is not an obligatory outcome of entry into stationary phase, and many stationary-phase events are attempts to access new sources of nutrients such that sporulation either will not take place or is at least delayed. These stationary-phase events include the following (Fig. 2.2) (1, 2, 5–7, 13, 15, 22, 27): (i) synthesis and secretion of degradative enzymes such as amylases and proteases; (ii) synthesis and secretion of antibiotics such as gramicidin and bacitracin; (iii) in some species, synthesis and release of protein toxins; (iv) development of motility; (v) killing and cannibalism of sister cells in the population; (vi) in a few species (e.g., *B. subtilis*), development of genetic competence; and (vii) biofilm development. Although these phenomena are not necessary for sporulation, they are often regulated by mechanisms that modulate gene expression during sporulation. Like sporulation, competence is also regulated in a cell density-dependent manner via the secretion of small peptides termed competence pheromones. There are also drastic changes in stationary-phase cell metabolism, with these changes extending into sporulation. Some of these changes include catabolism of polymers like poly-β-hydroxybutyrate formed in vegetative growth and initiation of oxidative metabolism due to synthesis of tricarboxylic acid (TCA) cycle enzymes. Many of these enzymes are not present in the developing forespore. Consequently, the mother cell and forespore have different metabolic capabilities.

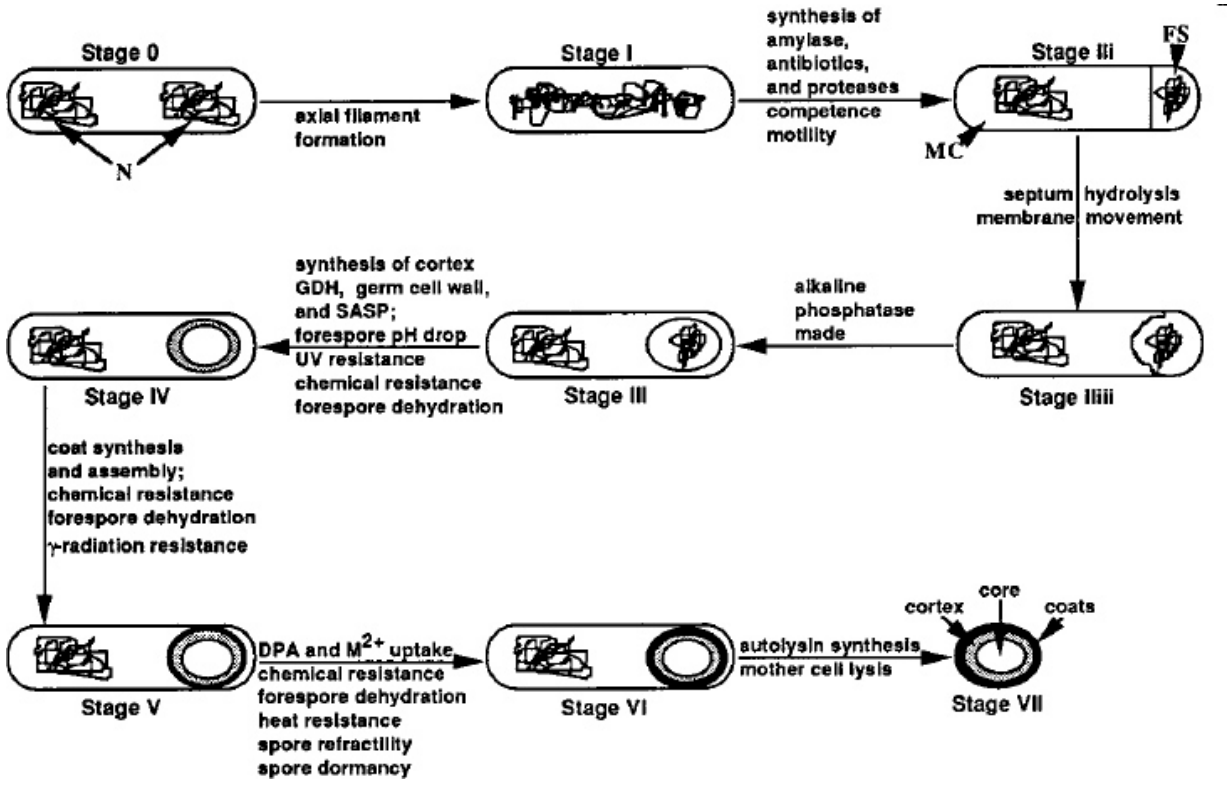


Figure 2.2 Morphological, biochemical, and physiological changes during sporulation of a rod-shaped *Bacillus* cell. In stage 0, a cell with two nucleoids (N) is shown; in stage IIi, the mother cell and forespore are designated MC and FS, respectively. Note that the forespore nucleoid is more condensed than that in the mother cell. Stage IIii is not shown in this scheme, and the forespore nucleoid is not shown after stage III for clarity. The time of some biochemical and physiological events, such as forespore dehydration and acquisition of types of resistance to different chemicals (alllumped together as "chemical resistance"), stretches over a number of stages.

Progression into sporulation generally requires regulatory signals that lead to the derepression of genes expressed only in stationary phase, although usually not the products of genes encoding degradative enzymes, antibiotics, toxins, and proteins involved in motility or competence. However, induction of sporulation in *Bacillus* species does require completion of chromosome replication, repair of DNA damage, and induction or synthesis of

TCA cycle enzymes. Entry into sporulation is also increased in cells growing at high cell density by small molecules secreted into the growth medium (28). In general, extracellular signals are extremely important in directing decisions about the immediate and ultimate fates of stationary-phase cells of sporeformers, as these cells have a variety of developmental pathways available, as noted above, and most of these pathways are not mutually exclusive (1, 5–7, 28).

Morphological, Biochemical, and Physiological Changes During Sporulation

In *B. subtilis*, sporulation can take as little as 8 h and is divided into eight stages based largely on morphological characteristics (Fig. 2.2). Growing cells are in stage 0, and in stage I, the sporulating cell's two nucleoids align in an axial filament that can be observed in electron micrographs. The first morphological feature of sporulation easily seen in the light microscope is the formation of an asymmetric septum dividing the sporulating cell into the mother cell and forespore compartments (3, 5–7). Immediately after asymmetric septation, only ca. 30% of the chromosome destined for the spore is present in the forespore; the remainder is subsequently transferred into the forespore, and then both mother cell and forespore compartments contain complete and apparently identical single chromosomes. However, the forespore chromosome is initially more condensed than the mother cell chromosome. A biochemical marker for late stage II is the synthesis of alkaline phosphatase. During stage II, genes in the two compartments of the sporulating cell may be expressed differentially.

Following septum formation, the mother cell plasma membrane engulfs the forespore, surrounding the forespore with two complete membranes, the inner and outer forespore (or spore) membranes (IM and OM). There are a number of events that occur in the transition from stage II to stage III, leading to the subdivision of stage II into three substages, two of which are shown in Fig. 2.2. In the transition from stage III to stage IV, a large PG structure termed

the cortex is laid down between the IM and OM (29). The cortex PG has a structure similar to that of cell wall PG, but with a number of differences (described below). The spore's germ cell wall is made at the same time as the cortex but appears to have the same structure as growing cell wall PG. During the transition from stage III to stage IV, the forespore synthesizes glucose dehydrogenase and SASP. The developing forespore acquires full UV resistance and some chemical resistance at this time, the forespore pH decreases by 1 to 1.5 units, and forespore dehydration begins.

In the transition from stage IV to stage V, the proteinaceous coat layers are laid down outside the OM (23), in a process that requires a protein made in the mother cell to recognize and bind to the curved engulfed forespore's OM (30). The coats of spores of *Bacillus* species contain up to ~80 proteins, almost all of which are unique to spores. The reason for the plethora of coat proteins is not clear, as many *B. subtilis* coat proteins can be lost with no apparent phenotypic effect. Forespore gamma radiation resistance begins to be acquired during this period, as is further chemical resistance, and forespore dehydration continues (8, 12). During the stage V-stage VI transition, the spore core's depot of DPA is accumulated following DPA synthesis in the mother cell. CaDPA is taken into the spore core by a SpoVV transporter in the OM and a SpoVA channel in the IM (4, 31); significant amounts of Mg^{2+} and Mn^{2+} are also taken up, almost all in the spore core (25). The precise state of CaDPA in the spore core is not known, although the amount in spores exceeds its solubility. During this period, the spore's central region or core undergoes the final process of dehydration. Because of the high ratio of solids to water in the spore core at this stage, the spore appears bright in phase-contrast microscopy. It has been suggested that the spore core is in a glass-like state, but this suggestion now appears incorrect (32–34). Because of permeability changes in the spore membranes, most likely the IM, the spore at this stage stains poorly, if at all, with common bacteriological stains, in particular nucleic acid stains. The spore also becomes metabolically dormant during this period and acquires further gamma radiation and

chemical resistance (8, 12). Finally, in the transition to stage VII, autolysins are produced in the mother cell, resulting in its lysis and release of the spore.

The eight stages are particularly useful in characterizing various asporogenous or *spo* mutants that have no defect in growth but are blocked in a particular stage in sporulation. These *spo* mutants are given an added designation denoting the stage in which they are blocked. Hence, *spo0* and *spoII* mutants are blocked in stages 0 and II, respectively. The various stages have also allowed correlation of biochemical changes with morphological changes (Fig. 2.2). However, the sporulation scheme outlined above is an oversimplification for several reasons. First, since the various stages are intermediates in a continuous developmental process, it is probably misleading to think of the stages as discrete entities. Second, the scheme is only for rod-shaped bacteria that sporulate without terminal swelling of the forespore. There are many sporeformers in which the forespore compartment swells considerably and the mother cell elongates, such as the toxigenic clostridia *Clostridium botulinum* and *Clostridium tetani* (13). Some sporeformers (e.g., *Sporosarcina* species) grow as cocci, and their sporulation septum is placed symmetrically; other quite different sporulation patterns have also been observed (13, 18, 19).

Regulation of Gene Expression during Sporulation

Much of our knowledge of regulation of gene expression during sporulation has been derived from analysis of *spo* mutants in *B. subtilis*, and asporogeny can be caused by mutations in >75 genes (2, 4–7). The identification of biochemical or physiological markers for the sporulation stages provides another aid to understanding gene regulation during sporulation (Fig. 2.2). Analysis of these markers in *spo* mutants indicates that sporulation is primarily a linear sequence of events, although development in the two cell types must be coordinated. In general, *spo0* mutants exhibit no sporulation-specific markers, *spoII* mutants exhibit only stage 0-specific events, etc.

While analysis of *spo* mutants has furnished a broad outline of regulation of gene expression during sporulation, molecular genetics has provided a detailed understanding of this process. Sporulation requires transcription of many new genes, as has been elegantly demonstrated by microarray and "-omics" technologies (2, 5–7). These changes in transcription during sporulation are directed in large part by changes in the specificity of the cell's RNA (E) polymerase due to synthesis of five new σ factors that have promoter specificities distinct from that of the main housekeeping sigma factor, σ^A. The sporulation-specific sigma factors direct transcription in the predivisional cell (σ^H), the mother cell compartment (σ^E followed by σ^K), and the forespore compartment (σ^F followed by σ^G). In addition, changes in sporulation-specific gene expression require modulation of transcription by (i) activation or inactivation of factors by covalent and noncovalent modification, (ii) regulatory communication to coordinate mother cell and forespore development, (iii) synthesis of DNA binding proteins to activate or repress transcription, (iv) degradation of DNA binding proteins and σ factors, (v) phosphorylation of serine/threonine residues by protein kinases (35), and (vi) synthesis of some small noncoding RNAs. This information has provided a detailed picture of the control of gene expression during sporulation that is striking not only in its complexity but also in the redundancy of its control mechanisms. The following discussion of these control mechanisms is simplified and concentrates on major regulatory gene products.

Initiation of *B. subtilis* sporulation requires expression of *spo0* gene products in vegetative cells (1, 2, 5–7, 22). The most important of these *spo0* gene products is Spo0A, the response regulator half of a two-component regulatory system. These signal transduction systems transmit signals, often by binding to DNA and affecting transcription, when an aspartyl residue in the response regulator is phosphorylated by a sensor kinase. In growing cells, Spo0A is primarily in the dephosphorylated state. However, under conditions that initiate sporulation, the level of Spo0A rises, as does its degree

of phosphorylation, the latter through the action of multiple sensor kinases. In *B. subtilis*, the majority of phosphate on Spo0A is derived via a phosphorelay initiated by phosphorylation of Spo0F (Fig. 2.3 and 2.4), and phosphate is then transferred from phosphorylated Spo0F (Spo0F~P) to a Spo0A by Spo0B. Note that this phosphorelay appears to be absent in at least some members of the *Clostridiales* (13, 14). In *Bacillus* species, there are two major kinases, KinA and KinB, as well other minor kinases that initiate the phosphorelay. Mutations in either *kinA* or *kinB* have little or no effect on sporulation, but a *kinA kinB* double mutant is asporogenous.

In addition to the kinases, there are many phosphatases that can dephosphorylate Spo0A~P, either directly or through dephosphorylation of Spo0F~P (Fig. 2.3). This multiplicity of kinases and phosphatases allows a variety of environmental signals to be integrated to determine intracellular levels of Spo0A~P, and a threshold concentration of Spo0A~P is needed to initiate sporulation by modulating the expression of ~100 genes (1, 2, 5–7, 22). A number of regulators of the kinases and phosphatases that modulate Spo0A~P levels have been identified, and overexpression of kinases that generate Spo0A~P triggers sporulation in growing cells. Not all cells in a population choose to sporulate, and the major factor influencing this decision is the Spo0A~P level, which is quite heterogeneous between individual cells in sporulating populations, and stochasticity in the overall phosphorelay appears to play a major role in the heterogeneity in Spo0A~P levels (1, 2, 27).

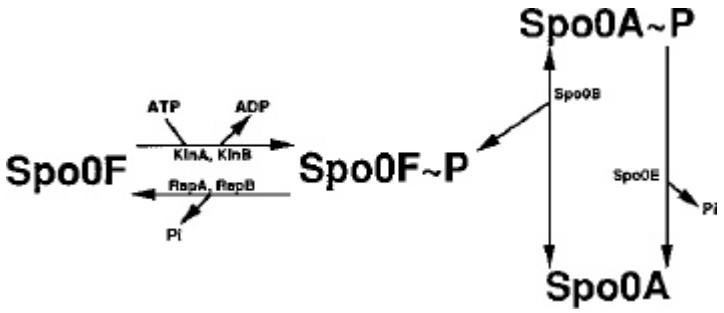


Figure 2.3 Some gene products and reactions that affect levels of Spo0A~P. Spo0E is a phosphatase that acts on Spo0A~P; RapA and RapB are phosphatases that act on Spo0F~P (6).

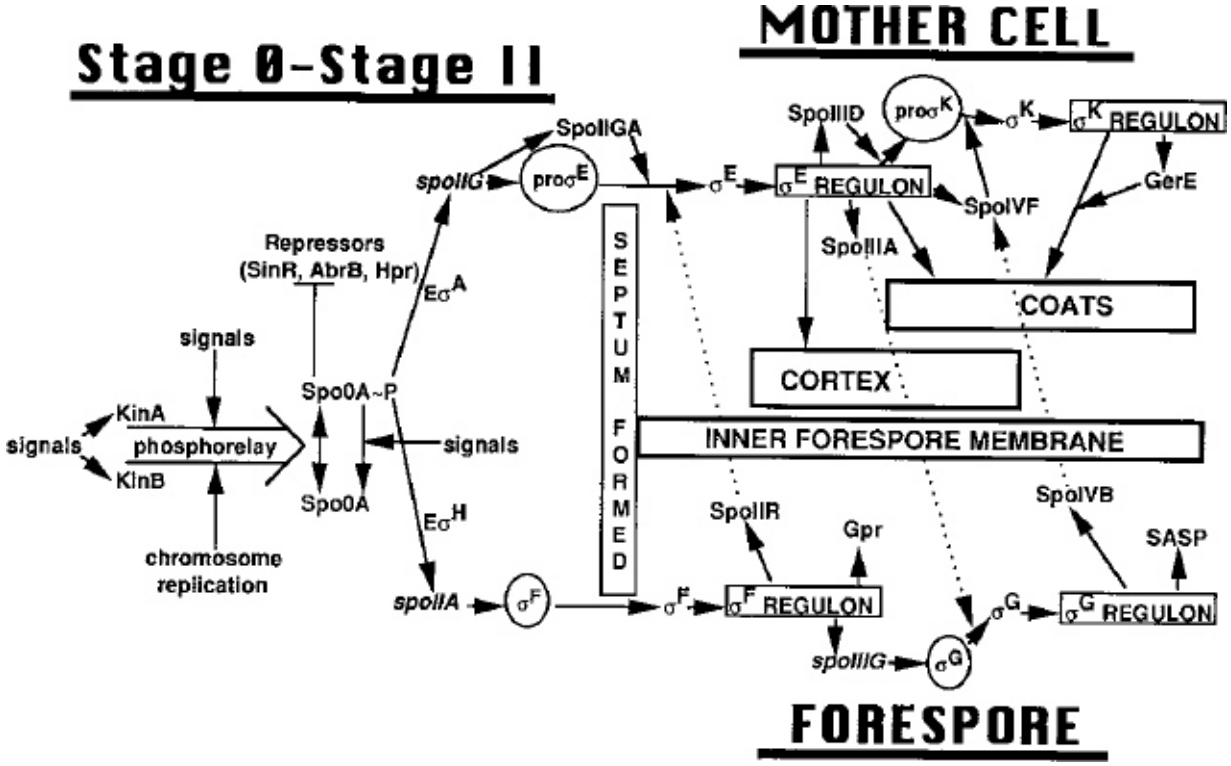


Figure 2.4 Regulation of gene expression during sporulation. The effect of Spo0A~P on repressors is negative; other effects of regulatory molecules on reactions are generally positive, although the effect of signals may be positive or negative. The enclosure of the pro-σfactors and σ factors indicates that at this time, these factors are inactive.

The phosphorylation of Spo0A increases its affinity for binding to sites upstream of several key genes, although different sites have

different affinities for Spo0A~P. The *abrB* gene has a high affinity for Spo0A~P, and binding of Spo0A~P decreases *abrB* transcription. Since AbrB is labile, a decrease in *abrB* transcription rapidly decreases intracellular AbrB concentration. AbrB is a repressor of a number of genes normally expressed in the stage 0-stage II transition, including *spo0H*, which encodes σ^H. AbrB is also an activator for synthesis of a second repressor, Hpr, that represses additional stage 0-expressed genes, especially genes for several proteases. In addition to AbrB and Hpr, there is a third repressor, SinR, that represses other genes expressed in the stage 0-stage II transition, including those of the *spoIIG* and *spoIIA* operons. SinR action is blocked by synthesis of a protein termed SinI that binds to SinR and blocks its action. Synthesis of SinI is stimulated by Spo0A~P and probably repressed by AbrB and Hpr.

There is also a fourth repressor, CodY, that represses many genes whose expression is needed early in stationary phase/sporulation, including genes whose products are involved in competence, motility, and the TCA cycle (36). CodY also represses genes required for sporulation, including *spo0A*. The repressor function of CodY requires branched-chain amino acids and GTP, and the latter effect likely explains why decoyinine, an inhibitor of guanine nucleotide biosynthesis, can induce sporulation in growing cells. The consequences of this regulation are that early in the stage 0-stage II transition, many genes normally repressed during vegetative growth are derepressed through a decrease in GTP levels and an increase in the level of Spo0A~P (Fig. 2.4). Among these stage 0-to-stage II genes is *spo0H*, encoding σ^H and early in sporulation, there is an increase in $E\sigma^H$ transcription. As Spo0A~P levels increase, concentrations become sufficient to bind the promoters of the *spoIIG* and *spoIIA* operons from which SinR has been removed, triggering the transcription of these two operons.

The *spoIIA* operon is transcribed by $E\sigma^H$ and encodes three proteins, with the third cistron (*spoIIAC*) encoding another sigma factor, termed σ^F The *spoIIAB* gene encodes an inhibitor of σ^F

function, whereas *spol IAA* encodes a SpoIIAB antagonist; SpoIIAB is also a protein kinase that can phosphorylate SpoIIAA. Prior to septation, σ^F is inactive in the sporulating cell due to its interaction with SpoIIAB, and interaction of SpoIIAB with SpoIIAA is blocked by phosphorylation of SpoIIAA by SpoIIAB.

The *spoIIG* operon is transcribed by $E\sigma^H$ and encodes two proteins, with the second cistron, *spoIIGB*, encoding another σ factor for RNA polymerase, σ^E Unlike σ^F and σ^H, σ^E is synthesized as an inactive precursor termed pro-σ^E The first gene of the *spoIIG* operon (*spoIIGA*) is the protease responsible for processing pro-σ^E to σ^E. Action of SpoIIGA on pro-σ^E requires gene expression under σ^F control in the forespore (Fig. 2.4). Although *spoIIG* is transcribed before septum formation, σ^E is not generated until after septation and then only in the mother cell. σ^F is also produced before septum formation and is maintained in an inactive state by interaction with SpoIIAB as noted above. However, following septation, σ^F becomes active in the forespore, as SpoIIAB leaves σ^F and interacts with SpoIIAA. This process is promoted largely by dephosphorylation of SpoIIAA~P by the SpoIIE phosphatase that resides in the sporulation septum (3, 5–7).

$E\sigma^F$ -transcribed genes include *rsfA*, *spoIIR*, *gpr*, and *spoIIIG* (2) (Fig. 2.4). RsfA is a DNA-binding protein that modifies the specificity of $E\sigma^F$ SpoIIR promotes pro-σ^E processing by SpoIIGA in the mother cell shortly after septation, and this requirement for *spoIIR* transcription in the forespore for pro-σ^E processing in the mother cell is the first example of cross talk between the two compartments of the sporulating cell that coordinate mother cell and forespore development. The *gpr* gene encodes a protease that acts on SASP in the first minute of spore outgrowth, and *spoIIIG* encodes another σ factor, termed σ^G, that is responsible for the bulk of forespore-specific transcription (2, 5–7). However, σ^G is not active following its synthesis in stage III, at least in part because of the product of the σ^F-controlled *csfB* gene.

Ultimately, mother cell-specific events, including transcription of the *spoIIIA* operon by $E\sigma^E$, are required for generation of active $E\sigma^G$ (Fig. 2.3 and 2.4). At least SpoIIIA proteins as well as SpoIIQ are essential components of a channel termed a feeding tube between the mother cell and forespore compartments, and although the molecules that pass through this channel have not been definitively identified, at least some are likely to be nutrients (3, 37). This is the second example of cross talk between mother cell and forespore, although the precise mechanism for the regulation of σ^G activity in the forespore and the identities of the molecules that pass through the feeding tube are not clear. The generation of σ^F only in the mother cell and the activation of σ^F and synthesis of σ^G only in the forespore now establish compartment-specific transcription during sporulation. As noted above, EG transcribes a number of genes expressed only in the forespore. As the third example of regulatory cross talk, one such gene (*spoIVB*) is responsible for communicating with the mother cell and coordinating gene expression in this compartment (Fig. 2.4; see below). The *ssp* genes are a large set of genes transcribed by $E\sigma^G$. These genes encode the SASP that are major protein components of the spore core (see below) (2, 8, 10–12). Transcription of genes by $E\sigma^G$ is modulated by the DNA binding protein SpoVT, and the *spoVT* gene is transcribed by $E\sigma^G$

In contrast to $E\sigma^G$, $E\sigma^E$ transcribes genes only in the mother cell, including genes needed for cortex biosynthesis and some genes (*cot* genes) needed for coat formation and assembly (2, 5–7, 23, 29). $E\sigma^E$ also transcribes *gerR* and *spoIIID*, which encode DNA-binding proteins that modulate $E\sigma^E$ action, resulting in transcription of different classes of $E\sigma^E$ -dependent genes. One such $E\sigma^E$ dependent gene that requires SpoIIID for transcription is *sigK*, which encodes a final new σ factor, termed. σ^K In some *B. subtilis* strains, the *sigK* gene has an intervening sequence that is removed only from the mother cell genome by a recombinase. Expression of the recombinase is regulated such that generation of an intact *sigK* gene

precedes *sigK* transcription. However, this intervening sequence is absent in the *sigK* genes of many other sporeformers (2, 5–7, 13). As is the case with σ^E, σ^K is synthesized as inactive pro-σ^K and is processed proteolytically about 1 hour after its synthesis. Conversion of pro-σ^k to σ^K in the mother cell requires expression of a gene (*spoIVB*) in the forespore (the third example of cross talk) and participation of the σ^E controlled *spoIVF* operon in the mother cell (Fig. 2.4). SpoIVFB is the protease that processes pro-σ^k, and SpoIVFA is an inhibitor of SpoIVFB function. Eσ^K also transcribes *sigK* (in conjunction with SpoIIID), the gene for DPA synthase, other *cot* genes, and *gerE*, which encodes a DNA-binding protein that modulates Eσ^K specificity (Fig. 2.4). Most knowledge of gene expression during sporulation ends at this point, including information about genes that may be required for mother cell lysis. However, the gene for one autolytic enzyme (CwlC) is transcribed by Eσ^K.

The preceding picture of regulation of gene expression during sporulation is derived predominantly from detailed studies with *B. subtilis*. However, as noted above, genomic sequence data indicate that many key sporulation regulatory genes, such as *spo0A*, *spoIIA* to *-C*, *spoIIGA* and *-B*, *spoIIIG*, *sigK*, etc., are present in other sporeformers, including *Clostridium* spp. (13–19). The conservation of the sequences of the shared proteins and the organization of these genes strongly suggest that regulation of sporulation across *Bacillus* and *Clostridium* species is relatively similar, and this has generally been determined to be the case, although details often differ.

THE SPORE

Spore Structure

The structure of the dormant spore is very different from that of a growing cell (Fig. 2.5), as some spore structures, including the

exosporium and coats, have no counterparts in growing cells. The outermost spore layer, the exosporium, varies significantly in size between species, being prominent in spores of *Bacillus cereus*, *B. anthracis*, *Bacillus thuringiensis*, and some members of the *Clostridiales*, and may not be present in spores of other species, such as *B. subtilis* (23, 24). This structure can exclude large molecules such as antibodies, can influence spore surface adhesion, and may play some role in spore pathogenesis. The exosporium is composed of lipid, carbohydrate, and protein, including glycoprotein, and the proteins are unique to spores.

Under the exosporium are the spore coats. In *B. subtilis* spores, a number of distinct coat layers are seen in electron micrographs. The coats are composed primarily of protein, with ~80 different proteins in *B. subtilis* spore coats, almost all of which are unique to spores. Some coat proteins are essential for assembly of the multilayered coat structure, but specific roles for many individual coat proteins are not known. Coat proteins for which roles are known or predicted include the following (2, 4, 8, 9, 12, 23): (i) proteins essential for assembly of another protein in the coat; (ii) enzymes in the coat that can detoxify reactive oxidative oxygen species; (iii) proteins that appear to facilitate nutrient germinant access to nutrient germinant receptors (nGRs); (iv) enzymes that can inactivate possible germinants; and (v) enzymes that can generate protein-protein cross-links in coat proteins. These cross-links include dityrosine and γ-glutamyllysine cross-links and may be important in spore coat structure. Spore coats also protect spore PG from attack by lytic enzymes, including those in many eukaryotic bacterivores, and the spore's inner layers against many chemicals. Spore coats can also play minor roles in UV and wet-heat resistance. Underlying the coats is the OM, a functional membrane in the developing forespore, but its functionality in the dormant spore is not clear (12, 38). The protein composition of the OM is different from that of the IM.

Underlying the OM is the cortex. Cortical PG is structurally similar to cell wall PG, but with several differences (29): cortical PG

contains diaminopimelic acid even if vegetative-cell PG contains lysine, and many of the muramic acid residues in cortical PG lack peptide residues. Although some muramic acid residues contain a single d-alanine, or in some species a dipeptide, much is present as muramic acid–δ-lactam (MAL), a compound not present in growing-cell PG. A number of genes involved in cortex synthesis have been identified and characterized, and the pathway for MAL synthesis during sporulation is understood. The timing of cortex biosynthesis and cross-linking during sporulation has been determined, and cortex PG is generally less cross-linked than growing-cell PG, although the precise distribution of crosslinks throughout the cortex is not known. The exact function of cortex PG cross-linking is also not known, but it likely plays roles in spore germination and resistance. Between the cortex and the IM is the germ cell wall, and germ cell wall PG appears to be identical to vegetative-cell PG.

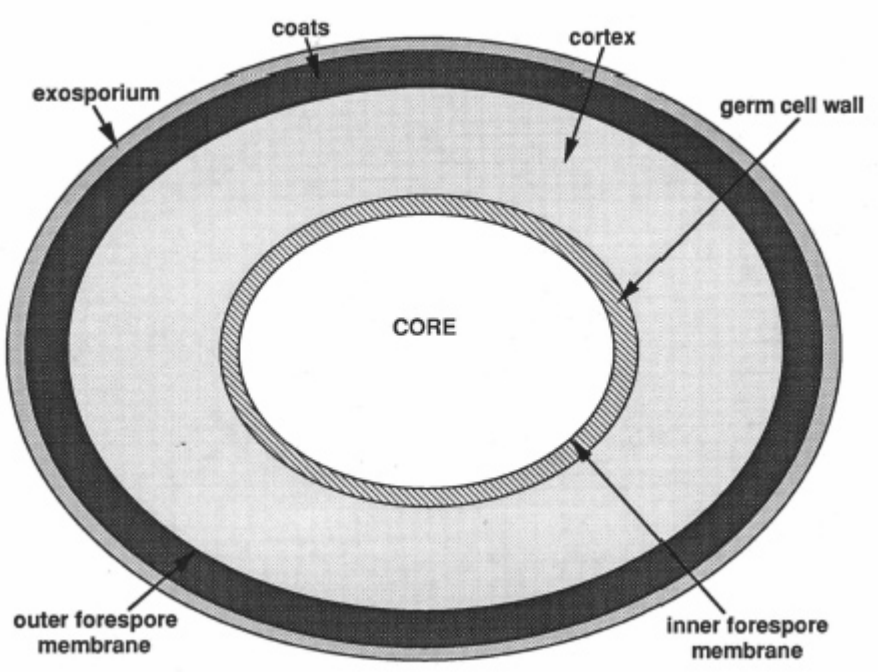


Figure 2.5 Structure of a dormant spore. The various structures are not drawn precisely to scale, especially the exosporium, whose size varies tremendously between spores of different species. The germ cell wall is also generally smaller than shown. The positions of the inner and outer forespore membranes, between the core and the germ cell wall and between the cortex and coats, respectively, are also noted.

The next structure, the IM, is a functional membrane and an extremely strong permeability barrier slowing the entry of almost all molecules, including perhaps even water, into the spore core (8, 12, 38). A lipid probe in the IM is largely immobile, suggesting that this membrane has a relatively “frozen” structure (8, 12, 39). However, the IM’s phospholipid content is similar to that of growing cells. The volume surrounded by the inner forespore membrane appears smaller than expected based on this membrane’s phospholipid content. However, the core’s volume expands significantly upon completion of spore germination and in the absence of membrane synthesis (39). A lipid probe in the IM also becomes fully mobile at this time. Notably, a number of crucial germination proteins are in the IM (see below).

Finally, the central region, or core, contains the spore's DNA, ribosomes, and most enzymes, as well as CaDPA and most other divalent cations. There are also unique gene products in the dormant spore, including the large SASP pool (ca. 10% of spore protein), much of which is bound to spore DNA (8, 12; also, see below). A notable feature of the spore core is its low water content (25). While vegetative cells have ca. 4 g of water per g of dry weight, the spore core has only 0.4 to 1 g of water per g of dry weight. The core's low water content likely plays a major role in spore dormancy and in spore resistance to a variety of agents. The core's low water content is also likely the reason for the immobility of ions and protein in the spore core (40). In contrast to the low water content in the spore core, the water content in other regions of the spore is similar to that in growing cells.

Spore Macromolecules

Some proteins in the spore core and outer layers are not present in growing cells. The SASP, SpoVV, SpoVA, nGR proteins, and cortex-lytic enzymes (CLEs) (see below) are particularly noteworthy, as some of these proteins play major roles in spore resistance and germination (4, 8–12, 31). SASP and SpoVA proteins are synthesized in the forespore during stage III of sporulation, when their coding genes are transcribed primarily by $E\sigma^G$, and are located in the spore core. There are two kinds of major SASP in *Bacillus* spp., the γtype and the α/βtype. The 75-to 105-residue γ-type SASP comprise ca. 5% of spore protein and do not bind other spore macromolecules. Their only known function is to be degraded during outgrowth by GPR, providing amino acids for metabolism and protein synthesis. γ-type SASP are encoded by a single gene in *Bacillus* spp. but are not present in spores of *Clostridium* species or in members of one clade in the *Bacillales* (41). The sequences of γ-type SASP are not homologous to those of other proteins, and these proteins' sequences have diverged significantly during evolution.

The α/β-type SASP make up 3 to 5% of total spore protein and are in all spore-forming *Bacillales* and *Clostridiales* species that have been studied (8, 12, 14, 17, 18). In contrast to γ-type SASP, α/β-type SASP are encoded by up to seven genes, all of which are expressed in parallel, although in some species two proteins are expressed at high levels and the remainder at lower levels. However, all α/β-type SASP have similar properties *in vitro* and *in vivo* (see below). The amino acid sequences of these small (60-to 75-residue) proteins are highly conserved both within and across species, although they too have no sequence homology to other proteins. The α/β-type SASP are also degraded to amino acids in the first minutes of spore outgrowth in a germination protease (GPR)-initiated process.

The α/β-type SASP are DNA-binding proteins, both *in vivo* and *in vitro* (8, 12, 14). *In vivo*, these proteins saturate the spore chromosome and provide much of the spore DNA's resistance to various treatments (see below). Binding of α/β-type SASP to DNA is not sequence specific but exhibits a preference for GC-rich regions, although AT-rich regions are bound. The proteins bind to the outside of the DNA helix, interacting primarily with the sugar phosphate backbone. This binding alters DNA structure, provides resistance to chemical and enzymatic cleavage of the DNA backbone, alters the DNA's UV photochemistry, and greatly slows DNA depurination (see below). A number of proteins present in vegetative cells are absent from *Bacillus* spores. These include amino acid and nucleotide biosynthetic enzymes that are degraded during sporulation and resynthesized during spore outgrowth (11). Other proteins, including enzymes of amino acid and carbohydrate catabolism, are present at similar levels in spores and cells, and spores also contain enzymes for RNA and protein synthesis and DNA repair. However, at least one protein needed for initiation of DNA replication may be absent. Enzyme activities common to both cell and spore proteins are almost always due to the same gene product. In general, spore and cell rRNAs and tRNAs are similar if not identical, although much tRNA in spores lacks the 3′-terminal A residue and little spore tRNA is aminoacylated. Spores do contain

some mRNA that has been identified by microarray and -omics technologies (42–44), although the function of this spore mRNA is not clear. Spore DNA appears identical to growing-cell DNA but has a different structure due to different associated proteins, as noted above and below.

Spore Small Molecules

Spores differ from growing cells in their small molecules (Table 2.1), which are located in the core. The small amount of core water and the huge depot of DPA and divalent cations are noted above. The ions in the spore core are immobile, as is at least one normally mobile protein (40), consistent with a dearth of free water in spores. The pH in the spore core is 1 to 1.5 units lower than in a growing cell or the mother cell compartment. Spores of most species accumulate a large depot of 3-phosphoglyceric acid (3PGA) shortly after and in response to the forespore pH decrease (11). However, spores have small amounts of nucleoside triphosphates and reduced numbers of reduced pyridine nucleotides or acyl coenzyme A (CoA), although the "low-energy" forms of these compounds are present. The high-energy forms are lost from the forespore late in sporulation (Fig. 2.2). Much of the CoA in spores is in disulfide linkage, some as a CoA disulfide and some linked to protein. The function of these disulfides is unknown, but they are reduced early in spore outgrowth. Spores also have low levels of most free amino acids but often have high levels of glutamate.

Spore Dormancy

Spores are metabolically dormant, catalyzing minimal metabolism of endogenous or exogenous compounds. However, incubation of dormant spores for extended times at 37 to 50°C or for shorter times at 60 to 70°C does lead to extensive degradation of at least spore rRNA (44, 45). However, this degradation itself does not lead to spore death, as rRNA is resynthesized early in outgrowth. Synthesis of some mRNAs has also been reported in spores incubated for long periods at 37 to 50°C (44), but detectable ATP

levels have not been found in spores incubated in this manner (46). The major Cause of spores' extremely low metabolism is undoubtedly the low water content of the spore core, which precludes protein mobility and enzyme action (25, 40). A reflection of this dormancy is that the spore core contains at least two enzyme-substrate pairs that are stable for years but interact in the early minutes of spore outgrowth, resulting in substrate degradation. These two enzyme-substrate pairs are 3PGA-phosphoglycerate mutase (PGM) and SASP-GPR (11). Although regulatory mechanisms other than dehydration initially stabilize 3PGA and SASP in the developing forespore despite the presence of PGM and GPR, the lack of PGM and GPR action on their substrates in dormant spores over very long periods at low temperatures and even during incubation at 37 to 50°C may be largely due to spore core dehydration.

Table 2.1 Small molecules in cells and spores of *Bacillus* species

Molecule(s)	Content (μ mol/g dry wt)	
	Cells[a]	Spores[b]
ATP	3.6	≤0.005
ADP	1	0.2
AMP	1	1.2–1.3
Deoxynucleotides	0.59[c]	<0.025[d]
NADH	0.35	<0.002[e]
NAD	1.95	0.1P
NADPH	0.52	<0.001[e]
NADP	0.44	0.018[e]
Acyl-CoA	0.6	<0.01[e]
CoASH[f]	0.7	0.26[e]
CoASSX[g]	<0.1	0.54[e]
3PGA	<0.2	5-18
Glutamic acid	38	24-30
DPA	<0.1	410-470
Ca^{2+}		380-916
Mg^{2+}		86-120
Mn^{2+}		27-56
H+[h]	7.6-8.1	6.3-6.9

[a] Values for *B. megaterium* in mid-log phase are from reference 6

[b] Values are the ranges for spores of *B. cereus*, *B. subtilis*, and *B. megaterium* and are from reference 6.

[c] Value is the total for all four deoxynucleoside triphosphates.

[d] Value is the sum for all four deoxynucleotides.

[e] Values are for *B. megaterium* only.

[f] CoASH, free CoA.

[g] CoASSX, CoA in disulfde linkage to CoA or a protein.

[h] Values are expressed as pH and are the ranges for *B. cereus*, *B. megaterium*, and *B. subtilis*.

SPORE RESISTANCE

Spores can survive for extremely long times, and claims have been made for spore survival for millions of years (19). While the latter claims are controversial, it is clear that spores can survive for at least hundreds of years. The spore's metabolic dormancy is undoubtedly one factor in its survival in the absence of nutrients. A second factor is the spore's extreme resistance to potentially lethal treatments, including heat, radiation, chemicals, desiccation, high pressure, and various types of high-energy plasma (8, 12, 14). Representative data on resistance or susceptibility of growing cells and spores of *B. subtilis* to different potentially lethal treatments are shown in Table 2.2. There are, however, some *Bacillales* and *Clostridiales* species which form much more resistant spores than *B. subtilis*. Spore resistance is due to a variety of factors, with core dehydration and α/β-type SASP being involved in many types of resistance, whereas the impermeability of the spore's inner membrane may be involved in only one.

Since different and sometimes multiple factors contribute to different types of spore resistance, it is not surprising that spore resistance to different treatments is acquired at different times in sporulation (Fig. 2.2). In recent years, some mechanisms of spore resistance to heat, UV, and chemicals have been elucidated. The following discussion of spore resistance concentrates on *B. subtilis* spores because of the detailed mechanistic data available for these spores. However, studies with spores of other *Bacillus* species as well as some with *C. perfringens* have found that factors involved in

resistance of *B. subtilis* spores are also involved in resistance of spores of other bacterial genera.

Freezing and Desiccation Resistance

Growing bacteria are killed during freezing and desiccation, unless special precautions are taken. The mechanism of this killing is not clear but may involve DNA damage. In contrast to the sensitivity of growing cells to freezedrying, spores are resistant to multiple exposures to freeze-drying (Table 2.2). The α/β-type SASP are one factor providing spore resistance to freeze-drying by preventing DNA damage (Table 2.2). The spore's DPA is another factor providing spore desiccation resistance, as spores deficient in DPA are more sensitive to desiccation than their DPA-replete counterparts, and killing of DPA-less spores by desiccation is also due to DNA damage (47). However, bacterial spores do not achieve desiccation resistance by accumulating sugars such as trehalose (12).

Pressure Resistance

Spores are more resistant to high pressures (>50 MPa) than are growing vegetative cells (48). Notably, spore killing by high pressure is probably due to initial induction of germination followed by the killing of the much less resistant germinated spores. The most effective way to kill high-pressure-germinated spores is by heat, and hence, high-pressure treatments are often carried out at temperatures that are elevated but not nearly as high as those needed to kill dormant spores. Spore germination induced by pressures of 100 to 350 MPa is due to activation of the spore's nGRs, and this decreases at elevated temperatures. In contrast, germination promoted by pressures of ≥ 450 MPa does not require nGRs but directly activates the SpoVA CaDPA channel, even at elevated temperatures (4, 48). The latter conditions are now used to reduce spore loads in foods or foodstuffs, as even if the high-pressure-germinated spore goes no further in germination than

CaDPA release, CaDPA-less spores are much more heat sensitive than dormant spores.

Gamma Radiation Resistance

Spores are more resistant to gamma radiation and high energy ions than are growing cells, and this resistance is acquired 1 to 2 h before acquisition of heat resistance (8, 12, 49). The α/β-type SASP are one factor contributing to spore gamma radiation and high-energy-ion resistance, as is DNA repair in spore outgrowth, although the precise damage caused by these agents is not known (50). Several mutations that result in increased spore core water also result in slightly lower spore resistance to high-energy ions and gamma radiation.

UV Radiation Resistance

Spores are generally 7 to 50 times more resistant than growing cells to UV radiation at 254 nm, the wavelength giving maximal killing (8, 12) (Table 2.2). Spores are also more resistant than growing cells at longer and shorter UV wavelengths. UV resistance is acquired by the developing forespore ca. 2 hours before acquisition of heat resistance (Fig. 2.2), in parallel with synthesis of α/β-type SASP. The α/β-type SASP are essential for spore UV resistance, but spore coats, cortex, and core dehydration are generally not. However, for some species, pigments in spores' outer layers may provide some UV protection (12, 23). Spore UV resistance is largely due to a different UV photochemistry of DNA in spores and in growing cells. The major photoproducts formed by UV irradiation of growing cells or purified DNA are cyclobutane-type dimers between adjacent pyrimidines. The most abundant of these are between adjacent thymine residues (TT) (Fig. 2.6A), with smaller amounts formed between adjacent cytosine and thymine residues (CT) or adjacent cytosine residues (CC). In addition, UV irradiation of cells or purified DNA generates various 6,4-photoproducts (64PP) also formed between adjacent pyrimidines. All these photoproducts can be lethal as well as mutagenic. In contrast, UV irradiation of spores

generates few cyclobutane-type dimers and 64PP but rather large amounts of a thyminyl-thymine adductinitially termed the spore photoproduct (SP) (Fig. 2.6B). The yield of the SP as a function of UV fluence in spores is similar to yields of TT as a function of UV fluence in growing cells, and SP is a potentially lethal photoproduct. Hence, the difference in UV photochemistry alone between DNA in growing cells and spores is insufficient to explain spore UV resistance, as there must be a difference in the capacity of cells and spores to repair TT and SP. Indeed, spores have two major mechanisms for SP repair, both of which operate in the first minute of spore outgrowth. One mechanism is an excision repair system that repairs TT and other lesions in growing cells. Spores lacking this repair system are 2-to 3-fold more UV sensitive than are wild-type spores. The second repair system, unique to both spores and SP, monomerizes SP to two thymines without excision of the lesion and makes very few errors. Spores lacking this SP-specific repair system are 5-to 10-fold more UV sensitive than are wild-type spores, and those lacking both repair systems are 20-to 40-fold more UV sensitive. SP-specific repair is catalyzed by the SP lyase (Spl), an iron-sulfur protein that uses *S*-adenosylmethionine (SAM) as a cofactor. Spl is a member of the “radical SAM” family of enzymes that use an [Fe-S] center plus SAM to generate a catalytic adenosyl radical. In contrast to enzymes of excision repair that are present in growing cells and spores, Spl is present only in spores.

Table 2.2 Killing and mutagenesis of spores and cells of *B. subtilis* by various treatments[a].

A. Freeze-drying			
		Survival (%)	
No. of freeze-drying cycles	Cells[b]	Wild-type spores	α–β– spores[c]
1 3	2	 100 (<0.5)	 7 (14)
B. 10% Hydrogen peroxide			
		Survival (%)	
Time of treatment (min)	Cells[b]	Wild-type spores	α–β– spores[c]
2.5 5 10 20 60	0.3	92 88 50 6(≤ 0.5)	 50 10(14) 0.1
C. UV[d]			
		Dose to kill 90% of the population (J/m^2)	
	Cells[b]	Wild-type spores	α–β– spores[c]
	40	315	25
D. Moist heat			
		D value	
Treatment temp (°C)	Cells[b]	Wild-type spores	α–β– spores[c]
95 85		14 min (≤0.5) 360 min(≤ 0.5)	 15 min

65 22	< 15s	105h 2.5 yr(≤ 0.5)	(13) 10h 2.8 mo(18)
		D value	
Treatment temp (°C)	Cells[b]	Wild-type spores	α–β–spores[c]
120 90	5min	33 min (12)	5 min

[a] Data are from reference 6. Values in parentheses are the percentages of survivors with asporogenous or auxotrophic mutations when spores undergo 30 to 99% killing.

[b] Cells in log-phase growth. Similar results have been obtained with wild-type and $\alpha^-\beta^-$ cells.

[c] These spores lack the two major α/β-type SASP and thus ca. 80% of the α/β-type SASP pool.

[d] UV irradiation with light predominantly at 254 nm.

The major factor causing the altered UV photochemistry of spore DNA is the saturation of DNA with α/β-type SASP; all α/β-type SASP appear relatively interchangeable in this regard. Spores lacking ca. 80% of these proteins (α–β–spores) are more UV sensitive than are growing cells (Table 2.2), and UV irradiation of α–β– spores generates significant amounts of TT and 64PP and reduced amounts of SP.UV irradiation of DNA saturated *in vitro* with any of a number of purified α/β-type SASP generates SP and minimal TT, CT, CC, or 64PP. However, yields of SP as a function of UV fluence in these complexes are ca. 10-fold lower than yields in spores. This difference is due to the spore's CaDPA, which acts as a photosensitizer. The change in the UV photochemistry of DNA upon binding of α/β-type SASP indicates that the DNA in this complex has an altered structure, and this is a structure between those of A- and B-DNA. During spore outgrowth, α/β-type SASP are degraded to amino acids, a process initiated by GPR (11). Because of the photosensitizing action of CaDPA, spores early in outgrowth are

more UV resistant than are dormant spores, due to CaDPA release prior to SASP degradation. However, as SASP degradation proceeds, UV resistance falls from this elevated level to that of vegetative cells.

Chemical Resistance

Spores are more resistant than growing cells to chemicals, including aldehydes, oxidizing agents (Table 2.2), phenols, chloroform, octanol, alkylating agents such as ethylene oxide, iodine, and detergents, as well as to pH extremes and lytic enzymes such as lysozyme (8, 12, 14). Resistance of spores to these agents is acquired at different times in sporulation (Fig. 2.2). For enzymes such as lysozyme, spores that lack coat protein due to chemical treatment or mutation are sensitive to cortex degradation by lytic enzymes, and coats protect spores against digestion by bacterivores (23). Coat-defective spores are more sensitive to many chemicals, including oxidizing agents, perhaps because coat proteins serve as a "reactive armor" that inactivates toxic chemicals before they reach more-sensitive targets further within the spore. The spore coats of some species also contain enzymes such as catalase and superoxide dismutase that can increase spore resistance to these enzymes' substrates (8, 12). The slow passage of hydrophilic molecules across the spore's IM is also important in spore resistance to chemicals (12, 38). Some chemicals, including formaldehyde, alkylating agents, and nitrite, kill spores at least in part by DNA damage, and the α/β-type SASP are essential in protection against inactivation of *Bacillus* and *Clostridium* spores by these agents. In contrast, many oxidizing agents kill spores not by DNA damage but rather by damaging the spore's IM such that even if the treated spores are germinated artificially, the IM ruptures, leading to spore death.

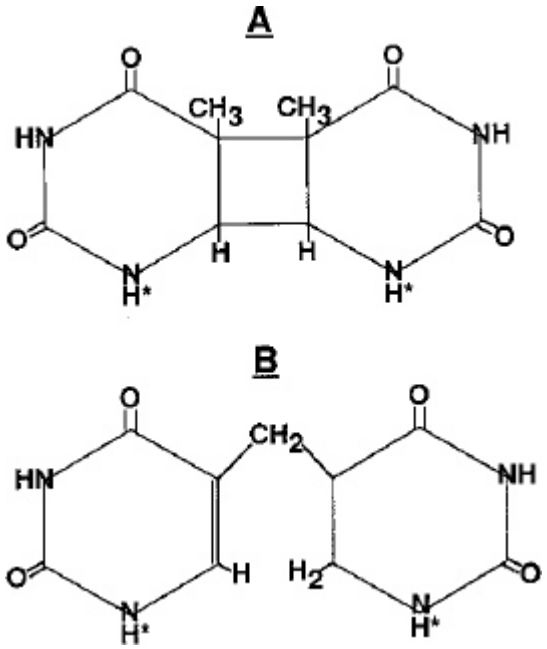


Figure 2.6 Structure of the cyclobutane-type TT dimer (A) and the 5-thyminyl-5,6-dihydrothymine adduct (spore photoproduct) (B). The positions of the hydrogens noted by the asterisks are the locations of the glycosylic bond in DNA

Some detailed information is available about the points raised above for oxidizing agents such as peroxides that kill growing cells by several mechanisms, a major one being generation of hydroxyl radicals that cause DNA damage. However, spore killing by hydrogen peroxide or organic hydroperoxides such as *t*-butylhydroperoxide (tBHP) is not accompanied by significant DNA damage or mutagenesis (Table 2.2) (8, 12). Consequently, DNA in spores must be well protected by these agents. While the spore coat, low core water content, and the low-permeability IM may play some role in resistance to H_2O_2 and tBHP, these factors do not directly protect spore DNA, and this protection is due to saturation of spore DNA with α/β-type SASP, as $\alpha^-\beta^-$ spores are much more sensitive to these peroxides (Table 2.2). In addition, wild-type spores are not appreciably mutagenized by H^2O^2 or tBHP, whereas $\alpha^-\beta^-$ spores are heavily mutagenized (Table 2.2). These results suggest that saturation of DNA with α/β-type SASP provides such good protection against DNA damage that killing of wild-type spores by

such peroxides is by other mechanisms. However, in α/β– spores, the rates of DNA damage caused by peroxides are greatly increased, such that DNA damage is a significant cause of spore death. Supporting this simple model is that spores of wild-type but not α/β– strains acquire one component of H^2O^2 resistance during sporulation in parallel with accumulation of α/β-type SASP. However, α/β-type SASP binding to DNA is not the only factor in spore hydrogen peroxide resistance. Indeed, an additional component of this resistance is acquired during sporulation at the time of final spore core dehydration. This may reflect roles in hydrogen peroxide resistance for spore core dehydration or the low permeability of the spore's IM or the fact that the loss of reduced pyridine nucleotides from the developing spore (which also occurs at this time) decreases production of hydroxyl radicals through the Fenton reaction. Unlike the situation in growing cells, spore core enzymes, such as catalases, alkylhydroperoxide reductases, and superoxide dismutase, and the DNA-binding protein MrgA play no role in spore resistance to peroxides.

Heat Resistance

The extreme resistance of spores to moist heat, the spore resistance most familiar to food microbiologists, has great implications for the food industry and is probably the most studied spore resistance property (8, 12, 25, 51). Spore moist heat resistance is remarkable, as spores of many species can withstand 100°C for many minutes. Heat resistance is often quantified as a *DT* value, which is the time in minutes at temperature *T* needed to kill 90% of a cell or spore population. Generally, *D* values for spores at a temperature of *T* + 40°C are approximately equal to those for their vegetative cell counterparts at temperature *T*. The extended survival of spores at elevated temperatures is paralleled by even longer survival times at lower temperatures, as spore *D* values increase 4-to 10-fold for each 10°C decrease in temperature (25). Consequently, a spore with a *D* value at 90°C (*D*90°C) of 30 min may have a *D*20°C value of many years. The spore cortex appears to play a major role in spore wet

heat resistance, as there is a good correlation between spores' cortex PG level and their wet-heat resistance, but how the cortex exerts its effect is not clear.

Knowledge of the identity of the target(s) that is damaged in spore killing by moist heat is crucial to an understanding of spore moist-heat resistance. This target is not DNA, as moist-heat killing of spores is associated with neither DNA damage nor mutagenesis (Table 2.2). Recent work has indicated that spore killing by moist heat occurs through damage to one or more proteins, although the identity of specific target proteins is not yet known (8, 12). Sublethal heat treatment can also damage spores in some way, with this damage being repairable during spore outgrowth (52). Although the specific damage is not known, it may well again be damage to one or more proteins. Perhaps not surprisingly, moistheat resistance can vary substantially between individual spores in populations (53), although the reason for this has not been elucidated. In contrast to our lack of knowledge about the mechanism(s) of killing spores by heat, there is much more information on factors that modulate spore heat resistance, as discussed below.

Sporulation Temperature

Elevated sporulation temperatures to an optimum increase spore moist-heat resistance (8, 12). Indeed, spores of thermophiles generally have much higher moist-heat resistance than do spores of mesophiles. Since spore proteins are generally identical to cell proteins, spore proteins are not intrinsically heat resistant. Presumably, proteins from thermophiles are more heat stable than those from mesophiles, accounting for the higher moist-heat resistance of spores of thermophiles. However, spores of the same strain are also often more heat resistant when prepared at higher temperatures (Table 2.3). This is probably due to the reduced core water content in spores prepared at higher temperatures (54) (Table 2.3), although how higher sporulation temperatures cause reduced spore core water content is not known. Elevated sporulation

temperatures do not alter spore resistance to dry heat or UV radiation but do increase spore resistance to a number of chemicals. The latter effect may be due to changes in levels of spore coat proteins as a function of the sporulation temperature. In contrast to the situation in growing bacteria, heat shock proteins play no role in spore heat resistance.

α/β-Type SASP

The killing of spores by moist heat is not by DNA damage, as neither general mutagenesis (Table 2.2) nor DNA damage accompanies spore killing, even though high temperatures could cause DNA depurination. Therefore, spore DNA is well protected against moist heat. Protection of DNA in both *Bacillus* and *Clostridium* spores against moist heat damage appears to be due to the saturation of spore DNA by α/β-type SASP (8, 12, 14) and spores of *B. subtilis* have *D* values 5-to 10-fold lower than those of wild-type spores (Tables 2.2 and 2.3). In addition, while moist-heat killing of wild-type spores generates <0.5% mutants in survivors, such killing of $\alpha^-\beta^-$ spores produces up to 18% mutants among survivors (Table 2.2). Killing of $\alpha^-\beta^-$ spores by moist heat is also accompanied by a large amount of DNA damage, including a basic sites, and α/βtype SASP markedly slow rates of DNA depurination in solution.

Table 2.3 Heat resistance of *B. subtilis* spores prepared at different temperatures with different cations and with or without α/β-type SASP[a]

Spore	Prepn temp (°C)	Mineralization	H^2O (g/g of spore core wet wt)	*D*100°C (min)
B. subtilis	50	Native	0.335	45
	37	Ca^{2+}	0.425	37
	37	Native	0.50	8.9
	37	H^+	0.571	2.7
	20	Native	0.55	4.9
B. subtilis 168 wild type	37	Native	0.37	360[b]
B. subtilis 168 $\alpha^-\beta^-$	37	Native	0.37	15[b]

[a] Data are from reference 6.

[b] D 85°C

Wild-type spores are much more resistant to dry heat than to moist heat and exhibit a high level of DNA damage and mutagenesis accompanying dry heat killing, whereas $\alpha^-\beta^-$ spores are more sensitive to dry heat than are wild-type spores and exhibit dry heat resistance similar to that of dry vegetative cells (Table 2.2). These findings suggest that (i)α/β-type SASP are a major factor in increasing the dry heat resistance of wild-type spores and (ii) α/β-type SASP provide significant DNA protection against dry heat damage, but (iii) wild-type dry spores are so well protected against mechanisms of heat killing other than DNA damage that at elevated temperatures, DNA damage does eventually kill wild-type dry spores. In support of these findings, α/β-type SASP slow the

depurination caused by dry-heat treatment of purified DNA, although not as much as for DNA in solution.

Spore Mineralization

Spores accumulate large amounts of divalent cations late in sporulation, approximately in parallel with DPA. Analyses of mutations in *B. subtilis* that eliminate DPA synthase have revealed that CaDPA-less spores are much less resistant to moist heat than are their CaDPA-replete counterparts (8, 12). Much of the latter effect is likely due to the increased core water content of CaDPA-less spores (see below). However, CaDPA also provides Significant protection to spore DNA against damage by either dry or moist heat when α/β-type SASP are absent (47). CaDPA is also required for dormant-spore stability, as CaDPA-less spores germinate spontaneously, although they can be stabilized by other mutations.

The amount and type of mineral ions accumulated also affect spore heat resistance (25, 54). Analyses of spores of several species from which mineral ions were removed by titration with acid and which were then back-titrated with a mineral hydroxide showed heat resistance with different cations in the following order: $H^+ < Na^+ < K^+ < Mg^{2+} < Mn^{2+} < Ca^{2+} <$ untreated. Despite the clear role for mineral ions in spore heat resistance, the mechanism of this effect remains unclear. Alteration of spore mineralization can alter spore core water content (Table 2.3), which presumably causes a significant effect on heat resistance. However, mineralization may also affect spore heat resistance independently of its effects on core water content, perhaps by modulating the heat resistance of specific spore proteins (Table 2.3).

Spore Core Water Content

Low core water content is the major factor contributing to spore heat resistance. Dehydration of the spore begins in the stage III-stage IV transition and continues through stages IV and V, with final dehydration taking place approximately in parallel with

acquisition of full spore heat resistance (8, 12, 25, 54). Synthesis of the spore cortex is essential both for effecting this dehydration and for maintaining the dehydrated state of the spore core. This is likely due to the ability of PG to change its volume upon changes in ionic strength and/or pH. If an expansion in cortex volume is restricted to one direction, i.e., towards the spore core, core water would be extruded via mechanical action. Although the precise mechanism of this process is unclear, the important role of the cortex in heat resistance is shown by the inverse correlation between spore heat resistance and the volume occupied by the spore cortex relative to that of the core (25). This correlates not only across species but also in a single species in which spore cortex biosynthesis has been altered by mutation (29). Presumably, the volume of the spore cortex influences the degree of core dehydration, and the amount of cortex and presumably its mechanical strength are crucial to the spore's ability to maintain core dehydration during heat treatment. Another factor determining spore core water content is CaDPA, as CaDPA-less spores have higher core water levels than otherwise identical CaDPA-replete spores (8, 12). Presumably, the core volume normally occupied by CaDPA is occupied by water in CaDPA-less spores. As noted above, CaDPA-less spores are more sensitive to moist heat than are CaDPA-replete spores.

Studies of spores from many species have revealed a good correlation between core water content and moist heat resistance over a 20-fold range of *D* values (25) (Fig. 2.7). However, at the extremes of core water contents, *D* values vary widely, presumably reflecting the importance of other factors such as sporulation temperature, cortex structure, etc., in modulating spore moist-heat resistance. One value that is unfortunately missing from analyses of core water content is the amount of core water that is free water. Studies of protein and ion movement in spores have indicated that these molecules are relatively immobile (8, 12, 33, 40), consistent with a dearth of free water in the core. Presumably, the low water content in the spore core causes heat resistance as well as long-term spore survival by slowing water-driven chemical reactions, such as

DNA depurination and protein deamidation. A low water content also stabilizes proteins against denaturation by restricting their molecular motion. It would be informative to know the precise amount of water associated with spore core macromolecules in order to calculate the degree of their stabilization by the low core water content.

DNA Repair

DNA repair is essential for spore resistance to UV and chemicals that kill spores by DNA damage (8, 12, 14). However, spore resistance to moist heat is not altered by loss of DNA repair functions, including those mediated by RecA. This is not surprising because moist heat does not kill wild-type spores by DNA damage. However, spore resistance to dry heat, a treatment that does kill spores by DNA damage, is markedly decreased by loss of various DNA repair activities, including RecA and other proteins.

Altered SpoVA Proteins

Recent work (51, 55–58) has found that some *B. subtilis*, *Bacillus amyloliquefaciens*, and *Bacillus lichenifor mis* food isolates that make very highly wet-heat-resistant spores contain transposons that carry a *spoVA* operon with only three of the highly conserved *spoVA* genes, *spoVAC*, *spoVAD*, and *spoVAEb*. The presence of this novel *spoVA* operon, termed *spoVA2*^{mob}, is the major cause of the spores of these strains' high wet-heat resistance. While the mechanism of the effect of SpoVA2^{mob} proteins on spore wet-heat resistance is not completely clear, these proteins also significantly slow CaDPA release during spore germination triggered by several types of germinants (see below).

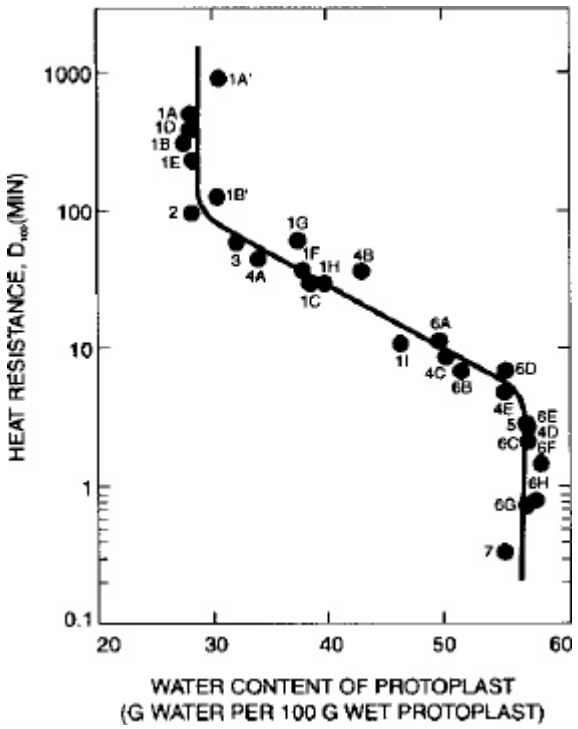


Figure 2.7 Correlation of spore heat resistance and protoplast (core) water content of lysozyme-sensitive spore types from seven *Bacillus* species that vary in thermal adaptation and mineralization. The numbers indicate species, as follows: 1, *G. stearothermophilus*; 2, "*Bacillus caldolyticus*"; 3, *B. coagulans*; 4, *B. subtilis*; 5, *B. thuringiensis*; 6, *B. cereus*; 7, *Bacillus macquariensis*. The letters denote the sporulation temperature or the mineralization of the spores of various species as described in the original publication. Reprinted from reference 25 with permission.

SPORE ACTIVATION, GERMINATION, AND OUTGROWTH

Activation

Although spores are metabolically dormant and can remain so for many years, if given the proper stimulus they return to active metabolism within minutes through spore germination (Fig. 2.8). Spore populations often germinate more rapidly and completely if

activated prior to germinant addition (4, 9, 11). However, the requirement for activation varies widely among spores of different species and between individual spores in populations and for the germinants used, as only nGR dependent germination requires activation (4). A number of agents cause spore activation, including low pH and some chemicals, although the most widely used agent is sublethal heat. The precise changes induced by spore activation are not clear but may involve reversible changes in protein structure, at least nGR structure, as different nGRs respond differently to heat activation (56, 59). However, it is also possible that heat activation affects the SpoVA channel for CaDPA release (56).

Germination

The precise period encompassed by spore germination, as distinguished from subsequent outgrowth, has been given a number of definitions. For the purposes of this review, we consider germination the events that take place without the need for metabolic energy. During germination, a dormant spore with a cortex and a large pool of DPA and mineral ions is transformed into a germinated spore in which the cortex has been degraded, DPA and most mineral ions have been excreted, and the core water content has become that of a growing cell (4, 6, 9–11). However, conversion of the germinated spore into a growing cell requires exogenous nutrients and the process of outgrowth. Notably, there is also one report that protein synthesis is essential for completion of germination of *B. subtilis* spores (60).

The initiation of spore germination can be triggered by many compounds, including nutrient germinants such as nucleosides, amino acids, sugars, and, for some spores, salts, and also CaDPA, long-chain alkylamines, and some PG fragments, although this germinant has not been well studied, in addition to high pressure (4, 6, 9–11). *C. difficile* spore germination is also triggered by various bile salts, presumably signaling the environment of the mammalian large intestine (4, 15, 61). CaDPA, alkylamines, and high pressure (described above) are relatively universal germinants,

although CaDPA is not a germinant for spores of *C. difficile* (61). In contrast, the nutrients that act as germinants vary from species to species, and metabolism of such germinants is not required. l-Alanine is a common nutrient germinant, and its action is sometimes inhibited strongly by d-alanine. Interestingly, l-alanine germination can be modulated by a spore-specific alanine racemase in spore coats that can generate d-alanine (4, 9, 62). The stereospecificity for nutrient germinants is due to these germinants' interaction with specific nGRs that are generally the products of tricistronic or tetracistronic operons. Sporeformers usually have between two and seven of these operons, which are expressed in the developing forespore during sporulation (4, 9).

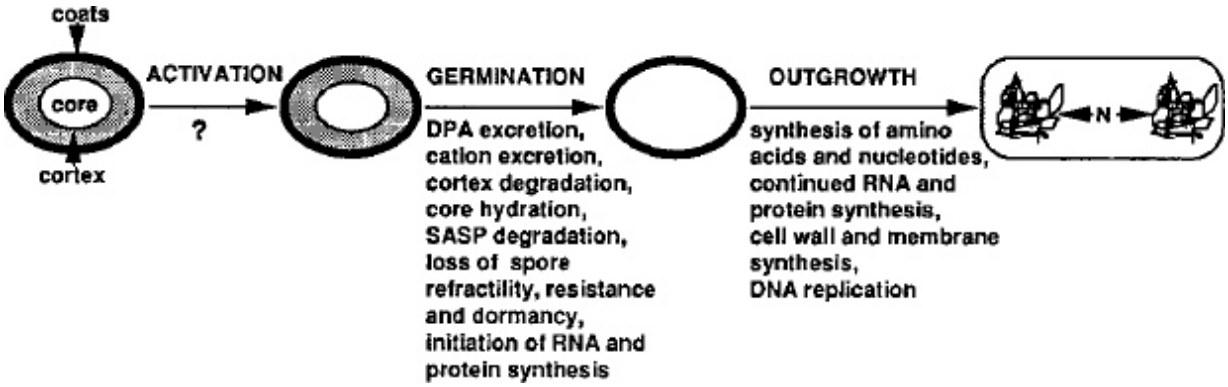


Figure 2.8 Spore activation, germination, and outgrowth. The events in activation are not known, hence the question mark. The loss of the spore cortex and the hydration and swelling of the core are shown in the germinated spore. Adapted from Fig. 3 in reference 10.

Different nGRs have different, although sometimes overlapping, specificities for nutrient germinants and can cooperate and even exhibit synergy in responding to germinant mixtures. In *B. subtilis*, allnGRs colocalize in the spores' IM in one or two foci, with the auxiliary IM germination protein, GerD, being essential for focus formation and rapid nGR-dependent germination (4, 9, 63). Loss of allnGRs almost completely eliminates spores' germination with nutrients, except for slow spontaneous germination (4, 9, 64). However, spores of *C. difficile* and close relatives have no IM GRs;

rather, its GRs are in spores' outer layer, and at least one such GR is a modified subtilisin-like protease that responds to bile salts (4, 9, 15). The nGRs are probably most often a complex of the three or four proteins encoded by individual *ger* operons, although variations in this arrangement exist (4, 9, 13, 14). Three nGR subunits appear likely to be integral IM proteins and one a peripheral IM protein, and nGRs are present exclusively in spores' IM with a number of domains outside the IM (4). Unfortunately, how nGRs function is not known, and analysis of the primary sequences or high-resolution structures of their subunits has not been informative in this regard. Notably, the *spoVA2*mob operons described above, which can greatly increase spore wet-heat resistance, also greatly decrease spore germination rates via nGRs and also via the cationic surfactant dodecylamine, which directly opens the SpoVA channel for CaDPA (56, 58, 65).

In addition to the genes encoding nGRs, there are other *ger* genes in *B. subtilis* mutations which alter spore germination (11). The functions of some of these additional *ger* genes are known, whereas those of others are not. In general, *ger* genes identified in *B. subtilis* have counterparts in other *Bacillus* species, and some of these genes, in particular those encoding nGRs, are also found in *Clostridium* species (4, 9, 13–15, 17). However, the *gerD* gene, which is essential for rapid *Bacillus* spore germination via nGRs, is not present in *Clostridium* species.

The earliest biochemical event in spore germination after nutrient germinant addition is the release of protons and other monovalent cations (4, 9–11). This event appears to be concomitant with commitment, in which spores are irreversibly committed to germinate, even if germinants are removed, and is paralleled by changes in IM permeability that in addition to allowing rapid monovalent cation release also allow slow leakage of ~10% of CaDPA and fast release of CaDPA at temperatures such as 75°C, which have no effects on dormant spores. However, the mechanism of these changes associated with commitment is not known. In *Bacillus* spores and spores of some *Clostridium* species, in

particular those that have the same CLEs as *Bacillus* spores, fast CaDPA release follows commitment and takes place via IM SpoVA protein channels. All CaDPA release can be completed in as little as 30 s for an individual spore, although individual spores initiate DPA release after very different lag times following germinant addition, lag times that can be minutes, hours, or even longer (4). Spores with extremely long lag times are termed superdormant and are of special concern to the food industry. Such superdormant spores have been isolated from populations of spores of a few *Bacillus* species, and factors contributing to spores' superdormancy have been identified (4). In *B. subtilis*, the replacement of released DPA and ions by water increases the core water content by ~30%. This reduces spore moist-heat resistance but has little effect on resistance to other agents and is not sufficient for resumption of enzyme action or protein mobility (8, 12, 40). The release of DPA and other ions is termed stage I of germination, and germination can be halted after stage I by mutations that block degradation of the spore cortex.

Initiation of cortex degradation is the next event in germination and allows a rapid approximately 2-fold increase in spore core volume through water uptake once the cortex that restricts core expansion is removed. This is termed stage II of germination and leads to spore outgrowth. The importance of cortex lysis in germination has focused attention on CLEs. A number of CLEs have been identified from spores of different species, including CwlJ, SleB, SleC, SleL, and SleM (4, 9–11, 14). These enzymes are located in the spore's outer layers and are active only on PG-containing MAL. This is consistent with the lack of cortex degradation during germination of spores of a *B. subtilis cwlD* mutant, as CwlD is required for MAL formation and thus for completion of spore germination. CLEs' specificity prevents these enzymes from hydrolyzing the germ cell wall during spore germination.

The function of the CLEs *in vivo* has been established in spores of several *Bacillus* species, in which SleB and one or two CwlJ homologs play redundant roles in cortex degradation (4, 8–11, 14).

However, *cwlJ sleB* spores of *Bacillus* species complete stage I of spore germination. CwlJ and SleB in spores are in a mature, potentially active form and thus must be regulated. While regulation of SleB is not understood, CwlJ activation requires either exposure to CaDPA released in stage I of germination or high concentrations of exogenous CaDPA, as in CaDPA triggered germination. In contrast to *Bacillus* spores, spores of some *Clostridium* species, including *C. difficile* and *C. perfringens*, contain a single essential CLE, SleC, present in spores as a zymogen that is activated by proteolysis early in germination (4, 9, 14, 15).

The pattern of germination noted above for spores of *Bacillus* and some *Clostridium* species is different in other *Clostridium* species. Thus, with spores, such as those of *C. perfringens*, that contain IM nGRs, or with *C. difficile* spores that lack IM nGRs but have outer layer GRs, GR activation does not directly activate CaDPA release. Rather, in these spores, activated GRs trigger pro-SleC cleavage by subtilisin-like proteases, and SleC then hydrolyzes cortex PG. This event then triggers CaDPA release by spore core osmotic pressure (66), much as lysozyme digestion of an nGR-deficient *Bacillus* spore triggers CaDPA release (4).

Outgrowth

Following completion of stage II of spore germination, core hydration is equivalent to that of a growing cell protoplast, and protein mobility is restored and enzymatic reactions begin in the core (11). These reactions include utilization of the spore's depot of 3PGA to generate ATP and NADH, degradation of SASP initiated by GPR, catabolism of some amino acids produced by SASP degradation, and catabolism of exogenous compounds. Carbohydrate metabolism early in outgrowth uses glycolysis and/or the hexose monophosphate shunt but may stop at acetate, as spores often lack enzymes of the TCA cycle. In *Bacillus megaterium*, endogenous energy reserves can support ATP production during the first 10 min of outgrowth.

RNA synthesis begins in the first minute of outgrowth, using nucleotides stored in the spore or generated by breakdown of spore RNA. The RNAs made at this time have been studied in *B. subtilis* and are composed of many mRNAs (11), although if dormant spores' rRNA has been degraded, massive rRNA synthesis begins early in outgrowth (45); protein synthesis begins shortly after mRNA synthesis. All components of the protein synthesizing machinery stored in the dormant spore appear functional, with the exception of some tRNA lacking the 3′-terminal adenosine residue that is repaired by tRNA nucleotidyltransferase early in outgrowth. Endogenous amino acids derived largely from SASP breakdown can support most protein synthesis in the first 25 min or so of outgrowth, but completion of outgrowth requires exogenous nutrients (4, 11). The spore regains the ability to synthesize amino acids, nucleotides, and other small molecules due to synthesis of biosynthetic enzymes at defined times in outgrowth, but regulation of gene expression throughout outgrowth has not been thoroughly studied. DNA replication is not generally initiated until late in outgrowth, although DNA repair can occur well before DNA replication, as spores contain deoxynucleoside triphosphates by the first minutes of outgrowth (11). However, the importance of DNA repair in the first minutes of outgrowth, other than repair of UV damage, has not been well studied. During spore outgrowth, the volume of the outgrowing spore continues to increase, requiring the synthesis of membrane and cell wall components. One question that remains intriguing is whether there are genes that are needed for outgrowth but no other stage of growth. A number of mutations affecting outgrowth (termed outmutations) have been isolated in *B. subtilis*, and most have been identified (11). While the functions of some of these genes have been established, to date, none has been shown to function solely in outgrowth.

PRACTICAL PROBLEMS OF SPORES IN THE FOOD INDUSTRY

Overview

Endospores of bacteria and heat-resistant spores of fungi pose safety and spoilage problems for the food industry. The primary sporeformers of importance in foods belong to the orders *Bacillales*, *Clostridiales*, and *Thermoanaerobacterales*. Three species of food-related sporeformers, *C. botulinum*, *C. perfringens*, and *B. cereus*, produce toxins that cause illness in humans and animals (51, 67, 68). Rare strains of *Clostridium butyricum* and *Clostridium baratii* may also produce botulinal neurotoxin (69). In the United States, *C. botulinum* is designated a select agent of the highest security level (tier 1) and can be studied only in strictly approved laboratories with qualified personnel (70, 71).

Sporeformers also cause spoilage of foods with drastic economic repercussions, contributing to the estimated 20 to 30% global spoilage of foods produced (72–74). The primary genera of nonpathogenic sporeformers causing food spoilage are *Anoxybacillus*, *Alicyclobacillus*, *Bacillus*, *Clostridium*, *Desulfotomaculum*, *Geobacillus*, *Paenibacillus*, *Thermoanaerobacter*, *Moorella*, and *Sporolactobacillus* (Table 2.1) (67, 68, 75, 76). Processing and formulation of foods to prevent foodborne illness and spoilage by sporeformers have become more challenging in the past decade due to consumer demands for foods that appear and taste fresh and lack chemical antimicrobials, thereby providing a “clean” label (77, 78). This consumer demand for fresh quality can compromise traditional methods of processing and formulation and can also change the microbial ecology of foods, creating the food microbiome (79). Moreover, the food supply is more international, with ingredients and processing of foods derived from various countries, which poses challenges in control of sporeformers (77, 78)

Sporeformers that pose health concerns are particularly important in low-acid foods with a pH of >4.6 (see below). To prevent foodborne illness and spoilage by sporeformers, many low-acid foods are processed by heat, which remains the cornerstone of food

preservation by physical technologies (80–82). Newer physiological technologies, such as high-pressure processing (HPP), as well as preventive food formulations are being evaluated and implemented in the food industry (83–87). Certain species of sporeformers also cause spoilage of high-acid and acidified foods (equilibrium pH ≤4.6) (75, 76). Thermophilic sporeformers (growth range of 50 to 110°C) are a cause of serious spoilage in certain food service applications, at elevated storage temperatures, and in warm climates. Psychrophilic (growth range, −5 to 20°C) and psychrotrophic (growth range, −5 to 30°C) sporeformers can grow and cause spoilage of refrigerated foods and of foods processed and stored in cold climates at temperatures of 0 to 20°C (75, 76, 88–92). Fungi that produce heat-resistant ascospores as well as many yeasts are an important cause of spoilage of raw materials, such as grains, and of processed food, often acidic beverages and fruits and vegetable products (92–94). Fungal spores and vegetative fungi and yeasts generally are more sensitive to heat than bacterial endospores. The spoilage properties and control of fungi and yeasts are described in several excellent sources (27–29, 95).

DISCOVERY OF PATHOGENIC AND SPOILAGE SPORE-FORMING BACTERIA AND DEVELOPMENT OF EARLY CONTROL PROCESSES

In the 1850s, Louis Pasteur discovered an anaerobic spore-forming bacterium during butyric acid spoilage in wines (95–97). Pasteur was able to isolate an obligately anaerobic bacterium he termed *Vibrion butyrique*, which is probably the same microorganism now classified as *C. butyricum* Prazmowski, the type species of the genus *Clostridium* (98). Trecul coined the name "*Clostridium*," following from the Greek word "kloth" (a small spindle) to describe a microorganism in its sporulating form; this term was then transformed to the Latin *Clostridium* (96). The first pathogenic

Clostridium species isolated in pure culture was *Clostridium tetani* by Kitasoto in 1889 (95–98). *C. perfringens* was isolated in pure culture in the 1890s (96) and was characterized by Welch and Nuttal in the 1890s. *C. perfringens* was found to be important in food poisoning and in wounds, sometimes causing gas gangrene (96). Pathogenicity and death of humans from *C. perfringens* were demonstrated in 1917 to be due not to blood invasion but rather to a poisonous substance produced by the bacterium (96). MacFarland and Knight (96) showed that the lethal factor was lecithinase C, the first bacterial toxin shown to have enzymatic activity (96). *C. botulinum* was discovered by van Ermengem in a remarkable series of experiments that was reported in a landmark publication in 1897 (99). Many of the biological and control concepts observed by van Ermengem are still relevant today and form the basis of food laws (such as low-acid canned foods). *C. difficile* was first reported by Hall and O'Toole in 1935 (96). *C. difficile* is a toxigenic sporeformer that has become one of the most common causes of human and animal intestinal disease during the past 2 decades (97) and raised concern about transmission in foods.

Endospores were discovered independently by Ferdinand Cohn and Robert Koch in the 1870s (100). Investigations by Pasteur and Koch led to the association of microbial activity with the safety and quality of foods and understanding of the pathogenicity of sporeformers. During investigation of the anthrax bacillus, *B. anthracis*, the famous Koch's postulates were born (101). These procedures provided proof that a disease is caused by a specific microorganism and also led to the development of pure-culture techniques. Diseases and spoilage problems caused by sporeformers have historically been mainly associated with foods that are thermally processed, because heat selects for survival and subsequent growth of spore-forming microorganisms. Growth of the sporeformer is primarily due to survival of exposure to heat and to the absence of competitor microbiota in the thermally processed food. Competitive microbiota in foods and in the intestinal tracts of

humans and animals is an extremely important factor in the prevention of growth of several pathogenic sporeformers (102–105).

The process of appertizing, or preserving food sterilized by heat in hermetically sealed containers, was invented in the late 1700s by Appert (106), who theorized that the elimination of air was responsible for the long shelf life of thermally processed foods. In the late 1800s and early 1900s, several scientists and engineers were instrumental in developing scientific principles and processes to ensure the safety and prevention of spoilage of thermally processed foods (107–111). In early studies of canned-food spoilage by sporeformers, Prescott and Underwood at the Massachusetts Institute of Technology and Russell at the University of Wisconsin found that endospore-forming bacilli caused the spoilage of thermally processed clams, lobsters, corn, and other foods (108, 109), and other seminal studies were performed in the United States and in Europe (110, 111). The classic studies of Esty and Meyer (112) in California provided definitive values of the heat resistance of *C. botulinum* type A and B spores and helped rescue the United States canning industry from its near demise as a result of botulism in commercially canned olives and other foods in the 1920s (113–117). The empirical use of thermal treatments gradually developed into modern-day thermal processing industries. As a result of these studies, thermal processing of foods in hermetically sealed containers became an important industry and remains the foundation of preservation of foods today.

Thermally processed commercial canned foods have an exemplary record of microbiological safety, particularly with regard to the agent of botulism as the primary organism of concern, with only seven major outbreaks of botulism involving several humans occurring from 1963 through 2013 (118–121; also see https://www.cdc.gov/botulism/surveillance.html for recent epidemiological updates). In 1975, Lynt et al. (122) estimated that during the 45-year period from 1940 to 1975, 775 billion cans of commercially canned foods were consumed, with only four known

deaths from botulism, documenting the safety of thermal processing.

Quantitative thermal processes, understanding of spore heat resistance, aseptic processing, and implementation of hazard analysis and critical control points (HACCP), food safety objectives (FSO), and other engineering concepts as well as establishment of microbiological criteria also resulted largely from developments in the canned-food industry (82, 123, 124).

MICROBIOLOGY OF SPOREFORMERS IN FOODS

Ecology and Distribution of Spores

The environments in which spores reside are mainly associated with selection for spore resistance phenotypes including temperature, acidity, and water activity. Common habitats include soils, sediments, vegetation, animal intestines, and rotting vegetation and animal carcasses (125–127). Sporeformers comprise saprophytic and proteolytic groups, and each group is associated with the habitat for optimal growth and survival. Dormant spores can exist in certain environments for hundreds to thousands of years (128), and many species are commonly found in soils and other relatively static habitats. Thermophilic sporeformers (growth at 50 to 110°C) are found in high-temperature environments and may survive and grow in thermally processed foods (80). Similarly, psychrophilic and psychrotrophic sporeformers reside in environments where they can become associated with foods present in a cold chain, such as cold packed meats, dairy products, and other foods that undergo refrigerated storage (75, 89, 90). Despite their differential distribution, it is often not feasible to predict the presence of sporeformers in foods, since many foods consist of blends of ingredients from diverse worldwide sources. Recent studies indicated that sporeformers are more prevalent in soils and in the human microbiome than previously assumed (129–131).

Characterization of Spores in the Environment and in Foods

Traditionally, the detection and characterization of sporeformers were performed using culture techniques and phenotypic tests (20, 132). Currently, molecular methods, including PCR, DNA sequencing, and -omics technologies, are used for detection and study of vegetative cells of sporeformers. Advanced genetic strategies and methods, particularly genome sequencing, and gene designations and polymorphisms are currently used for study of species designations, phylogeny, and evolution, as well as metabolic activities (133–138). These techniques have been employed to detect pathogens and spoilage organisms in food microbiomes (79, 138). However, techniques such as PCR and metagenomics and other methods do not necessarily detect living microorganisms, and many organisms cannot be cultured. Therefore, these techniques are not suitable for quantitation and for regulatory purposes (138). Considering the virtual solid state of bacterial endospores, extraction of nucleic acids would be challenging. Quantitative aspects of microbiological food testing were recently reviewed (123).

CONTROL OF SPOREFORMERS IN FOODS

Importance of Sporeformers in Low-Acid Canned Foods

C. botulinum is the central sporeformer of primary importance in many foods due to its resistance to environmental stresses, processing procedures, and chemical compounds compared to vegetative pathogens (80–82). *C. botulinum* produces the most poisonous toxin known and is the cause of the severe disease botulism (139). An outbreak of botulism is considered a public health emergency (140). Processing methods, product formulations, and other strategies of food preservation have been designed to control it in foods. The U.S. Food and Drug Administration (FDA)

and the U.S. Department of Agriculture (USDA) Food Safety Inspection Service define a low-acid canned food as a product with a finished equilibrium pH of >4.6 and a water activity (aw) of >0.85, excluding tomatoes and tomato products having a finished equilibrium pH of <4.7 (82–84). Certain countries in the European Union define a low-acid food as one having a pH of >3.5. Thermal processing is the cornerstone of food processing to control pathogenic and many other sporeformers. The regulations regarding thermal processing of canned foods are described in the U.S. *Code of Federal Regulations* (21 CFR, parts108 to 114 [www.govinfo.gov/help/cfr]). In the United States, the USDA has regulatory oversight of products that contain at least 3% raw red meat or 2% cooked poultry, and the FDA regulates other food products. Detailed descriptions of the thermal process, including the environment within the facility, equipment specifications, and formulations, must be filed with the proper governmental agency prior to commercial processing. The processor must also report process deviations or instances of spoilage to the FDA or USDA. Processing facilities are also inspected regularly. Guidance for industry in the United States and Europe can be found at https://www.fda.gov, https://www.usda.gov, www.fao.org, and https://www.who.int.

Low-acid foods are packaged in hermetically sealed containers, often cans or glass jars but also plastic pouches and other types of containers. These “cans” (as collectively defined in this chapter) containing low-acid food products must be processed by heat to achieve commercial sterility (141), which is a condition achieved by application of heat that inactivates microorganisms of public health significance, as well as any microorganisms that are not of health significance but are capable of reproducing in the food at the temperature of storage and distribution. Commercial sterility is an empirical term to indicate a low level of microbial survival and provision of shelf stability. Preservation procedures such as acidification or lowering water activity by brining or other means can also be used to attain commercial sterility. Chemical

preservation procedures are often combined with a reduced-heat treatment in processing of many foods (82, 83).

In thermal processing of low-acid canned foods to control *C. botulinum*, the degree of heat treatment applied varies considerably according to the class of food, the spore numbers, pH and acidity of the food, storage conditions, and other intrinsic and extrinsic factors. For example, canned low-acid vegetables and uncured meats usually receive a 12*D* process or "botulinum cook" (see below). Lower-heat treatments are applied to shelf-stable canned cured meats as well as to foods with reduced water activity or other antimicrobial factors that inhibit growth of spore-forming bacteria. In practice, the spore content of ingredients and the cleanliness of the cannery environment are of major importance for successful heat treatment of low-acid canned foods. Certain foods and food ingredients, such as mushrooms, potatoes, spices, sugars, and starches, can contain high numbers of spores of *C. botulinum* and other species (142, 143), which may be monitored for their spore input in a process (144).

Researchers in the early and mid-1900s designed thermal processes to prevent botulism and spoilage in canned foods (106–111, 145, 146), and these processes have theoretically and technically advanced during the past several decades. Pflug (146–148) refined the semilogarithmic microbial destruction model and designed a strategy to achieve the required heat process *FT* value. He also introduced the concept of the probability of a nonsterile unit on a one-container basis. This reasoning logically explains the traditional term 12*D*, which designates the time required in a thermal process for a 12-log reduction of *C. botulinum* spores. In thermal processing (or other processing methods, such as HPP), two values have traditionally been used to describe the thermal inactivation of an organism: the *D* value is the time required for a 1-log reduction of a microorganism at a defined temperature, and the *z* value is the temperature increment required to change the *D* value by a factor of 10, or 1 $\log_{10}$ (79–81). While the *D* value represents the resistance of a microorganism to a specific temperature, the *z* value represents

the relative resistance of a microorganism to inactivation at different temperatures. The thermal process for the 10^{-11} to 10^{-13} level of probability of a botulism incident occurring will depend on the initial number of *C. botulinum* spores present in a container. This initial spore number can be relatively high in certain food, for example, 10^4 for a container of mushrooms, or very low, such as 10^1 or less for a container of a meat or dairy product (142–144). Pflug (146–148) emphasized that the thermal processing industry should prioritize process design to protect against (i) a public health hazard (botulism) from *C. botulinum* spores, (ii) spoilage from mesophilic spore-forming microorganisms, and (iii) spoilage from thermophilic microorganisms in containers stored in warm climates or environments. Generally, low-acid foods in hermetically sealed containers are heated to achieve 3 to 6 minutes at a temperature of 121°C (250°F) or equivalent at the center or most heat-impermeable region of a food. This ensures inactivation of the most heat-resistant *C. botulinum* spores with a *D*121°C (250°F) of 0.21 min and a *z* of 10°C (18°F). *C. sporogenes* spores, which have an approximately10-fold-higher heat resistance (*D*121°C of ca. 1 min) than those of *C. botulinum*, are frequently used as a surrogate in thermal processing for inactivation of *C. botulinum* spores (149–151). Spoilage with economic impact is also avoided by achieving ca. 5*D* killing of mesophilic spores that typically have a *D*121°C of ca. 0.1 to 1 min. Recently, Schill et al. (151) reported that there are two clades of *C. sporogenes* PA3679 that differ in heat stability. Therefore, it is important to use the correct strain of *C. sporogenes* PA3679 as a surrogate and to ascertain purity periodically by single-colony isolation and formation of fresh spore stocks. Foods that are distributed in warm climates of tropical or desert areas require a particularly high thermal treatment of ca. 20 min at 121°C to achieve a 5*D* killing of *Thermoanaerobacterium* (*Clostridium*) *saccharolyticum*, *Geobacillus stearothermophilus*, and *Desulfotomaculum nigrificans* because these thermophilic organisms have a *D*121°C of ca. 3 to 4 min (Table 2.4) and can cause spoilage with severe economic effects (68, 89). High thermal

treatments can have a detrimental impact on nutrient content and organoleptic qualities, but they ensure a shelf-stable food (68, 75, 76). Consequently, other processing technologies are in development to preserve the organoleptic properties of certain thermally processed foods.

The concept of semilogarithmic or first-order kinetics of heat inactivation of spores and the use of D and z values have been carefully reevaluated over the years (152–154).have studies have led to the finding that populations within the same crops of spores often differ in heat sensitivities and that non-log-linear death kinetics more accurately explain spore inactivation of most spore populations. Analysis using non-log-linear kinetics can explain the tailing and curving that are commonly observed in heat inactivation of spore populations. The kinetics of heat inactivation of spore populations closely followed a cumulative Weibull distribution, i.e., $\log S = b(\mathrm{T})^{tn(\mathrm{T})}$, where S is the survival ratio, $b(\mathrm{T})$ and $n(\mathrm{T})$ are temperaturedependent coefficients, and t is time (minutes) (155–157). Kinetics models for inactivation and recovery of bacterial spores have been valuable in evaluating and developing improved procedures within the food industry (80–82,152–154). Advanced kinetics and predictive models of spore inactivation have been outlined to simulate growth of proteolytic *C. botulinum* during cooling of heated foods (155).

Table 2.4 Heat resistance of sporeformers of importance in foods[a]

	Approx D value[b] at temp (°C)						
Bacterium	80	85	90	95	100	110	120
Spores of public health significance							
Group I C. botulinum types A and B		50		7–30	1–3	0.1–0.2	
Group II C. botulinum type B		1–30	0.1–3	0.03–2			
C. botulinum type E	0.3–3				0.01		
B. cereus					3–200	0.03–2.4	
C. perfringens			3–145	0.3–18	2.3–5.2		
Mesophilic aerobes							
B. subtilis					7–70	6.9	0.5
B. licheniformis					13.5	0.5	
B. megaterium					1		
B. polymyxa			4–5		0.1–0.5		
Bacillus coagulans subsp.			11–30		2–3		
thermoacidurans							
A. acidoterrestris			16	2.6			
Thermophilic aerobes							
G. stearothermophilus				100–1,600	1–6		
B. coagulans				20–	2–3		

				300			
Mesophilic anaerobes							
C. butyricum	4–5	0.4–0.8					
C. sporogenes					80–100	21	0.1–1.5
C. tyrobutyricum	13						
Thermophilic anaerobes							
D. nigrificans					<480		2–3
T. thermosaccharolyticum				400		3–4	

[a] Sources: see text for references.

[b] At pH ca. 7 and an aw >0.95.

Thermal processing is often applied to aseptically processed low-acid canned foods, whereby manufactured and usually processed product is used to fill presterilized containers, followed by aseptic hermetic sealing with a closure in a sterile environment (156, 157). This technology was initially used for commercial sterilization of milk and cream in the 1950s (157) and then expanded to other food products, such as soups, eggnog, cheese spreads, sour cream dips, puddings, and high-acid products such as fruit and vegetable drinks. Aseptic processing and packaging systems have the potential to reduce energy, packaging, and distribution costs (156).

Spores of *C. botulinum* can survive for long periods in high-acid foods with pH values of ≤4.6 (158, 159). Growth of *C. botulinum* and outbreaks of botulism in high-acid foods under special conditions have been reported (89, 158, 159). Some outbreaks in high-acid foods have been associated with inadvertent increases in pH, such as by growth of molds with catabolism of organic acids, referred to as metabiosis (89). Recently, there has been increased interest in using nontraditional lower-heat treatments, often combined with HPP, for infant foods (157). Such changes to the

thermal process for infant foods must be performed with caution and with appropriate validations to eliminate spores of *C. botulinum*, since if spores survive, they could cause infant botulism. Recent evidence of plasmid-mediated transfer of genes encoding botulinum neurotoxin type A and complexing proteins to nontoxigenic clostridia, including *C. sporogenes* (160, 161), could result in botulinum neurotoxin-producing strains with greater thermotolerance than group I (proteolytic types A, B, and F) *C. botulinum*. However, the frequency of genetic transfer of botulinum neurotoxin genes appears to be very low under laboratory conditions, and such transfer seems to occur infrequently in the environment, intestinal tracts of animals and humans, and foods (160, 161).

Properties of Pathogenic and Spoilage Sporeformers Relevant to Food Processing

The principal microbial hazard in thermally processed foods and in minimally processed refrigerated foods is *C. botulinum*. Other toxigenic sporeformers, including *C. perfringens* and potentially *C. difficile* and *B. anthracis*, are of concern in certain thermally processed foods (162). The biological and pathogenic properties of toxigenic species of *Clostridium* and *Bacillus* are described in other chapters, and only properties related to spores and foods are described here. *C. botulinum* and certain other pathogenic bacteria produce spores that swell the mother sporangium, giving it a spindle or club shape (Fig. 2.9). Spores of *C. botulinum* types B and E frequently possess an exosporium (163), and serotypes types A and E and *C. sporogenes* characteristically produce appendages (Fig. 2.10) (163). *B. cereus* and *B. anthracis* also possess an exosporium and bear appendages on the spore surface (163).

C. botulinum has traditionally been divided into four physiological groups (I through IV) on the basis of phenotypic properties (69, 97, 132, 164, 165). Group I consists of proteolytic strains that produce neurotoxin types A, B, and F, and group II comprises nonproteolytic

strains that produce neurotoxin serotypes B, E, and F. Strains in groups I and II are the causes of human botulism and are of concern in food safety. They have certain phenotypic properties that affect their resistance to processing and their ability to grow in foods (97, 166, 167) (Table 2.5). In particular, group I spores have high tolerance to acid and salt and produce spores with high heat resistance. Group II strains have considerably less heat resistance than group I spores and are more sensitive to acidity and salt (69, 97, 132, 164, 165).

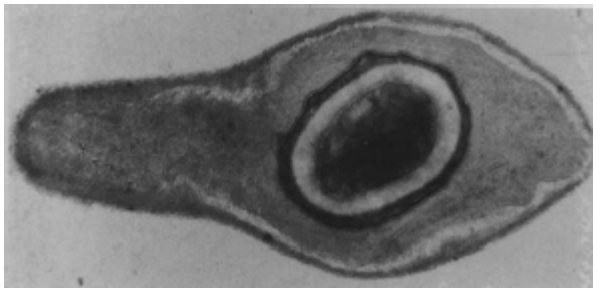


Figure 2.9 Transmission electron micrograph (magnification, × 50,000) of a longitudinal section through a spore and sporangium of *C. botulinum* type A, showing the characteristic club shape.

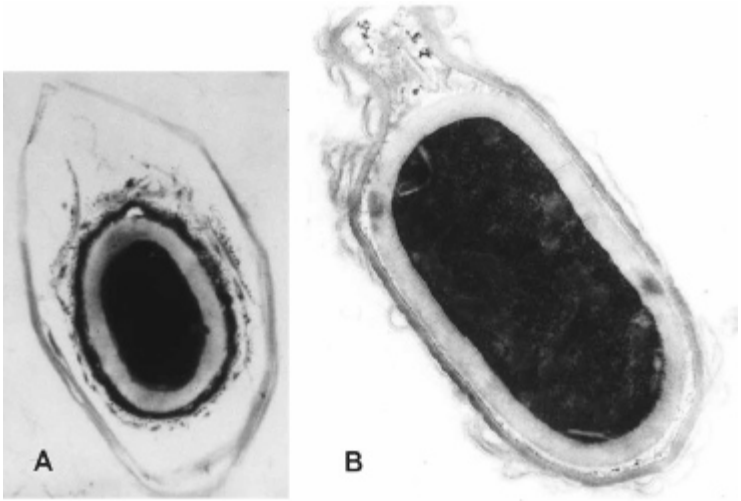


Figure 2.10 Electron micrographs of *C. botulinum* type B (A) and E (B) spores, showing characteristic exosporium in types B and E and appendages in type E. Courtesy of Philipp Gerhardt from spores produced in E.A.J.'s laboratory. *Botuli*

As described in detail in chapter 18 of this volume, nearly all(>98%) foodborne, infant, and wound botulism cases in the continental United States are caused by proteolytic group I *C. botulinum* strains that form type A and B botulinum toxins. The most common event that leads to foodborne botulism by group I strains is temperature abuse of a food product that contains *C. botulinum* spores and has a permissive acidity (pH >4.6) and water activity (>0.93) (97, 105, 168, 169). Improper commercial processing has also led to global outbreaks of botulism (165, 168). Group I strains can grow rapidly at temperatures of 25 to 40°C, particularly if the food is not formulated with antimicrobials and a competitive microbiota is not present due to processing or the nature of the food. It has been estimated that 1 to 10 ng of botulinum toxin per kg body weight is sufficient to kill an adult human by the oral route (139, 170, 171).

The ability of group II *C. botulinum* to grow at low temperatures has generated concern in the United States and certain other countries that refrigerated food products could lead to botulism outbreaks (172–174). Type E has caused several outbreaks of foodborne botulism (169, 175), particularly from consumption of fish, marine mammals, and other substrates associated with marine and terrestrial water environments as well as some terrestrial foods in which *C. butyricum* producing type E botulinum toxin is formed (169). In the United States and most other countries, nonproteolytic types B and F have rarely caused confirmed foodborne botulism. Nonproteolytic strains of *C. botulinum* serotypes B are also not a significant cause of infant or wound botulism globally (103). Nonproteolytic botulism caused by types B and F appears to be more common in the United Kingdom and in certain other countries (106–108). In experimental food substrates, group II *C. botulinum* strains can grow and produce botulinum toxin at refrigeration temperatures (≥3.3°C) during extended storage, generally requiring a minimum of 10 days but usually several weeks to months to produce sufficient botulinum neurotoxin to cause mouse lethality (51, 144). Most strains of *C. botulinum* grow very slowly below 5°C, and the levels of botulinum toxin produced by

nonproteolytic strains are often very low (142). To control nonproteolytic *C. botulinum* serotypes B, E, and F, it has been recommended that refrigerated products with a shelf life greater than 5 days receive a heat treatment to kill 6 log10 of psychrotrophic or psychrophilic spores of *C. botulinum* and that additional intrinsic or extrinsic preservation factors be included to ensure safety (105, 173, 174).

Table 2.5 Growth requirements of sporeformers of public health significance[a]

	Inhibitory condition			
Bacterium	Minimum pH	NaCl concn (%)	Minimum a_w	Temp range for growth (°C)
Group I *C. botulinum*	4.6	10	0.94	10–50
Group II *C. botulinum*	5.0	5	0.97	3.3–45
C. butyricum (toxigenic)	4.8	12		
C. *baratii* (toxigenic)				10-45
B. cereus	4.35–4.9	ca. 10	0.91–0.95	5–50
C. perfringens	5.0	ca. 7	0.95–0.97	15–50

[a] See text for references.

In recent years, advances have been made in the genomics of *C. botulinum*, including the discovery of traits related to sporulation and spore resistance. The first strain to have its genome sequenced was *C. botulinum* type A 3502, which yielded considerable insights into the genes affecting biological properties (reviewed in references 176 and 177). Genomic analyses of *C. botulinum* groups I and II have revealed several features regarding sporulation, spore resistance, and germination. The genome sequences of ca. 200

strains of *C. botulinum* as well as numerous strains of *C. perfringens* and *B. cereus* are currently available in GenBank. The properties garnered from the genome sequences related to *C. botuli num* and botulism are mainly described in chapter 18 [*C. botulinum*]. Genome analyses have shown that components of the well-defined *Bacillus* phosphorelay signal transduction pathway, including Spo0F and Spo0B, are absent from essentially all *Clostridium* species, indicating that the initiation of sporulation occurs through direct phosphorylation of the Spo0A transcription factor by orphan sensor histidine kinases (177–180). Moreover, there is not conservation of amino acid sequence or structure for sensor domains of these kinases, indicating that each species may respond to different initiating signals (111–114). Bioinformatics analyses of the genome sequence are beginning to reveal characteristics underlying spore activation, germination, spore resistance, and properties governing growth and toxin formation in foods (111–114).

An important sporeformer among the pathogenic clostridia is *C. perfringens*, which is widespread in soils and is a normal resident of the intestinal tracts of humans and certain animals (181) (see chapter 19). The properties of *C. perfringens* related to foods were recently reviewed (181). *C. perfringens* spores are large, oval, and centrally or subterminally located and swell the rod-shaped cells (181). *C. perfringens* can grow extremely rapidly in high-protein foods, such as meats, that have been cooked to eliminate vegetative bacteria and yeast competitors and are inadequately cooled, allowing this pathogen to produce an enterotoxin that causes diarrheal disease. It also produces a diversity of other extracellular toxins and degradative enzymes (182), which can cause animal and human illness, and at least one toxin (epsilon) of *C. perfringens* acts on neuronal tissue (183). *C. perfringens* is very important in infectious diseases in animals (see chapter 19). *C. perfringens* differs from many other clostridia in being nonmotile, reducing nitrate, and carrying out a stormy fermentation of lactose in milk.

The ability of *C. perfringens* to cause foodborne illness is related to its ubiquitous distribution in foods and food environments, the formation of endospores that can survive cooking of foods, and extremely rapid growth in warm foods (doubling time of 6 to 9 minutes at 43 to 45°C) (181) (see chapter 19). The optimum temperature for growth of vegetative cells is ca. 43 to 45°C (109 to 113°F). Due to the rapid growth of *C. perfringens* in many foods, cell numbers sufficient to cause foodborne illness can be produced rapidly (see chapter 19). Germination of spores and growth of cells can occur at temperatures up to 50°C (ca. 122°F). *C. perfringens* does not generally grow below 15°C (68°F), and true psychrotrophic strains of the bacterium have not been isolated. *C. perfringens* can grow over a pH range of 5.0 to 8.5 to 9.0, and the optimum is ca. 6.5 (see chapter 19). At temperatures below 45°C (113°F), some strains will grow at pH 5. As with most bacteria, organic acids and their salts, including acetate, lactate, and citrate, are much more effective than are mineral acids for inhibiting growth of *C. perfringens*. Growth of most strains is also inhibited by 5 to 6% sodium chloride (Table 2.5). Sodium nitrite in cured meat products also inhibits growth. Conditions for growth and toxin formation of *C. perfringens* in many foods have been reviewed (181) (see chapter 19).

The heat resistance of *C. perfringens* spores is strain dependent and varies considerably among different isolates (181). In general, two classes of heat sensitivity of spores are observed. Heat-resistant spores have $D_{90°C}$(194°F) values of 15 to 145 min and z values of 9 to 16°C (16 to 29°F), in contrast to heat-sensitive spores, which have $D_{90°C}$ values of 3 to 5 min and z values of 6 to 8°C (11 to 14°F). The resistance of *C. perfringens* spores is considerably less than those of proteolytic *C. botulinum* group I (181). The spores of the heat-resistant class generally require a heat shock of ≥75°C (≥167°F) for 5 to 20 min in order to germinate. The basis of the wide variation in heat resistance of *C. perfringens* spores is not currently understood. The spores of both classes may survive cooking of foods and may be stimulated by heat shock for germination during heating procedures. Both classes can cause diarrheal foodborne illness,

although it would be expected that the heat-resistant forms would be a more frequent cause of illness.

Foodborne illness caused by *C. perfringens* nearly always involves temperature abuse of a cooked food, and the vast majority of foodborne illnesses caused by *C. perfringens* could be avoided if cooked foods were eaten immediately after cooking or rapidly chilled and reheated before consumption to inactivate vegetative cells. The objective in prevention of foodborne illnesses by *C. perfringens* is to limit the multiplication of vegetative cells in the food prior to ingestion. Since the spores are widespread and are moderately resistant to heat, they often survive the cooking procedure, germinate, and rapidly outgrow to large vegetative-cell populations if the rate of cooling is inadequate. This property has led the USDA to develop performance standards for cooling in the production of ready-to-eat meat and poultry products (184, 185). The USDA-FSIS draft guidelines state that cooked meat products should be cooled at a rate adequate to prevent a 1-log10 increase of *C. perfringens* (181, 184, 185). If foods are not rapidly cooled, they should be held at 60°C (140°F) or higher. Modeling studies have been conducted to evaluate and prevent *C. perfringens* growth on cooling of certain foods (184, 185). For cooled foods, the maximum internal temperature should not remain between 130°F and 80°F for more than 1.5 hours, and not between 80°F and 40°F for more than 5 hours. For products that undergo a pasteurization step, the entire process must not allow growth greater than 1 log10 in the product during processing and cooling. Methods for inactivation and control of *C. perfringens* spores in foods were recently reviewed (181).

Several *Bacillus* species are important in food safety and food spoilage (186, 187). *Bacillus* is a large genus, but only *B. cereus* and *B. anthracis* are definitive human pathogens. *B. cereus* produces a heat-labile enterotoxin causing diarrheal illness, as well as the toxins hemolysin HL and cytotoxin K, and a heat-stable lowmolecular-weight toxin(s) causing an emetic response in humans (186, 187) (see chapter 20). The emetic toxin has been

identified as cereulide, a non-ribosomally produced peptide (186, 187). Generally *B. cereus* must grow to very high numbers (>106/g of food) to cause human illness. *B. cereus* is closely related to *B. megaterium*, *B. thuringiensis*, and *B. anthracis*, but *B. cereus* can be distinguished from these species by biochemical tests, the absence of toxin crystals, and more recently by genetic typing and genomic methods. Other bacilli, including *B. licheniformis*, *B. subtilis*, and *Bacillus pumilus*, have been reported to rarely cause foodborne disease outbreaks, primarily in the United Kingdom (186–188).

B. cereus spores occur widely in foods and are commonly found in milk, cereals, starches, herbs, spices, and other dried foodstuffs (186–188). They are also frequently found on the surfaces of meats and poultry, probably because of soil or dust contamination. Investigators in Sweden reported isolation of *B. cereus* from 47.8% of 3,888 different food samples. In the United Kingdom, *B. cereus* was isolated from 98 of 108 (91%) rice samples. The bacterium causes spoilage of raw and unpasteurized milk (186–188), and foods containing dried milks, such as infant formulas, may possess high levels of spores or cells.

B. cereus grows over a temperature range of approximately 10 to 48°C (50 to 118.4°F), with an optimum of 28 to 35°C (82.4 to 95°F) (Table 2.5). Psychrotrophic strains that produce enterotoxin in milk have been isolated (186, 187). The doubling time at the optimum temperature in a nutritious medium is 18 to 27 minutes. Several strains can grow slowly in high-protein foods containing 7.5% sodium chloride. The minimum water activity for growth is 0.95. The bacterium grows over a pH range of approximately 4.9 to 9.3, but these environmental limits for growth are dependent on water activity, temperature, and other interrelated intrinsic and extrinsic parameters.

Spores of *B. cereus* are ellipsoidal and central to subterminal and do not distend the sporangium. Spore germination can occur over the temperature range of 8 to 30°C (46.4 to 86°F). Spores from strains associated with foodborne illness had a heat resistance of

*D*95°C(203°F) of ca. 24 minutes. Strains not associated with foodborne illness have a wider range of heat resistance. It is likely that strains involved in foodborne illness have higher heat resistances and are more apt to survive cooking.

Since spores of *B. cereus* are widespread in nature and survive extended storage in dried food products, it is not practical to eliminate low numbers of spores from foods (186, 187). Control against foodborne illness should be directed at preventing germination of spores and multiplication of large populations of the bacterium. Cooked foods should rapidly and efficiently be cooled to less than 7°C (45°F), or maintained above 60°C (140°F) and should be thoroughly reheated before serving (186). Other factors affecting food safety and spoilage by *B. cereus* are described in chapter 20.

The virulent pathogen *B. anthracis* has occasionally been associated with gastrointestinal anthrax from consumption of foods, particularly meats and animal byproducts, such as skins (188–190). Anthrax also results from occupational exposure, such as in tanning of hides. In late 2001, the deliberate release of *B. anthracis* spores revealed that the pathogen could also cause disease and death as a bioterrorism agent, mainly by aerosolization but also by dissemination in foods (189, 190). The resistance properties of spores of *B. anthracis* have been reviewed and provide a framework for control (177, 178, 182). Certain other species of *Bacillus*, such as *B. licheniformis*, *B. subtilis*, and *B. pumilus*, have also been reported to sporadically cause foodborne disease through production of toxins, including lipopeptides (186–188), but their incidence and importance in food safety are not well understood.

TECHNOLOGIES FOR INACTIVATION OF SPOREFORMERS IN FOODS

Thermal Inactivation of *C. botulinum* Spores

C. botulinum is the primary concern for public health safety from thermally processed foods, as well as other common processing methods, such as HPP. Severe outbreaks of botulism in canned olives and certain other commercial foods in California in the 1920s and 1930s led to concepts and practices for control of *C. botulinum* in canned foods (113–117). *C. botulinum* is categorized in four groups (I to IV) based on physiological properties and spore resistance (69, 97, 169). Group I *C. botulinum* type A and B strains can produce spores with remarkable heat resistance, with thermal resistances greater than those of all other foodborne pathogens except prions. The control of *C. botulinum* in foods is of paramount importance in food safety, and adequate processing and formulation are integral to the control of its growth and toxin formation in foods. From the 1920s to the present, thermal processing has been used to control *C. botulinum* in canned and many categories of foods. The classic investigation on the heat resistance of *C. botulinum* spores was carried out by Esty and Meyer in the 1920s (112) in California as a result of the commercial outbreaks of botulism in canned foods. They examined 109 type A and B strains at five heating temperatures over the range 100 to 120°C (212 to 248°F). They found that the initial inactivation rate was logarithmic between 100 and 120°C and depended on the spore concentration, the pH, and the heating menstruum. As with thermal treatments of other organisms, tailing and a plateau are typically observed after the initial several logs of inactivation. The use of a reproducible system is valuable in periodically determining the heat resistance of existing and new spore isolates and spore crops prepared in the laboratory. Extrapolating the data of Esty and Meyer gives a maximum value of $D_{121.1°C}$ of 0.21 min for *C. botulinum* type A and B spores in phosphate buffer (112, 149). Owing to these seminal studies, the thermal processing industries have used $D_{121°C}$ as a standard in calculating process requirements. Spores of nonproteolytic *C. botulinum* types B, E, and F have much lower heat resistance (100-to 1,000-fold) than spores of proteolytic type A and B strains. Typically, type E spores have a $D_{70°C(158°F)}$ varying from 29

to 33 min, a $D_{80°C(176°F)}$ of 0.3 to 3 min, and a $D_{100°C(212°F)}$ of <0.1 to 0.5 min depending on the strain. The *z* value ranges from 13 to 15°F. Spores of nonproteolytic *C. botulinum* type B have heat resistance higher than that of type E spores yet lower than that of spores from proteolytic strains. Scott and Bernard (191) determined that the $D_{82.2°C}$ of spores of nonproteolytic type B strains ranged from 1.5 to 32.3 min, compared with a $D_{82.2°C}$ of 0.33 min for a type E strain. Media containing lysozyme can enhance recovery of group II *C. botulinum* spores, because lysozyme substitutes for spore lytic enzymes that are inactivated by heat (192). *D* values at 85 and 95°C were 100 and 4.4 min, respectively, for spores of strain 17B and 45.6 and 2.8 min, respectively, for spores of strain Beluga E recovered on media containing lysozyme.

The thermal resistance of *C. botulinum* spores is strongly dependent on environmental and recovery conditions. Heat resistance is markedly affected by acidity, and spores have lower heat resistance at low pH values (149). Esty and Meyer (112) found that spores had maximum resistance at pH 6.3 and 6.9, and resistance decreased markedly at pH values below 5 or above 9. Increased levels of sodium chloride or sucrose resulting in decreased aw increased the heat resistance of *C. botulinum* spores (166). Sugiyama (193) found that spores grown in media containing fatty acids exhibited increased heat resistance. *C. botulinum* spores coated in oil were more resistant to heat (194). As with *Bacillus* spp., sporulation of *C. botulinum* at higher temperatures results in spore crops with greater heat resistance (25, 195), possibly related to acquired thermotolerance due to heat shock proteins (195). Compared to *Bacillus*, the compositional factors contributing to heat resistance of *C. botulinum* spores are not well defined. The factors described for heat resistance for *Bacillus* outlined in the first part of this chapter are found in *C. botulinum*, i.e., reduced water content, SASP, mineral deposition and matrix, and DPA content; however, undefined factors also likely contribute to sporulation and heat resistance. Zinc ions in the medium enhanced the sporulation of proteolytic *C. botulinum* type B (196). As a strict anaerobe, *C.*

botulinum grown in medium with a low redox potential can accumulate reduced transition metals such as $Fe^{2+}$and Cu^{2+}, which resulted in enhanced heat sensitivity in laboratory conditions (197). A supply of hydrogen gas in the heating menstruum led to more rapid spore inactivation (198). It is likely that production of gases, including hydrogen and carbon dioxide, and organic acids in anaerobic metabolism also affects heat resistance (198).

Recently, several studies have described the diversity in thermal resistance of *C. sporogenes*, which is closely related to group I *C. botulinum*. Although closely related to proteolytic *C. botulinum* type A, *C. sporogenes* spores usually have a much higher heat resistance (approximately 7-to 10-fold-higher resistance depending on the strain). Consequently, *C. sporogenes* is often used as a conservative surrogate for *C. botulinum* in thermal processing. In a recent study, two clades of *C. sporogenes* were identified based on pulsed-field gel electrophoresis and whole-genome sequencing (151). Clade I had a lower heat resistance than clade II, while strain PA3679 in clade 2 produced spores that have about a 10-foldincreased heat resistance relative to *C. botulinum* type A spores, and strains from this clade have been widely used in validation of thermal food processes. A meta-analysis of data collected from 38 studies indicated that PA3679 had significantly higher heat resistance than proteolytic *C. botulinum*. The *D* values at 121.1°C were estimated to be 0.19 min for *C. botulinum* type A and 1.28 min for *C. sporogenes*, while the *z* values were very similar at 11.3 and 11.1°C for the two species (151). Genomic studies were conducted to reveal genes responsible for heat resistance in *C. sporogenes* compared to *C. botulinum* (25, 199). The most significant difference was the acquisition of a second *spoVA* operon, which is responsible for transport of DPA into the spore core during sporulation. Advanced genetic and genomic tools previously not available for *C. botulinum* and related clostridia, including clustered regularly interspaced short palindromic repeat (CRISPR)-Cas systems and transcriptomics and genome mining, should lead to a greater

understanding of heat resistance in this critical group of sporeformers playing a role in food safety.

Nonthermal Processing Methods for Control of Sporeformers

Several nonthermal methods and sporicidal chemicals have been evaluated for control of sporeformers in foods and food contact surfaces (83–85, 87, 200–202). One of the most common newer technologies being used in industry is HPP and especially HPP combined with thermal processing (HPTP). HPP and HPTP are being widely introduced into the food industry and are emphasized here. Other methods of control of sporeformers have been thoroughly described in expert treatises (201, 203).

Fundamental studies of high pressure have been performed with the model organism *B. subtilis*, as described in the first section of this chapter. Applied studies for food safety and prevention of spoilage have mainly been directed at spores of *C. botulinum* groups I and II and certain spoilage organisms, such as *Alicyclobacillus acidoterrestris*, as described in this section. Sporicides such as chlorine compounds have been evaluated for inactivation of spores, and most fundamental studies have been conducted with *B. subtilis*. Several expert treatises have addressed sporicides for food preservation (201–203). This section emphasizes inactivation of spores of *C. botulinum*, since it is the most important sporeformer with regard to severity of foodborne illness and regulatory actions.

Traditional Physical Methods and Sporicidal Chemicals

Gamma Irradiation and Oxidizing Chemicals Spores of *C. botulinum* groups I and II, as well as other sporeformers, are highly resistant to gamma irradiation, compared to vegetative cells of most microorganisms (201–204). In many foods, it is impractical to inactivate spores by irradiation. Moreover, due to consumer concerns about irradiation and its required labeling, its application

is limited to certain foods or ingredients, such as spices (205). *C. botulinum* spores have a *D* value of 0.1 to 0.45 megarads (2.0 to 4.5 kGy). *C. botulinum* spore gamma radiation resistance varies depending on the serotype and strain; proteolytic types A, B, and F are most resistant (202). UV irradiation has been used to control vegetative bacterial and fungal spoilage on certain food products, including fruits. However, UV treatment needs to be used with caution, as it was shown to enable rapid growth of *C. botulinum* and toxin formation on melons treated with UV to prevent mold spoilage (206), highlighting the importance of the food microbiota in controlling this pathogen.

C. botulinum spores can be inactivated by ethylene oxide, halogen sanitizers, and hydrogen peroxide (201–203). Hydrogen peroxide is commonly used for sanitizing surfaces in aseptic packaging, and halogen sanitizers are used in cannery cooling waters. Alternatives to hydrogen peroxide such as peracetic acid have received consideration due to the deleterious effects of hydrogen peroxide on packaging equipment and materials. The assessment of biocides and food preservatives in sporicidal efficacy has been reviewed (201–203).

Biological Control of Sporeformers

In many food systems, sporeformers, including *C. botulinum*, do not compete well with the inherent microbiota in the food microbiome. The mechanisms governing the inability of *C. botulinum* to compete with inherent microbiota in foods and in the adult intestinal tract are not well defined, but they likely include competition for nutrients, inherent inhibitory substances such as bacteriocins and defensins in foods and the gastrointestinal tract, and in foods the lowering of the pH to a point at which *C. botulinum* cannot grow and form botulinum neurotoxin (207–210). In addition to these factors, in the gas trointestinal tracts of animals and humans, immune responses are elicited in response to pathogens, which adds an important dimension not observed in foods.

One of the most effective methods for control of *C. botulinum* and other pathogens in foods is the addition of or production of lactic acid and other fermentative inhibitory metabolites, including proteinaceous bacteriocins (105, 207, 209, 210). The production of acids generally requires a source of fermentable carbohydrates in the food which is converted to organic acid end products, lowering the pH and preventing growth and toxin formation. When foods are processed to inactivate vegetative lactic acid bacteria and other inhibitory bacteria (e.g., by pasteurization and HPP), this can lead to a permissive environment for pathogens to grow. The ability of competitor bacteria and fungi to inhibit toxin formation has been illustrated in various food systems (207, 208, 210). Competitor organisms, their succession and growth in foods, and the inhibitory mechanisms are an important area for development of food safety and quality systems. With advanced methods, including denaturing gradient gel electrophoresis-PCR, length heterogeneity-PCR, metagenomics, and other advanced genetic methods, potentially combined with traditional culture techniques, there is the potential to determine changes in microbiota in foods and reach a more in-depth understanding of the organisms important in competitive control (91, 102, 138, 211–214).

Innovative Processing Technologies

Newer preservation procedures are increasingly being adapted and employed by industry (83–87). These technologies include high pressure with or without thermal treatment, pulsed electric fields, radiofrequency, ultrasound, acoustic technologies, cold plasma, membrane filtration, and new chemical sporicidal chemical systems. The description and efficacy of allof these technologies are outside the scope of this chapter, and the emphasis here is on HPP, as this technology has widely been incorporated into food processing (86).

HPP and HPTP

The most common high-pressure system combines pressure and thermal processes (HPTP). HPTP processors typically use temperatures from ambient to >100°C and pressures of 100 to 900 MPa (86, 87). Exposure times can range from a few seconds to over 20 min but are usually 3 to 5 min. HPP generally kills vegetative cells of yeast, molds, and bacteria and can extend the shelf life of the foods and avoid destruction of nutrients and organoleptic properties, which may occur with thermal processing. The product is usually packaged in a flexible container such as a patch or plastic bottle, although spoil multilayer plastic and aluminum packaging is also used. The product is processed in a high-pressure chamber filled with a pressure-transmitting hydraulic fluid (usually water).

Vegetative pathogens and yeasts and molds in foods can generally be eliminated with process pressures of 200 to 800 mPa at ambient temperatures (85, 86). HPP and HTPT inactivate pathogens and spoilage organisms, including *Salmonella*, *Escherichia coli* O157:H7, *Listeria* spp., *Campylobacter* spp., and *Staphylococcus aureus* (85–87). Overall, HPP and HPTP inactivate microorganisms in the following order: mold and yeast vegetative cells >Gram-negative bacteria >Gram-positive bacteria >bacterial spores. The mechanisms of inactivation probably include cytoplasmic membrane and cell wall damage, essential enzyme inactivation by noncovalent disruptions, and DNA damage. Similar to thermal processing, inactivation of organisms by HPP and HPTP usually exhibits nonlinear kinetics, possibly due to a heterogeneous population of organisms. The efficacy of inactivation of vegetative cells and spores is dependent on the food commodity.

Research on HPP and HPTP for inactivation of bacterial sporeformers has mainly been performed with species of *Bacillus*, *Clostridium*, and *Alicyclobacillus*. The primary food safety target of HPP and HPTP in many foods is the inactivation of spores of *C. botulinum*, and several studies in laboratory media and in foods in contained facilities have been performed (215–218). Interestingly, inactivation of spores by HPP and HPTP does not correlate with inactivation by thermal processing for *C. botulinum* and certain

other sporeformers. For example, *C. botulinum* type B spores are more resistant to HPTP than type A spores (218), which contrasts with susceptibility to thermal processing. *C. botulinum* type E spores are less resistant than nonproteolytic type B and F spores to HPTP (219). In addition to vegetative spoilage organisms, spores of the important spoilage organism *A. acidoterrestris* can be inactivated by HPTP (220). Further experiments are necessary to evaluate the inactivation of other spoilage sporeformers by HPP and HPTP.

Currently, the processing factors used in HPP and HPTP are mainly empirical in the inactivation of sporeformers. In contrast to thermal processing, concise models and practical parameters are not yet precisely defined. With certain pathogens, particularly *C. botulinum*, it is not permitted in the United States to conduct research on HPP except in facilities that are approved for work with select agents, which limits the availability of data for quantitative analysis and modeling. Nonpathogenic surrogates of *C. botulinum* would be useful for evaluation of pathogen survival of HPP and HPTP processes. However, unlike the utility of established surrogate systems for thermal processing (for example, the use of defined strains of *C. sporogenes*), surrogates are only in development for HPP treatments. The ideal surrogate would be a strain that is isogenic except for the deletion of pathogenicity genes, e.g., deletion of the gene encoding botulinum toxin in *C. botulinum*. With the development of new genetic tools, such as CRISPR-Cas9 systems, these surrogates should be attainable in the near future and could be evaluated in facilities that do not handle select agents.

Additional Innovative Technologies for Control of Sporeformers

Other technologies are being explored and have been implemented in sectors of the food industry. These technologies include electric and electromagnetic fields, pulsed electric fields, plasma, and ultrasonics (84, 85, 87). Generally, these methods are effective in

inactivating vegetative cells but are relatively ineffective compared to heat in killing spores.

IMPORTANCE OF BIOFILMS IN CONTROL OF SPOREFORMERS IN FOODS AND MANUFACTURING

Biofilms have been increasingly recognized as an important area for food safety and quality (221–223). Bacterial spores are hydrophobic and attach to food contact surfaces, which can lead to biofilms (224). These structures have enhanced resistance to inactivation by chemicals and physical processes. Biofilms of pathogens and spoilage bacteria are generally more resistant to cleaning and sanitation procedures than planktonic cells (221, 222). Fundamental studies on the formation and properties of biofilms have been conducted with *B. subtilis* (223), and these can serve as a conceptual model for biofilms of food-related sporeformers.

SPOILAGE OF FOODS BY SPOREFORMERS

Bacteria and fungi cause enormous economic losses of foods by spoilage (72, 73). It has been estimated that 20 to 30% of commercial foods undergo microbial spoilage with associated loss of consumption. With the global increase in population and the need for sustainable food resources, technologies to prevent spoilage would greatly help to alleviate food shortages and spoilage and contribute to food security and sustainability. The physiological states of sporeformers contribute to their ability to grow in and spoil foods (137). Spore-forming bacteria and fungi are responsible for spoilage of several classes of foods. The primary genera of sporeformers causing spoilage of foods are *Anoxybacillus*, *Alicyclobacillus*, *Bacillus*, *Clostridium*, *Desulfotomaculum*, *Geobacillus*, *Paenibacillus*, *Thermoanaerobacter*, *Moorella*, and *Sporolactobacillus* (68, 75, 76, 89).

Spoilage sporeformers can be divided into various genetic and physiological groups depending primarily on their growth temperature, acid resistance, and spoilage manifestations. The principal groups of sporeformers causing spoilage are thermophilic organisms causing flat sour spoilage, thermophilic anaerobes not producing hydrogen sulfide, thermophilic anaerobes forming hydrogen sulfide, putrefactive anaerobes, facultative *Bacillus* mesophiles, butyric clostridia, lactobacilli, and heat-resistant molds and yeasts (68, 88, 89, 93). Spoilage sporeformers have certain physiological properties that affect their propensity to spoil foods. *Bacillus* species are aerobic and generally not osmotolerant. Some *Bacillus* species and strains are psychrotrophic, including *B. cereus*, which can spoil refrigerated dairy products; others, such as *B. subtilis*, are mesophilic and can spoil bakery products. Psychrophilic strains of *Bacillus* have been isolated from spoiled dairy products (225). Thermophilic sporeformers can spoil canned foods and foods in hermetically sealed packages (89).

Clostridia range from being strict anaerobes to mildly aerotolerant, and most species are not osmotolerant, although *C. sporogenes* can grow at an aw of ≥0.93. Clostridia generally spoil foods with low oxygen-reduction potential, such as canned or vacuum-packaged foods. However, the microbiota of a food can rapidly utilize oxygen and lower the oxidation potential, allowing clostridia to proliferate in many foods, including products produced in an aerobic atmosphere. The main *Clostridium* species causing spoilage are *C. sporogenes*, *C. butyricum*, and *T.* (*C.*) *saccharolyticum* (89).

Table 2.6 Spoilage of canned foods by sporeformers[a]

Type of spoilage	pH	Major sporeformer(s) responsible	Spoilage defects
Flat sour	≥5.3	*B. coagulans*, G. *stearothermophilus*	No gas, pH lowered. May have abnormal odor and cloudy liquor.
Thermophilic anaerobe	≥4.8	*T. thermosaccharolyticum*	Can swells, may burst. Anaerobic anaerobe and products give sour, fermented, or butyric odor. Typical foods are spinach and corn.
Sulfide spoilage	≥5.3	*D. nigrificans, C. bifermentans*	Hydrogen sulfide produced, giving rotten egg odor. Iron sulfide precipitate gives blackened appearance. Typical foods are corn and peas.
Putrefactive anaerobe	≥4.8	*C. sporogenes*	Plentiful gas. Disgusting putrid odor. pH often increased. Typical foods are corn and asparagus.
Psychrotrophic clostridia	≥4.6		Spoilage of vacuum-packaged chilled meats. Production of

			gas, off flavors and odors, discoloration.
Aerobic sporeformers	≥4.8	*Bacillus* spp.	Gas usually absent except for cured meats; milk is coagu-lated. Typical foods are milk, meats, and beets.
Butyric spoilage	≥4.0	*C. butyricum*, *C. tertium*	Gas, acetic and butyric odor. Typical foods are tomatoes, peas, olives, and cucumbers.
Acid spoilage	≥4.2	*B. coagulans* subsp. *thermoacidurans*	Flat (*Bacillus*) or gas (butyric anaerobes). Off odors depend on organism. Common foods are tomatoes, tomato products, and other fruits.
	≥4	*A. acidoterrestris*	Flat spoilage with off flavors. Most common in fruit juices and acid vegetables and also reported to spoil iced tea.

Acidic canned foods often receive a pasteurization treatment to inactivate vegetative spoilage organisms. Spoilage of acid canned foods is carried out by relatively few species of sporeformers (89). The primary organisms of concern in high-acid foods (≤pH 3.8) are yeasts and *A. acidoterrestris*, particularly in juices (68, 76). Moderately acidic canned foods in the pH range of 3.8 to 4.5 include foods such as canned fruits in syrups and tomatoes. Certain species

of *Clostridium*, including *Clostridium pasteurianum*, *C. sporogenes*, *C. tyrobutyricum*, and *C. beijerinckii*, as well as the species *Paenibacillus polymyxa*, *Bacillus coagulans* subsp. *acidurans*, and *Bacillus coagulans* (*Lactobacillus sporogenes*), have been isolated from moderately acidic canned foods (76, 89). Certain strains have been characterized as being extremely acid tolerant (89). Prominent bacterial thermophilic (>55°C) sporeformers include *G. stearothermophilus*, *D. nigrificans*, *Thermoanaerobacterium thermosaccharolyticum*, and *Moorella thermoacetica* (68, 73, 88) (Table 2.6).

Alicyclobacillus species are highly acid tolerant and grow at low pHs of 2.0 to 6.0 (220). They are also moderately thermophilic but not osmotolerant, growing only above a water activity of 0.98. *Alicyclobacillus* has been recognized as a prominent spoilage organism of acidic products, such as pasteurized fruit or vegetable juices that are improperly cooled or stored at high temperatures (75, 76, 88).

Acidic and acidified foods with an equilibrium pH of ≤4.6 are generally not processed sufficiently to inactivate all spores, since most species of sporeformers do not grow under acid conditions and inactivation of allspores would be detrimental to food quality and nutritional composition. Certain foods, such as cured meats and hams, do not undergo a thermal process sufficient to inactivate sporeformers and thus must be kept under refrigerated conditions for microbial stability. These classes of foods present opportunities for the growth of sporeformers which do not present a public health hazard but which can cause spoilage that results in economic losses (68, 76). Most of the spoilage organisms and their characteristic spoilage patterns have been recognized for many years (89).

Heat-resistant fungi and yeasts are spoilers of acidic foods with economic significance, particularly fruit-derived and high-sugar products (92, 93). While most filamentous fungi and yeasts are killed by heating for a few minutes at 60 to 75°C, heat-resistant fungi produce thickwalled ascospores that survive heating at ca.

85°C for 5 minutes (94). The most common genera of heat-resistant fungi causing spoilage are *Byssochlamys*, *Neosartorya*, *Talaromyces*, and *Eupenicillium* (93, 94). Certain heatresistant fungi also produce toxic secondary metabolites collectively referred to as mycotoxins (89, 93).

Practical control of spoilage sporeformers includes monitoring of raw foods entering the cannery, particularly sugars, starches, spices, soil-borne foods such as onions, garlic, and mushrooms, and dried foods (76, 89). Dry ingredients such as sugar, starches, flours, and spices often contain high levels of sporeformers (89, 205). Spore populations can also accumulate in a food plant, such as thermophilic spores on heated equipment and saccharolytic clostridia in plants processing sugar-rich foods, such as fruits. A goal is to limit the initial spore load in a food product. Adequate thermal processing depends on rapid cooling of products, chlorination of cooling water, and implementing and maintaining good manufacturing practices within the food plant; appropriate storage and distribution conditions are also required. In practice, the inherent spore contamination of foods and food ingredients and of the cannery environment contributes to spoilage problems (76, 89). Development of processing methods to prevent or minimize spoilage depends on physical and chemical methods to inactivate the target organisms and on formulation of foods and the use of microbial inhibitors to prevent growth.

SPOILAGE OF SPECIFIC FOOD CLASSES BY SPOREFORMERS

Dairy Ingredients and Finished Products

Thermophilic sporeformers can be a majorcause of spoilage in the manufacture of dairy ingredients (68, 76, 89, 134). Of particular Importance in dairy ingredient manufacture are the sporeformers *G. stearothermophilus*, *Geobacillus thermoleovorans*, and *Anoxybacillus flavithermus*. The presence of these spoilage

thermophiles can lead to off flavors and textures through their formation of degradative enzymes, and these organisms are indicators of poor hygiene, particularly in heated areas of the plants, such as evaporators. Many thermophilic Bacilli form biofilms that are difficult to control by cleaning in place and other hygienic procedures. *C. tyrobutyricum* and related organisms can cause gas formation "blowing" and butyric acid odors in hard cheeses. The presence of the pathogens *C. botulinum*,*C. perfringens*, *B. cereus*, and potentially *C. difficile* is a concern in finished dairy products, particularly infant formulas and medical foods (68). Heat-resistant clostridia, Including *C. sporogenes* and *T. thermosaccharolyticum*, can cause swelling through gas production and off flavors through production of degradative enzymes in canned milk products that have undergone ultrahigh-temperature treatment (89). Fermented milk and products such as cheeses have been spoiled by clostridia, including *C. tyrobutyricum*, *C. butyricum*, *C. sporogenes*, and *C. beijerinckii*. *Paenibacillus* and *Bacillus* can cause spoilage of pasteurized milk. Sterilized milk can be spoiled by *Bacillus stearothermophilus*, and dehydrated milk by *G. stearothermophilus*.

Refrigerated Meat Products

Psychrotrophic clostridia can spoil refrigerated meat products in several ways, including via the production of noxious gases such as hydrogen sulfides, formation of putrefactive amines, formation of slime, and protein and carbohydrate degradation (135, 226–228). They can also cause swelling of cans and packages. The primary Species involved in ready-to-eat meat and poultry spoilage are *Clostridium laramie*, *C. estertheticum*, *C. gasigenes*, *C. butyricum*, and *C. pasteurianum*. In particular, *C. estertheticum* and *C. gasigenes* can cause "blown-pack" spoilage, which occurs in refrigerated batches of vacuum-packaged meats and involves the production of large quantities of gas, putrid odors, and a metallic sheen on the meat. Me sophilic bacilli such as *B. coagulans* can also cause off odors described as medicinal and phenolic.

Fruit and Vegetable Juices

Sporeformers are a serious cause of spoilage in several types of fruit and vegetable juices (76, 89, 229). *Alicyclobacillus* spp. have been recognized as a particular problem, leading to medicinal off flavors. Souring and gas formation also occur in dairy-based products by thermophilic bacilli and clostridia. Shelf-stable high-acid products can be spoiled by various clostridia and bacilli, including *C. pasteurianum*. These sporeformers can also carry out gas formation and acid production in fruitcontaining products, such as pastries.

Bakery Products

Various *Bacillus* spp., including *B. amyloliquefaciens*, *B. subtilis*, *B. pumilus*, and *B. cereus*, are recognized as causing a defect in bakery products known as ropiness, in which fruity odors such as that of overripe melons are produced (68, 76). This is followed by enzymatic activities leading to discoloration, changes in texture, and stickiness due to the production of extracellular polysaccharides. Spores are commonly present in flours and other raw ingredients and introduced into the dough, and they survive the baking process and then proliferate and cause the ropiness. This type of spoilage can mostly be prevented by careful monitoring of raw ingredients for the presence of aerobic sporeformers.

Detection of Food Spoilage Sporeformers

Physical, chemical, and microbiological methods for determination of food spoilage have been described (68, 75, 76, 89, 134). Safety procedures used in working with spore-forming pathogens, including *C. botulinum* and *B. anthracis*, need to be carefully controlled and tested in an approved laboratory (70). There was a tremendous impact on the industry from a massive recall of infant formula that may have contained *C. botulinum* spores (unpublished data). This incidence of the presumptive presence of *C. botulinum* spores in dried infant formula produced in New Zealand and the

resulting massive recall of products highlight the importance of rapid and definitive detection methods for high-risk pathogens.

Traditionally, culture methods have been used for detection of spores, but culture-independent molecular methods are being adapted for detection of sporeformers in foods (133, 134). These include protein-based methods for surface molecules, including immunobased methods such as enzyme-linked immunosorbent assays. Currently, there is a trend of utilizing approaches linked to nucleic acid detection, such as PCR and high-throughput and next-generation DNA sequencing. Most protein and DNA methods generally require germination of spores, as intracellular proteins and nucleic acids are difficult to isolate from intact dormant spores, which may limit the utility of *in situ* detection methods.

SPORES AS PROBIOTIC AGENTS

Species of *Bacillus* and *Clostridium* have been used for decades as probiotic agents to purportedly prevent disease and improve health of animals and humans (230–237). Their primary benefits have been stated to be inhibitory actions towards pathogens in the gastrointestinal tract, and the sporeformers may also stimulate beneficial immune responses. The mechanisms of persistence and inhibitory action towards pathogens have not been elucidated, but they may include competition for adherence and nutrients, production of inhibitory metabolites, and avoidance of innate and antibody-mediated immune responses. For use in animals, including poultry and monogastric animals, spore probiotics have been demonstrated to enhance food conversion and probably affect pathogen infection. The safety of aerobic endosporeforming bacteria is critical for their use as probiotics (234, 235, 237, 238). Methods for safety assessments have been outlined. Antibiotic resistance, genetic modification, and gene transfer in the gastrointestinal tract are among major concerns. Several commercial companies are investigating *Bacillus* spp. as probiotics for humans and animals.

MODELING OF SPOREFORMER GROWTH IN FOODS AND ASSESSMENT OF SAFETY

Abundant information exists on the behavior of microorganisms in foods, and it is often useful to generate statistical models to quantify safety risks in foods. Two general types of models have mainly been used in food microbiology: (i) those analyzing experimental growth and survival data using simple and higher-order polynomials and (ii) theoretical models derived from basic scientific principles and computer analysis, used to predict microbial survival. Statistical models can be particularly useful to define important variables and predict microbial behavior in advance of practical testing.

The safety of thermally processed low-acid foods can be enhanced by application of risk management programs, including HACCP, FSO, and stepwise and interactive evaluation of food safety by an expert system, among others (123, 144, 239). The Food Safety Modernization Act incorporates measures of safety and spoilage by sporeformers. These systems entail a systematic and quantitative risk assessment program to ensure the safety of foods. They were designed to have strict control over all aspects of the safety of food production, including raw materials, processing methods, the food plant environment, personnel, storage, and distribution. In practice, the identification of potential hazards for a given process and meticulous control of critical control points are required. Methods for HACCP and FSO quality assurance programs for thermally processed foods have been expertly reviewed (123, 142).

Various modeling strategies have been used in assessing microbiological growth and phenotypic properties of microorganisms in foods (123, 144). Certain companies and institutions use advanced models such as neural nets. These can be valuable, since they are adaptive and improve in precision and predictive capability over time. An example of a model used extensively in the dairy industry is that of Tanaka et al. (240) for

preventing growth of *C. botulinum* in processed cheese. Although models can provide guidelines for food safety, the assurance of sterility generally needs to be ascertained by laboratory challenge studies (105, 123, 144).

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3
Microbiological Criteria and Indicator Microorganisms

The concepts and principles for the establishment of microbiological criteria (MCs) were elaborated in the mid-1980s by the International Commission on Microbiological Specifications for Foods (ICMSF) (1). These concepts have been used to develop recommendations for criteria for foods in international trade or for criteria specific to pathogens, such as *Listeria monocytogenes*, in foods (2, 3). They are also the basis of the Codex Alimentarius Commission document *Principles and Guidelines for the Establishment and Application of Microbiological Criteria Related to Foods*, first issued in 1997 (4) and revised in 2013 (5). This document defines an MC as a risk management metric which indicates the acceptability of a food or the performance of either a process or a food safety control system following the outcome of sampling and testing for microorganisms, their toxins and metabolites, or markers associated with pathogenicity or other traits at a specified point of the food chain.

While the Codex Alimentarius Commission currently recognizes only the general category "microbiological criterion," other national, transnational, and international organizations, trade associations, and other stake holders in the food chain often describe microbiological standards, guidelines, or specifications that can be differentiated as follows:

- *Microbiological standard* is a mandatory criterion included in a law or a regulatory ordinance.

- *Microbiological guideline* is an advisory criterion issued by either authorities, industry associations, or food manufacturers. Such guidelines are indicative of what can be expected for certain microbiological parameters when a food is manufactured according to best hygienic or manufacturing practices.
- *Microbiological specification* is an element of purchasing agreements between a buyer and a supplier of a raw material or a food product. Its use may be mandatory or advisory depending on the agreements between the two parties.

The general category "microbiological criterion" recognized by the Codex Alimentarius Commission covers both MC utilities related to the safety and acceptability of discrete lots of food products and MC applications concerning food hygiene and process assessment and monitoring (6, 7). Interestingly, the European Commission (EC) has split these two types of utility and recognizes "food safety" criteria next to "food hygiene process" criteria (8). The consequences in case of noncompliance are in principle similar for the two different utilities in both the Codex Alimentarius Commission and EC contexts. Lots that fail to meet MCs that target human pathogens specifically (i.e., food safety criteria) are considered not suitable to be placed on the market, while food lots and/or food operations failing criteria for microorganisms or groups of microorganisms that are not human pathogens or may include such pathogens as a subfraction are not deemed unsafe as such, but they would require, for instance, increased scrutiny of hygiene and processing measures and of raw materials as well as finished goods as appropriate to ensure operational control over food safety. The term "MC" is used throughout this chapter, since the underlying principles for their establishment and application are valid for all three subcategories mentioned above as well as for criteria specific to safety or hygiene/process control.

MCs have traditionally been developed around significant pathogens, relevant commensals, and hygiene indicators as

reflected in the ICMSF cases, which were first published in 1986 (1) and more recently updated (9, 10). They are widely used today to discriminate between acceptable and unacceptable lots of food products.

In order to address the need for science-based risk assessment, the ICMSF has explored the potential applications of risk assessment techniques to microbiological issues (11). This initial work has triggered an evolution from hazard-based to risk-based food safety management, as observed during the last decade. Numerous discussions have since then taken place to develop the concepts further. These activities have culminated in the publication of the document *Principles and Guidelines for the Conduct of Microbiological Risk Management* (MRM) by the Codex Alimentarius Commission (12).

The evolution of the traditional metrics, including MCs, to include additional risk-based metrics has taken place over many years and has allowed a better understanding of the utility, performance, and limitations of MCs. These elements are discussed below, along with the role of microbiological indicators.

MRM METRICS

Details on the establishment and implementation of microbiological risk management (MRM) metrics are outlined in Annex II of *Principles and Guidelines for the Conduct of Microbiological Risk Management (MRM)*, adopted by the Codex Alimentarius Commission in 2007 (12). The scope of this document was to provide a framework for the MRM process to the Commission as well as to Commission members and member organizations. The purpose was also to provide guidance to the food industry and other stakeholders who design, validate, and implement control measures ensuring the manufacture of safe food that consistently achieves the targets defined in the MRM metrics.

The principles for their establishment and implementation are outlined as follows in the document (12) and are applied in addition to those described in the guidelines themselves.

- The establishment and implementation of MRM metrics should follow a structured approach, with both the risk assessment phase and the subsequent risk management decisions being fully transparent and documented.
- MRM metrics should be applied only to the extent necessary to protect human life or health and set at a level that is not more trade restrictive than required to achieve an importing member's appropriate level of protection.
- MRM metrics should be feasible, appropriate for the intended purpose, and applied within a specific food chain context at the appropriate step in that food chain.
- MRM metrics should be developed and appropriately implemented so that they are consistent with the requirements of the regulatory and/or legal system in which they will be used.

Metrics used by competent authorities to express the expected level of control have been based traditionally on the following criteria:

- The *product criterion* (PdC) specifies chemical or physical characteristics of a food (e.g., pH and water activity), which affect the safety of a food product. Different elements are related to (and impact) a PdC, such as the frequency and level of contamination, the effectiveness of control measures, the conditions under which the product will be used, and other parameters ensuring that it will not contain the pathogen at an unacceptable level when the product is consumed. Each of the factors that may impact the effectiveness of a PdC has to be considered in a transparent manner.

- The *process criterion* specifies the conditions of treatments to be applied at a specific step of the manufacturing process to achieve the targeted level of control of a microbiological hazard. Again, there are a number of parameters, such as the initial level of the target pathogen, the type and resistance of the target pathogen, and the impact of the food matrices, that influence the expected effect.
- The MC is used for the examination of foods at a specified point of the food chain to determine the compliance of a food with a pre-established limit. Such a criterion can be used as a direct control measure for manufactured lots or, in the framework of a food safety control system, to periodically verify that the system is functioning as intended. Elements pertaining to an MC are defined in "Introduction to MC and Sampling Plans" below and also require the definition of necessary actions to be taken should a tested (lot) product exceed the established MC.

These metrics have existed for a long time but have been, and are still, evolving over time. This is linked to the shift of the management of food safety issues from a hazard-based to a risk-based approach. Thus, the evolving focus for managing such issues has led to the establishment by the Codex Alimentarius Commission of more-quantitative risk-based metrics outlined below.

Food Safety Objectives

The food safety objectives (FSO) metric expresses the maximum frequency and/or concentration of a pathogen in a food at the time of consumption that provides or contributes to the appropriate level of protection defined by a government. This risk-based limit, established by competent authorities, should be achieved operationally within the food chain, while flexibility is given to individual elements such as primary production or manufacturing.

While the FSO represents the final outcome, it does not specify how individual steps will contribute.

Performance Objectives

The performance objectives (PO) metric represents an operational risk-based limit at a specific point in the food chain, i.e., the concentration of a microbiological hazard in a food at that point that should not be exceeded in order to achieve the established FSO. Since it contributes to achieving an FSO, a PO should be considered and established in the light of previous and subsequent steps and their corresponding POs. An industry may find it beneficial to establish its own POs, taking into account its position within the food chain, the impact of subsequent steps, including the intended use of the end product, and the degree of risk it is willing to tolerate.

Performance Criterion

The performance criterion (PC) expresses the outcome achieved by a single control measure or a combination of control measures, usually microbicidal and/or microbiostatic control measures. This metric articulates the desired reduction and/or maximum acceptable increase of the hazard. The PC usually describes the required outcome to achieve the defined PO. The PC needs, therefore, to take into account variability of pathogens, food composition, and processing parameters along the segment of the food chain for which the PO has been established.

INTRODUCTION TO MC AND SAMPLING PLANS

The groundwork for the establishment of MCs within the global regulatory food safety management context was initially developed during joint consultations of the World Health Organization (WHO) and Food and Agriculture Organization (FAO) in the 1990s. The Codex Alimentarius Commission has a role in recommending MCs

at the international level. National governments may choose to adopt Codex MC into their national systems or use them as a starting point for addressing their intended public health goals. National governments also may establish and apply their own MCs. Food business operators may establish and apply MCs within the context of their food safety control systems. Importantly, according to the Codex Alimentarius Commission (5), the need for an MC should be demonstrated, e.g., by epidemiological evidence that the food under consideration may represent a significant public health risk and that a criterion is meaningful for consumer protection.

The outcome of the above long-term consultations has been continuously evolving with the input of, for instance, the ICMSF, national governments, and academic researchers. These efforts resulted in the preparation of a document on the principles for the establishment of MCs, first issued by the Codex Alimentarius Commission in 1997 (4) and revised in 2013 (5). The revision of this document was initiated in 2010 to take into account new developments in food safety management systems and in particular the MRM concepts. General overviews of sampling plans and MCs in the context of microbiological testing, including differences between MCs and FSO/PO, as well as how to operationalize MCs regarding these MRM metrics, have been published (10, 13–16).

Traditionally and historically, MCs have been established to determine whether a lot of product is suitable for commercial distribution and consumption. Acceptability of such a product was defined as the compliance with requirements for certain microorganisms (including parasites) and/or their toxins or metabolites. Requirements have been expressed as absence or as maximal numbers or concentrations of these parameters per unit of mass, volume, area, or lot. MCs have been and are still widely used to make a decision upon analysis of a food for defined parameters (9, 10, 14). The analytical results obtained are compared to the established requirements and serve as a basis to decide whether a lot is acceptable or needs to be rejected.

Importantly, the thinking of the Codex Alimentarius Commission is that the microbiological safety of foods is best managed by the effective implementation of control measures that have been validated, where appropriate, throughout the food chain to minimize contamination and improve food safety. This preventive approach, according to the Commission (5), offers more advantages than sole reliance on microbiological testing through acceptance sampling of individual lots of the final product to be placed on the market. Nevertheless, the establishment of MC may be appropriate for verifying that food safety control systems are implemented correctly.

Whether an MC's utility is for lot acceptance or verification of control, the design of a meaningful MC is essential. It must be considered that the development of such criteria is a complex process requiring the appropriate knowledge and information, resources, and efforts. They should therefore be developed only when there are clear benefits and justified need and when they are effective and practical in serving their purpose (5).

MCs, if deemed necessary and justified, are established to meet one or more objectives, such as those listed below (5, 9, 10):

- evaluating a specific lot of food to determine its acceptance or rejection, in particular if its history is unknown (i.e., port-of-entry assessment)
- providing information to food business operators on microbiological levels which should be achieved when best practices are applied (i.e., good hygiene practices [GHPs], good agricultural practices [GAPs], and good manufacturing practices [GMPs])
- assessing the suitability of a food or raw material for a particular use, such as its consumption or further processing
- verifying the performance of a food safety control system or its elements along the food chain, e.g., prerequisite programs,

HACCP (hazard analysis and critical control point) systems, and/or environmental monitoring schemes

- evaluating the adequacy of particular processing applied to deliver an acceptable product and/or to signal the need for action to avoid loss of control

- verifying the microbiological status of foods in relation to acceptance criteria specified between food business operators and/or for due diligence assessments

- verifying that the selected control measures are meeting risk-based metrics such as POs and FSOs

- detecting potential unforeseen problems in the design and/or operation of a food safety control system

To fulfill these objectives, an MC needs to be established for a specific food or a defined category of food showing similar characteristics, taking into account a number of elements, including the following:

- evidence of recognized or potential hazards to health, e.g., based on epidemiological evidence or reasoning

- microbiological status of the raw materials used to manufacture the food

- effect of processing and other handling on the microbiological status of the food

- likelihood and consequences of microbial contamination during and after processing

- likelihood and consequences of microbial growth during subsequent handling, storage, and use of the food

- intended use of the food as well as possible unintended use

- intended category of consumer as well as others likely to be consuming the food

- costs and benefits associated with the application of the criterion
- point of the food supply chain at which the criterion will apply
- changes (e.g., decrease or increase in numbers) in the levels of the target microorganism likely to occur after the point at which the MC has been set
- relationship to PO/POs established by authorities or industries for particular food supply chains and/or to FSO metrics and/or a defined appropriate level of protection specified by a competent authority

Considering its intended purpose, an MC is then established by translating the general principles above into specific elements of an MC and associated sampling plan, specifying at least the following eight components (5):

- the purpose of the MC
- the food, process, or food safety control system to which the MC applies
- the specified point in the food chain where the MC applies
- the microorganism(s) and the reason for its selection
- the microbiological limits (m or M; see below) or other limits (e.g., a level of risk)
- a sampling plan defining the number of sample units to be taken (n), the size of the analytical unit, and, where appropriate, the acceptance number (c)
- depending on its purpose, an indication of the statistical performance of the sampling plan
- analytical methods and their performance parameters

Consideration should also be given to the corrective action to be taken related to the purpose of the MC when it is not met. Actions

to consider typically include more frequent sampling, inspection, and auditing, further processing, diversion to an alternate use, rework, rejection, containment and disposition, withdrawal, and/or recall. The specific action decided on should be based on an assessment of the risk to the consumer where relevant, the point in the food chain, and the food specified and may consider history of conformance. Food business operators should reevaluate their food safety control systems, including GHPs and operational procedures, and/or carry outfurther investigation to determine appropriate preventive actions to be taken.

In all cases and for any purpose, the microorganisms specified in MCs need to be relevant for the food in question and its process; they may encompass bacteria, viruses, yeasts and molds, algae, parasitic protozoa, and helminths as well as their toxins or metabolites. MCs typically include relevant pathogens, indicators of process hygiene and/or control, and utility or spoilage organisms. The current approach in the formatting of recently issued MCs is to make a clear distinction between food safety-related parameters and process hygiene/control indicators (7, 8, 17).

Given the many relevant objectives and parameters to consider when an MC is being established, it is evident that this is a rather complex task requiring specific expertise as well as good insight and supportive data regarding the food safety management system at hand. One of the overarching important principles of the Codex (5) approach to using MCs for food safety management is that their establishment and application should be based on scientific information and analysis. When sufficient data are available, a risk assessment may be conducted on foodstuffs and their use. However, MCs may also be developed based on empirical knowledge related to GHPs or using scientific knowledge of food safety control within the context of HACCP.

The different elements constituting an MC define the requirements and their stringency. They are usually called sampling plans and are characterized by the parameters n, c, m, and M, which are defined as

follows: *n* is the number of samples that must be analyzed; *c* is the maximum allowable number of defective sample units in a two-class plan or of marginally acceptable sample units in a three class plan; *m* is a microbiological limit that in a two-class plan separates good from defective quality or in a three class plan separates good quality from marginally acceptable quality; and *M* is a microbiological limit that in a three-class plan separates marginally acceptable quality from defective quality.

According to the Codex Alimentarius Commission (5), the required stringency of food safety control systems, including the MC used, should be appropriate to protect the health of the consumer and ensure fair practices in food trade. The MC used should be capable of verifying that the appropriate level of control is achieved.

Sampling plans can be categorized as attributes plans or variables plans (9, 10). The first are the most appropriate when no or little information is available on the performance of the process and hence its impact on the microbial flora. This is typically a situation encountered by regulatory authorities at a port of entry or by manufacturers purchasing raw materials from a new and thus poorly known supplier. Variables plans are suitable only when there is sufficient knowledge on the nature and frequency distribution of microorganisms within lots of a particular food. This type of plan is suitable for manufacturers having a deep understanding of their process and thorough monitoring of their production, such as would be expected from a well-established HACCP program.

Attributes plans can be further subdivided into two class and three-class plans (9, 10, 14). Two-class plans are used to accept or reject individual lots based on the analysis of a specified number of sample units (*n*) using qualitative or quantitative criteria to discriminate lots and to make a decision regarding, for example, populations of a microorganism(s) above or below a defined concentration or presence/absence of a microorganism(s). While two-class plans are usually used for pathogens (6–8, 10, 14), this is not exclusive, and this type of plan exists as well for indicators. An

example is sampling plans for *Enterobacteriaceae* in infant and follow-up formula with the following parameters: $n = 10$, $c = 2$, and $m = 0$ (in analytical units of 10 g) (7). The statistical analyses for evaluating two-class attribute-based sampling plans are different for presence/absence data and for stratified quantitative data (18).

Three-class plans are used in situations where the quality of the product can be divided into three different classes according to the concentrations of microorganisms within the sample units. While analytical results exceeding the limit m are undesirable, in a three-class plan, a limited number of samples as defined by c can nevertheless be accepted. However, results exceeding the upper limit M make the product unacceptable. This is typically used for criteria where quantitative data are stratified into three groupings. The statistical analyses for three-class plans are different from those of two-class plans (9, 10, 14)

PERFORMANCE OF SAMPLING PLANS

The statistical performance of a sampling plan is usually illustrated by its operating characteristic curve, which describes the probability of acceptance as a function of the actual proportion of nonconforming analytical units or concentration of the microorganisms in the food (9 10, 14, 16, 17,19–21). An operating characteristic curve can be used to evaluate the influence of individual parameters of the sampling plan on the overall performance of the plan and as such can be a basis to determine alternative sampling plan approaches delivering equivalent performance.

Sampling plans for finished products are used by authorities or by manufacturers to detect noncompliant lots of products, i.e., lots presenting a defect. The defect rate of manufactured food products relates to the actual fraction of servings that are contaminated (9, 10, 14). This defect rate is an important element to understand in

the context of microbiological sampling and testing activities, whether they are applied to within-lot or to lot-by-lot testing.

Attributes plans have the ability to detect a given level of contamination and thus to reject a nonconforming lot of product with a certain probability. This ability is defined as the performance of the sampling plan and can be calculated using calculation sheets such as the ones published by the ICMSF (http://www.icmsf.org/wp-content/uploads/2018/01/Standard-Program.xlsm), and sampling plan performances are illustrated for the ICMSF cases in Table 3.1. Other organizations have developed sampling tools and guidance to evaluate the performance of sampling plans generally or for specific applications, such as those developed by the Joint Expert Meetings on Microbiological Risk Assessment under the auspices of the FAO and the WHO (22, 23). The characteristics of some of these have been compared (17).

As illustrated in Table 3.1, the performances of sampling plans show that their ability to detect defects at very low rates (i.e., very low levels of contamination) requires quite an investment in resources. Generally, pathogens occur infrequently and at low levels in food. The more effective the implemented hygiene control measures are, the lower the defect rate will be, and the greater the number of samples that would need to be tested in order to increase the probability of detection. However, due to the destructive nature of microbiological testing, the required laboratory infrastructure and capacity, and the associated high costs, testing cannot be expanded indefinitely. A limitation of within-lot testing as performed by authorities—for example, at the port of entry or during distribution—is frequently a lack of information on the conditions under which a particular food was manufactured. In addition, such testing is performed only sporadically, and data are not necessarily used and managed in the most effective way. A useful review on the impact of distribution of microorganisms in foods on the utility and performance of MCs has been published (19), illustrating some limitations as well as misconceptions associated with the statistics for food safety sampling.

Examples of recently issued MCs with attendant probabilities have been published by the Codex Alimentarius Commission for ready-to-eat foods, powdered infant formula, and bottled water (6, 7, 24). Details as well as additional examples are provided elsewhere (14–16, 18, 20, 23). A recent report provides an overview of various MCs and microbiological limits recommended by the European Food Safety Authority (17) in its scientific opinions.

MCs formulated as "absence" of one or more pathogens have been established by some governments and companies and represent a special case. Occasionally, such requirements do not even specify the target pathogen or, even more frequently, do not associate them with any specific sampling plan. As a consequence, such requirements do not express any tangible target or numerical tolerance.

These types of criteria are not compatible with current MRM metrics and therefore do not provide any quantifiable target against which the effectiveness of implemented food safety measures could be compared and measured. The simple term "absence" does not take into account that even sampling plans with c values of 0 and an extremely high number of samples (n) do not ensure the complete absence of a pathogen. A "$c = 0$" parameter also does not take into account the discriminatory power of a sampling plan but rather emphasizes the wish to not detect a specific pathogen in a given set of samples. For example, in the case of a lot defect rate limit of 5%, a sampling plan with the parameters $n = 95$, $c = 1$, and $m = 0$ (in analytical units of 25 g) would be more stringent than a traditional sampling plan of $n = 60$, $c = 0$, $m = 0$ (in analytical units of 25 g), rejecting a greater number of defective lots. However, in addition to increasing the analytical burden, it would give the impression that the presence of the pathogen is being "tolerated," despite the fact that the 95-sample plan is actually more protective.

Additional factors such as the nonhomogeneous distribution of contaminants and lack of technologies or procedures applied during processing to eliminate pathogens do not yet allow the manufacture

of products such as raw meat or poultry to be totally free of pathogens. Considering these limitations, there is a need for a compromise between the desire to achieve “absence” of pathogens and the feasibility of reaching this goal as well as the ability to detect pathogens through the application of sampling plans.

The limitations of sampling plans for finished products to ensure their safety as illustrated in this section emphasize the need to place microbiological testing in the broader framework of the overall food safety management system. To do so, it is certainly important to have a more complete picture of elements and factors contributing to the contamination of food products, as discussed in the following section.

Table 3.1 Examples of the ICMSF cases approach (9, 10), with sampling plans and stringency (case) aligned with degree of risk and conditions of use[a]

Degree of concern relative to utility and health hazard type	Examples	Conditions under which food is expected to be handled and consumed after sampling in the usual course of events which may result in:		
		Reduced risk	No change in risk	Increased risk
Utility: general contamination, reduced shelf life, incipient spoilage	Aerobic colony count, yeasts and molds	**Case 1** (3 class) *n* = 5, *c* = 3, *m* = 1,000/g, *M* = 10,000/g Mean concn, 5,105/g	**Case 2** (3 class) *n* = 5, *c* = 2, *m* = 1,000/g, *M* = 10,000/g Mean concn, 3,282/g	**Case 3** (3 class) *n* = 5, *c* = 1, *m* = 1,000/g, *M* = 10,000/g Mean concn, 1,829/g
Indicator: low, indirect hazard	*Enterobacteriaceae*, generic *E. coli*	**Case 4** (3 class) *n* = 5, *c* = 3, *m* = 100/g, *M* = 1,000/g Mean concn, 511/g	**Case 5** (3 class) *n* = 5, *c* = 2, *m* = 100/g, *M* = 10,000/g Mean concn, 328/g	**Case 6** (3 class) *n* = 5, *c* = 1, *m* = 100/g, *M* = 10,000/g Mean concn, 183/g
Moderate hazard: not usually life-	*Staphylococcus aureus, Bacillus cereus, C.*	**Case 7** (3 class) *n* = 5, *c*	**Case 8** (3 class) *n* = 5, *c*	**Case 9** (3 class) *n* = 10, *c*

threatening, usually no sequelae, normally of short duration, symptoms self-limiting, can result in severe discomfort	*perfringens, Vibrio parahaemolyticus*	= 2, *m* = 10/g, *M* = 100/g Mean concn, 33/g	= 1, *m* = 10/g, *M* = 100/g Mean concn, 18/g	= 1, *m* = 10/g, *M* = 100/g Mean concn, 6/g
Serious hazard: incapacitating but not usually life-threatening; sequelae are rare, of moderate duration	*Salmonella* spp., *L. monocytogenes*	**Case 10** (2 class) *n* = 5, *c* = 0, *m* = 0/25 g Mean concn, 1/55 g	**Case 11** (2 class) *n* = 10, *c* = 1, *m* = 0/25 g Mean concn, 1/178 g	**Case 12** (2 class) *n* = 20, *c* = 1, *m* = 0/25 g Mean concn, 1/495 g
Severe hazard: for the general population or in foods intended for susceptible populations, causing life-threatening or substantial chronic sequelae or illness of long duration	For the general population, *E. coli* O157:H7, *C. botulinum* neurotoxin For restricted populations, *Salmonella* spp., *Crono-bacter* spp.; *L. monocytogenes*	**Case 13** (2 class) *n* = 15, *c* = 0, *m* = 0/25 g Mean concn, 1/328 g	**Case 14** (2 class) *n* = 30, *c* = 1, *m* = 0/25 g Mean concn, 1/854 g	**Case 15** (2 class) *n* = 60, *c* = 1, *m* = 0/25 g Mean concn, 1/2,034 g

[a] Relative performance values of ICMSF cases are illustrated in terms of the mean concentration that will result in rejection with at least 95% probability, assuming hypothetical criteria and a standard deviation of 0.8. Calculations were performed with ICMSF Microbiological Sampling Plan tool, version 2.08 (http://www.icmsf.org/wp-content/uploads/2018/01/Standard-Program.xlsm).

MICROBIOLOGICAL PROFILE OF A FOOD PRODUCT

The microbiota of a food product, including commensals, hygiene indicators, and pathogens, is a result of the history of the product along the whole food chain. It is a dynamic system that evolves through the different steps of the food chain, from primary production through finished product to final preparation and consumption by the final user. The composition of the microbiota of the finished product as well as the levels of individual microorganisms or groups thereof is a function of the initial flora of individual raw materials, which is influenced and evolving at each step of the food chain due to the conditions and factors contributing to their decrease or their increase.

Elements contributing to changes of the microbiota are illustrated in Fig. 3.1 for industrially processed foods. Similar schemes and considerations can also be established for unprocessed or minimally processed foods as well as for other segments of the food chain, such as primary production, distribution, marketing, or the preparation and handling by the final consumer in catering or institutional facilities or even at home.

The fate of individual pathogens along a processing line as illustrated in Fig. 3.1, or any other type of food preparation scheme in the food chain, can be described using the conceptual formula defined by the ICMSF (9, 10, 14):

$$H_0 - \Sigma R + \Sigma I \leq \text{FSO or PO}$$

In this conceptual formula, $H0$ is the initial level of the pathogen under consideration, ΣR is the total (cumulative) reduction that is achieved through one or several consecutive bactericidal steps, and ΣI is the total (cumulative) increase that can occur due to growth and/or recontamination. All elements of the formula are expressed in $\log_{10}$ units.

While this conceptual formula is usually used to describe the fate of pathogens, it can very well be used to describe the fate of other microorganisms, such as relevant hygiene indicators or spoilage organisms, and hence reflect the hygienic performance of the process in question and the resulting microbiological quality of the product.

With respect to the illustrated example, the impact of the raw materials subjected to a killing step, such as a heat treatment, on the microbiota of the finished product will be determined by the type and levels of the initial microbiota and the magnitude of the killing step. In the case of raw materials added after the heating process, no further reduction will occur other than a possible quantitative modification related to the addition ratio of the different raw materials.

Microorganisms such as pathogens or hygiene indicators present in the processing environment can lead to contamination of the intermediate product. This can occur either indirectly, from the production premises and external surfaces of equipment, or directly, through contaminated food contact surfaces. While direct contamination by food handlers is unlikely to occur in industrially manufactured products, events involving human interventions in the processing line can result in contamination. Such direct contamination may be significant in productions requiring important human handling, such as food service or catering operations. Production processes being dynamic, the occurrence of such contaminations may be continuous in certain cases and sporadic in others, and their importance will depend on the levels of microbial contaminants in the environment or in the processing lines. These elements will determine the occurrence and the distribution in the finished products.

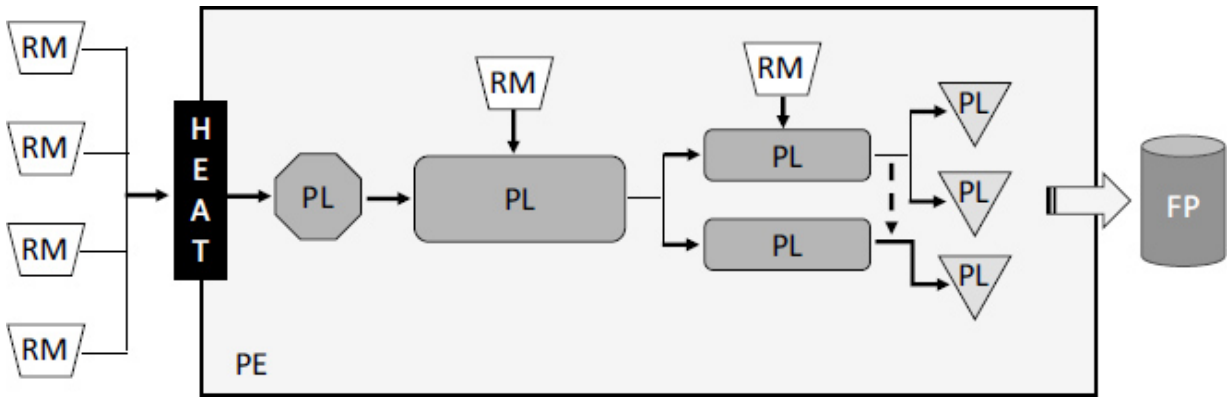


Figure 3.1 Schematic flow diagram of a food process, including raw materials (RM), a heat treatment, and food contact surfaces in the processing line (PL) as well as the processing environment located after the heat treatment (PE) up to the finished product (FP).

The consequences of such cross-contaminations will be the presence or an increased concentration of these microorganisms in intermediate and finished products. Microorganisms surviving a killing step or being introduced after this step may, given favorable intrinsic or extrinsic conditions, further increase due to growth at specific steps of the process, during distribution, or during preparation by the final consumer.

Although POs and FSOs are generally not intended to be verified by analytical means, POs may be suitable for such verification, and the use of MC for verification can be useful (13, 16). Verification that a PO is met on the basis of compliance with an MC may be very meaningful regarding due diligence documentation of ongoing safety and/or process control, trend analysis, and early warning for the need of corrective action. Compliance with the MC derived for a particular PO needs to be validated as part of the design of the overall food safety management system. The various measures implemented at the particular step, individually verified by other means, may include the following:

- establishment of a suitable MC for the end product, testing and statistical analysis

- verification (including monitoring and record keeping) of a pertinent and validated PC relating to the PO or of specific control measures underlying the PC and suitable for verification
- surveillance or screening programs on the prevalence of a microbial hazard in a food on the marketplace, which may be relevant mainly when a PO is established by a competent authority

In a concept paper by Zwietering et al. (16), three different cases of deriving an MC for a PO or FSO were developed, providing explicit and detailed guidance on various steps to be taken, the specific data needed, and assumptions and decisions to be made in the process.

PERFORMANCE OF CONTROL MEASURES

MCs have played and still play an important role in defining whether a food is acceptable and whether an individual lot complies with established limits. Sampling and testing are, however, rarely considered effective tools to control microbial hazards.

The safety of food products, and hence the protection of consumers, can be ensured only through the implementation of effective control measures. These control measures encompass actions and activities used to prevent, eliminate, or reduce food safety hazards to an acceptable level.

Control measures falling under GHPs applied by food manufacturers are usually generic and therefore applicable to the manufacture of different product categories. They are qualified as prerequisite programs (PRPs) and designed to minimize contamination from the processing line and the processing environment (Fig. 3.1). Examples of control measures that are typically considered GHP measures are given below:

- layoutof processing lines and zoning to control flows of air, pressure conditions within premises, movement of personnel, and mobile equipment as well as raw and packaging materials or intermediate products
- equipment design and installation for cleanability
- general cleaning and sanitization procedures
- preventive maintenance to minimize breakdowns and risks associated with unscheduled interventions
- appropriate waste management
- appropriate pest management
- training and behavior of personnel in PRPs, in particular, those related to microbiological considerations

The application of PRPs is valid as well for practices applied in different parts of the food chain such as good agricultural practices upstream and good distribution practices downstream. These programs represent a mandatory basis for the implementation of specific control measures as defined in hazard analysis and critical control point programs such as operational and critical control points. Specific control measures require a thorough validation in order to demonstrate their effectiveness in controlling relevant pathogens to the desired levels. Their performance during processing is determined through monitoring of relevant process parameters, such as temperature, time, flow rates, pressure, etc., against critical limits established during validation. In this context, testing of finished products against microbiological limits is normally performed during the validation procedure as well as during the ongoing verification of the overall performance of the food safety management system.

General control measures such as PRPs, as well as certain operational PRPs, such as specific cleaning and sanitization measures, can be verified through microbiological testing. Such measures are usually designed to prevent postprocess

contaminations occurring after specific control measures such as a heat treatment (critical control point). PRPs are combinations of measures aiming, in the first place, at preventing the ingress of pathogens into areas close to the processing line. In case ingress has occurred, the second line of defense is avoiding their establishment in processing areas, their subsequent multiplication, and finally their dissemination into different parts of the plant and the processing line, thus posing a direct threat to the finished product.

The establishment of testing plans for an appropriate verification of the effectiveness of control measures is more complex than the ones limited to finished products. Such plans need to take into consideration all elements contributing to the presence of microorganisms and, in particular, of pathogens in the finished products. They therefore have to include plans for sampling raw materials added after the control measure(s) and the processing environment and processing lines, i.e., product contact surfaces or intermediate product as appropriate.

ICMSF has published in detail on risk-based approaches to develop and apply environmental testing programs (14) and has provided case examples for a number of different food products (9, 10). Codex has elaborated the principles and guidelines for ready-to-eat food products and for powdered infant formula (6, 7)

The first step is usually to determine a baseline of the microbiota, focusing on the relevant pathogen(s) and hygiene indicator microorganism(s) present in the processing environment and processing lines, i.e., food contact surfaces and/or intermediate product. If no or only few historical data are available, it is important to collect this information through investigative sampling. Data for indicators can be obtained rapidly and at reduced costs and will provide sufficient initial information on the general hygiene status of the premises to allow development of a complementary sampling program for pathogens.

This baseline represents the situation and levels at which the plant and process are considered to be under control and at which the risk

of a product contamination is therefore minimal or negligible. This baseline is used as a benchmark to evaluate results and data generated through the sampling and testing activities defined in the sampling program against the established internal limits. This is done to verify and confirm that control is consistently maintained over time. If the findings demonstrate deviations, such as the presence of a pathogen in one or more sampling points in areas critical to the process and exposure to contamination or levels of the hygiene indicator exceeding internal limits, the actions need to be taken to restore control. Under such conditions, the testing frequency of the finished product is usually increased, and it becomes essential to perform a thorough investigative sampling to determine the source and origin of the contamination in order to take the appropriate measures.

INDEX AND INDICATOR MICROORGANISMS

Attempts to use certain microorganisms or groups of microorganisms requiring only simple, relatively rapid, and inexpensive methods to predict the presence of pathogens and to avoid expensive and lengthy testing have been made for more than 30 years. Initial work to define microorganisms able to “represent” pathogens was focused on water, and the concepts of model organisms or surrogate organisms (25) have been put forward by different authors, such as Havelaar and Pot-Hogeboom (26) and Payment and Franco (25). Over time, two different terms have been used to describe and define such microorganisms, namely, “index organisms” and “indicator organisms,” and they are well established today.

The distinction between index and indicator organisms was discussed initially by M. Ingram in 1977 (27, 28). Index microorganisms have been defined as microorganisms, groups of microorganisms, or microbial metabolites whose presence in numbers exceeding a specified limit indicates the possible presence of pathogens with similar behavior and ecology. Index organisms

are therefore to be used as direct predictors of the presence of specific pathogens. Such a prediction, however, is possible only if a statistically valid correlation between the organism used as index and the “associated” specific pathogen has been established. This correlation is, however, difficult to establish, in particular for pathogens that are found only sporadically and at low levels of index organisms.

Indicator microorganisms, on the other hand, have been defined as microorganisms, groups of microorganisms, or microbial metabolites whose presence in numbers exceeding specific limits indicates a failure in the adherence to GHPs. The intended role and purpose of indicator organisms are thus to serve as an indirect predictor of the presence of a pathogen. The presence of such organisms is therefore an indication of an increased risk related to a deviation from the implemented hygiene control measures. The direct testing for the specific pathogen cannot, however, be replaced by the testing for indicators, and combined programs including both of them are the rule.

It should be noted that several publications discuss, along with hygiene indicators, microorganisms, groups of microorganisms, or metabolites described as quality indicators. However, such quality indicators are usually related to physicochemical or organoleptic parameters and, hence, to quality attributes of a food product. While their presence and growth can be associated with defective control measures leading to spoilage of food products, they are, to our knowledge, not used to predict the presence of pathogens and hence are not within the scope of this chapter.

The use of indicators has been discussed by many authors, in particular in trying to define appropriate or ideal properties that allow them to fulfill their role in raw materials, the processing environment, and processing lines as well as in the end product. Several key properties are listed below (11), and these should be evaluated for any organism or metabolite being considered for use as an indicator:

- The history of the concomitant presence of the indicator(s) and the target pathogen or its toxin should be verified.
- The indicator(s) should usually be present at higher levels than the target pathogen.
- The presence of the indicator(s) should be indicative of an increased risk for faulty practices and/or faulty processes contributing to control of the target pathogen.
- The survival or stability of the indicator(s) should be similar to or greater than that of the target pathogen.
- The proliferation of the indicator(s) should be similar to or faster than that of the target pathogen.
- The indicator(s) should be easily detectable and/or quantifiable. • Identifiable characteristics of the indicator(s) need to be stable.
- Methods for indicator organisms need to fulfill the same requirements as the one for pathogens, i.e., they need to be reliable and validated; in addition, they should be more rapid and less expensive.
- Quantitative results on prevalence and/or concentration should show correlations between indicator and target pathogen.
- Results need to be applicable to process control or other control measures that indicate adequate control of the food safety system over the target pathogen or flag the need for corrective action.
- Analyst health should not be at risk and the analytical method should be suitable for in-plant use.

Because the behavior of a hygiene indicator should be similar to that of the associated pathogen, both will follow similar if not parallel patterns in the same processing facility. Although the outcome will be different, the conceptual formula of the ICMSF

illustrated in “Microbiological Profile of a Food Product” above can be used to describe their fate and provide useful information and insight on whether the targeted FSO and PO are likely to be achieved.

Numerous publications have been devoted to trying to determine the most appropriate hygiene indicator(s) for relevant pathogens, and it is not the purpose of this chapter to provide an exhaustive list of “ready-to-use” indicators. *Enterobacteriaceae* and *Listeria* spp. are certainly the most frequently used hygiene indicators in food processing and are associated with *Salmonella* and *L. monocytogenes*, respectively. This is also reflected in their inclusion in numerous MC. They are, however, not the only ones, and others, such as coliforms, fecal coliforms or *Escherichia coli*, fecal enterococci, sulfite-reducing clostridia (e.g., *Clostridium perfringens*), aerobic mesophilic counts, and certain metabolites, such as ATP, thermonuclease, and alkaline phosphatases, have been described as being suitable under certain circumstances.

However, even when there is knowledge that there is no consistent, systematic relationship between a target pathogen and an index or indicator organism, it may still be the best available option to use routine monitoring of the latter. As one example, the ICMSF has issued an opinion paper (29) on the use of sulfite-reducing clostridia (SRC) in powdered infant formula or associated ingredients in response to inquiries from industry and regulatory authorities to the ICMSF to provide advice on the appropriateness of testing for *Clostridium botulinum* in these products. Given that *C. botulinum* is not considered a hazard in powdered infant formula (7) and that the analysis of the pathogen is much constrained by its probably being heterogeneously distributed and occurring only at very low levels in powdered dairy products, the ICMSF did not recommend routine testing for this pathogen. Despite the fact that there is no direct mathematical correlation between levels of spores of SRC and those of *C. botulinum*, the ICMSF concluded that testing for SRC would be appropriate as an indicator of process hygiene with respect to the control of anaerobic sporeformers. ICMSF

considered that SRC levels exceeding a limit for *m* of 100 CFU/g, for example, would point to conditions potentially conducive to the multiplication of anaerobic clostridia in processing lines, or some source of external contamination (29). Considering the characteristics and behavior of various types of clostridia, such levels would then also point to conditions possibly supporting multiplication of *C. botulinum*, when present, beyond normal low levels. In contrast, SRC levels below this limit would confirm the effectiveness of the established hygiene control measures, such as adherence to GHPs and good manufacturing practices, and hence a negligible risk for *C. botulinum*.

The choice of an appropriate hygiene indicator depends on the knowledge of a number of factors related to the products manufactured and, in particular, its microbiota, the microbiota of the raw materials, the processing conditions applied, and their effect on the microbiota, the food's composition and characteristics and their effect on the behavior of microorganisms, the microbial ecology of the processing environment and lines, the aim of implemented control measures (such as PRPs), and the effects of different types of cleaning and sanitization procedures, among others.

An important but frequently underestimated or poorly managed aspect of environmental verification programs is the use and interpretation of generated data. Since hygiene indicators are associated with specific pathogens, their use is also associated with "predicting" and "anticipating" deviations and defects. It is therefore of utmost importance that the data generated, often at a higher frequency and in higher numbers than the ones for pathogens, be used in the most appropriate and beneficial way to fulfill their task. Failures in the interpretation and management of these data will jeopardize the usefulness of verification testing and, in particular, the ability to correct deviations identified at an early stage.

In any event, MCs for nonpathogens such as indicator organisms or even utility organisms do play an important role in their own right

in hygiene/process control, including environmental monitoring. In addition, the Codex Alimentarius Commission, for instance, has adopted the practice of recommending criteria for process hygiene where the safe production of particular products is dependent on maintaining a high level of hygienic control (7), specifically stating that these are not intended to be used by competent authorities but by manufacturers as a means of verification of their hygiene programs.

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Francisco Diez-Gonzalez

4
Responses in Foodborne Bacteria

Bacteria are the most abundant living forms on our planet and are capable of inhabiting all types of environments in nature, even those that would be considered inhospitable for all other living organisms. As a result of millions of years of evolution, bacteria have developed diverse catabolic and anabolic capabilities (1). Among the different bacterial taxa, heterotrophic bacteria often occupy niches associated with eukaryotic organisms, such as plants and animals. These heterotrophic bacteria have developed very close relations through commensal, mutual, symbiotic, or parasitic associations (2). Since most human foods are derived from plants and animals, large numbers and diverse taxa of heterotrophic microorganisms are naturally present in raw and fresh foodstuffs.

The microbiome associated with fresh and raw foods includes not only bacterial groups that are natural inhabitants of plants and animals but also those which can result from the attachment and colonization of other microorganisms to food matrices at different stages after harvesting or slaughtering (3). Very few foods are completely devoid of any bacterial population, and the transient or established presence of microorganisms is considered normal and frequently unavoidable. The association of microorganisms with foods often leads to spoilage and undesirable changes in products with intrinsic characteristics that permit bacterial growth. A more serious concern is contamination with organisms that may cause illnesses.

One of the major challenges that humans have faced since ancient times is the preservation of food materials in order to nourish large populations and to avoid ingesting pathogenic bacteria. The development of beneficial food processing was critical in history for

civilizations that were capable of sustaining large numbers of people with a supply of abundant and safe foods. As a result, a variety of technologies have been developed to modify or treat foods for the control of microorganisms (4). Early technologies such as fermentation with innocuous bacteria, cooking, drying, and salting allowed the preservation of foods for long periods after harvest. Over the last century, multiple effective preservation techniques have been developed in addition to those traditional methods.

The technologies of food preservation use different forms of energy to change the characteristics of the raw materials, to kill microorganisms, and/or to delay their growth. The food technologies developed over many centuries change the intrinsic properties of foods, affecting acidity, redox potential, water content, solute concentration, and presence of antimicrobial substances (5). Preservation technologies subject bacterial cells to different levels of stress, which in the most effective cases lead to their inactivation and death (3). For the purpose of this chapter, the term “stress” refers to any extracellular influence that threatens the ability of microorganisms to perform optimally.

Food preservation technologies aimed at rapidly inactivating microbial cells include thermal processes, irradiation, high-pressure processing, and the use of strong oxidant compounds. Other technologies accomplish the preservation of foods by inhibiting growth, and the most extensively used methods involve low-temperature storage (refrigeration and freezing), reduction of moisture content (concentration and drying), control of ox-redox potential (use of controlled atmospheres and vacuum packaging), and acidification (fermentation and addition of organic acids) (6).

The ability of heterotrophic microorganisms to survive multiple stressful conditions evolved over millions of years of natural selection. The stress responses that developed throughout evolution can be effective in overcoming different food preservation technologies (7). In nature, microorganisms are constantly exposed to changes in temperature, oxygen, moisture, light, pH, and

chemical composition, caused by abiotic and biotic factors threatening their viability. Unicellular microorganisms, especially bacteria, are able to survive those natural challenges as a result of their ability to adapt and/or acquire genes from other microorganisms that allow them to counteract those natural assaults (2, 8). The acquisition of stress response genetic mechanisms has proven quite useful in assisting heterotrophic microorganisms to attach to and colonize plants and animals in order to withstand physical and chemical insults advanced by food processing technologies. The process of developing stress responses is very sophisticated, and microbes employ complex networks of genetic responses to combat stress, with different levels of regulation (9).

These multifactorial networks of stress responses are often capable of protecting the cells, or in allowing their recovery, if the magnitude and type of stress are not lethal (9). Stress mechanism responses frequently lead to tolerance to increased stress after an initial exposure to lower stresses that were not severe enough to kill cells (stress adaptation). For example, the key mechanisms that allow *Escherichia coli* O157:H7 to survive extreme acid exposure are typically induced by previous growth at a mildly acidic pH (10). In addition, the presence of globally regulated stress tolerance systems often leads to increased resistance to more than one type of stress, referred to as cross-resistance or cross-protection. For example, the acid resistance of *E. coli* O157:H7 has been reported to be augmented following pretreatment at high temperatures (11).

Throughout millions of years of adaptation and gene uptake, bacteria have developed complex genetic regulatory systems that involve key components capable of controlling the expression of multiple operons. These key genes encode proteins referred to as global regulators (12). Almost every bacterial species has a globally regulated stress response system, and some of the key components are very well conserved throughout different phyla. In addition to these common global regulators, a number of bacterial species possess unique specific stress response genes. The objective of this

chapter is to provide a general overview at the molecular level of the different stress response mechanisms that have been characterized in foodborne pathogenic bacteria. In particular, responses to exposure to acid, osmotic, oxidative, pressure, light, and temperature stresses are covered. The focus is on Grampositive (*Listeria monocytogenes* and *Staphylococcus aureus*) and Gram-negative (*E. coli*, *Salmonella*, and *Campylobacter*) bacteria that have been thoroughly studied. The chapter first describes global regulator systems and then reviews the molecular mechanisms of tolerance to specific environmental stresses.

GLOBAL STRESS RESPONSES

Technologies intended to control microorganisms in food are effective because they are capable of causing serious damage to bacterial cells. High-temperature processes such as cooking and pasteurization inflict irreversible functional and structural destruction of multiple cell components (13). Heating of foods causes extensive bacterial death because of the denaturation of proteins, inactivation of ribosomes, and disruption of membrane integrity, as well as damage to DNA. Irradiation of foods is also an effective technology intended to cause widespread bacterial death. Bacterial inactivation caused by ionizing irradiation is largely due to DNA damage resulting from the generation of reactive oxygen species (ROS) from water (14). Most bacterial cells have DNA repair mechanisms, but if the DNA damage is very extensive, bacterial cells may be irreversibly inactivated and killed.

Because of its serious impact on viability, significant damage to the molecular structure of DNA triggers a series of extensive molecular responses in bacterial cells (15). Bacterial species have developed several global stress responses systems, and at least three such systems have been extensively characterized. The SOS stress response system is a universal cellular protection network induced by the presence of single-stranded DNA (ssDNA) that has been studied comprehensively in *Enterobacteriaceae* (16). The SOS

system evolved to counteract the generation of mutations caused by DNA disruption (16, 17). Under low-DNA-damage conditions, the SOS regulon is normally repressed, but large accumulations of ssDNA trigger the induction of more than 30 genes that make up this complex response (16, 18).

LexA is the natural repressor of SOS when the concentration of ssDNA is low. In the presence of large amounts of ssDNA, a bacterial recombinase protein, RecA, binds to the DNA strands and the self-proteolysis of LexA occurs (18). The degradation of LexA triggers the expression of multiple genes that encode enzymes that restore DNA structure and function via repair, recombination, and replication (15). Two examples of enzymes involved in DNA replication after LexA inactivation in *E. coli* are the DNA polymerases Pol IV and Pol V, encoded by *dinB* and *umuDC*. These polymerases have a high rate of synthesis, which can lead to high error rates and increased mutation frequency (19). The SOSregulated genes involved in DNA repair can be classified based on three DNA pathways in several enterobacteria: homologous recombination, nucleotide excision repair, and translesion synthesis (16). Other SOS-dependent genes are *sulA*, which encodes a protein involved in the inhibition of cell division, and *recN-ruvAB*, which encode enzymes that restore breaks in double-stranded DNA (19).

Despite advances in rapid genomic and proteomic analysis, most of our understanding of bacterial stress mechanisms to date originates from extensive studies with *E. coli* and *Salmonella* as model organisms. In those bacteria, additional global stress response systems have been identified and characterized: the general stress response, the stringent response, the heat shock response, and the polyphosphate-mediated nutrient limitation response (16–18). Other global stress responses reported in *Salmonella* include PhoPQ, Fur, and OmpR-EnvZ (20). Since the early 2000s, the important role of small RNA molecules as part of the complex network of bacterial stress responses has been increasingly recognized, and recent evidence indicates their importance for cell survival (21, 22).

General Stress Regulator

One of the main biological challenges for bacterial cells occurs during logarithmic growth, when a complex and rapid activation of genes and protein synthesis occurs (19).

During exponential growth, DNA polymerase III catalyzes DNA replication and RNA polymerase is responsible for transcription. The RNA polymerase in *E. coli* is a very large holoenzyme composed of five protein subunits: two α subunits, one β subunit, one β´ subunit, and a σ chain (1). The RNA polymerase catalytic core is maintained under most conditions and consists of a tightly bound $\alpha_2\beta\beta'$ complex. The σ factor is the subunit that varies depending on the cell cycle, and its binding to the rest of the holoenzyme is relatively weak. The σ factor plays a central role in targeted transcription of genes and is responsible for recognizing promoter sequences to initiate the transcription of targeted genes (23). Bacteria have multiple σ factors that are activated under different conditions. As many as seven σ factors have been reported in *E. coli*, and recent genomic analyses of almost 1,000 genomes have confirmed the conservation of at least six of them (23, 24). In Gram-positive organisms such as *Bacillus subtilis*, more than 15 sigma factors have been identified (24).

$\sigma^{70}$is the main σ factor in *E. coli* directing the transcription of housekeeping genes under most conditions. σ^{70} is encoded by *rpoD*, and it is responsible for coordinating protein synthesis during the exponential growth phase (25). Rapidly growing *E. coli* cells expressing σ^{70} initiate the activation of an alternate sigma factor, σ^{S} the most important regulator of the general stress response when the cells are approaching stationary phase (26). σ^{S} coordinates cell survival under stressful conditions and regulates protein synthesis during subsequent growth (27). In some of the first reports, the *rpoS* gene was referred to as *katF* because its induction was linked to the production of a catalase (18). The full transcription of *rpoS* takes place only once the cells have reached stationary phase, and

the binding of σ^S to the RNA polymerase holoenzyme directs the transcription of multiple genes involved in metabolism and regulation (26, 28).

The extent of the influence of RpoS on *E. coli* metabolism is reflected in the fact that it regulates at least 10% of the genome, including over 85 genes or regulons with multiple metabolic or regulatory functions (29). Multiple loci encoding systems involved in stress responses, stationary-phase metabolism, and according to recent evidence even virulence factors are controlled by σ^S (24, 30). The genes controlled by σ^S are distributed across the entire *E. coli* genome, but there are some regions of higher concentration of RpoS-regulated operons. Weber et al. have classified all the RpoS-regulated genes into six major categories based on their functions: metabolism, regulation, transport and membrane function, adaptation to stress, protein processing, and unknown function (29). Despite the recent advances in genomic analysis, a relatively large proportion of all the functions of genes regulated by RpoS have yet to be characterized (29).

Multiple σ^S-regulated genes are part of the SOS global stress response system, and a network of specific stress response genes are involved in protecting the cells against osmotic, acid, and oxidative effects (31). An example of the interconnection between SOS and σ^S-dependent stress responses is the observation that an *rpoS* mutation caused a marked reduction in the concentration of the DNA polymerase Pol IV (32). This reduction in Pol IV levels is due to the reduction in σ^S-regulated transcription of *dinB*, which is required for *de novo* synthesis of Pol IV (18). RpoS is also involved in the oxidative stress response, as it regulates the expression of HPI and HPII, two catalases encoded by *katG* and *katE* (33). RpoS is also involved in osmoprotection via the regulation of the expression of *otsA* and *otsB*, whose products catalyze the synthesis of trehalose (34). In addition, the glutamate decarboxylase operon (*gadABC*, which protects *E. coli* and other bacteria from extreme acid conditions) is indirectly regulated by σ^S by means of *gadX* and

gadW (10, 29). These specific stress response systems are described in more detail below.

Because σ^S is crucial for cellular functions, its concentration is tightly regulated by a series of mechanisms involving both synthesis and degradation (Fig. 4.1) (26). The key signal that initiates *rpoS* transcription is a reduction in growth rate; however, a steady decline in growth is necessary to activate rapid transcription (26, 31). The regulation of *rpoS* transcription is dependent on several promoters and small molecules. The promoters *nldD*p1, *nlpD*p2, and *rpoS*p are located upstream of the *rpoS* coding region (28). When cells are replicating rapidly, the transcription of *rpoS* is largely repressed by a complex formed by the catabolite repression protein and cyclic AMP (29, 35). In contrast, one of the small molecules involved in activating *rpoS* mRNA synthesis is guanosine tetraphosphate (ppGpp) (26).

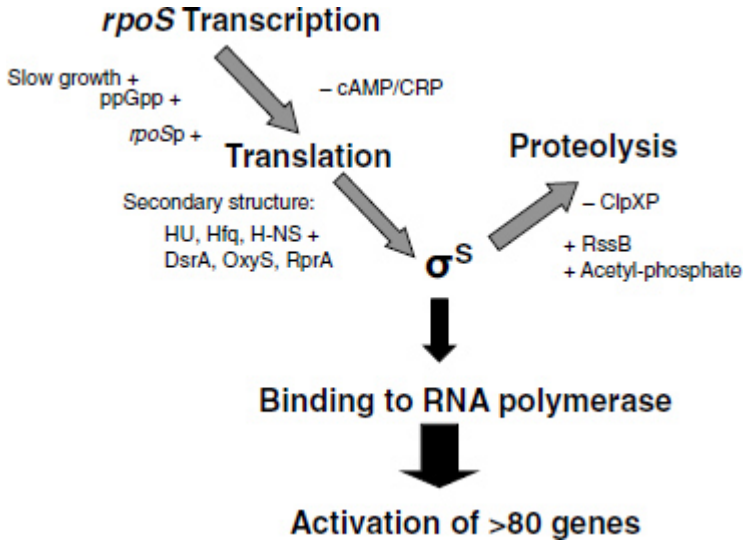


Figure 4.1 Diagram of σ^S regulation and some of the main factors involved in its activation (+) or inhibition (–). cAMP, cyclic AMP; CRP, cyclic AMP receptor protein.

The translation of *rpoS* mRNA is influenced by multiple external stimuli. Changes in aspects of the environment such as pH, osmolarity, temperature, and cell concentration enhance the rate of translation. However, a reduction of carbon source availability

during stationary phase strongly inhibits increases in σ^S concentration (26, 31). The development of secondary structures of *rpoS* mRNA that inhibit ribosome binding is another negative regulatory mechanism. External stress conditions cause the loss of those mRNA structures and then allow transcription. Additional *rpoS* mRNA regulators include a nucleoid protein (HU), the histone-like protein (HN-S), and the small regulatory RNAs DsrA, OxyS, and RprA (21, 22, 26). The σ^S concentration is also dependent on protein degradation pathways. The growth stage of bacterial cells influences the rate of σ^S protein hydrolysis, which has a half-life between 1 and 20 min (36). ClpXP is an ATP-dependent protease that is involved in σ^S proteolysis, but it needs a phosphorylated recognition factor, RssB, to be able to bind RpoS (26). Acetylphosphate is an indirect regulator of σ^S proteolysis because it is the preferred substrate for RssB phosphorylation (37).

The σ^S equivalent general stress regulator in Grampositive bacteria is known as σ^B (or SigB), and it has been reported in *Listeria*, *Bacillus*, and *Staphylococcus* (38, 39). More than 150 genes are regulated by σ^B. Multiple extrinsic and environmental conditions trigger the activation of σ^B, such as lack of carbon source, phosphate, and oxygen. Environmental stresses, including acidity, alkalinity, high solute concentrations, ethanol, and nitric oxide, also elicit enhanced synthesis of σ^B (38). All of the conditions that lead to σ^B induction can be classified into two types: energy limitations and environmental stresses (6). The SigB-dependent stress response regulation is not present in every Gram-positive bacterium. SigB stress responses have been observed in *Bacillus*, *Mycobacterium*, *Listeria*, *Streptomyces*, *Staphylococcus*, and *Ralstonia*, but other strictly anaerobic and facultative anaerobic Gram-positive bacteria, such as *Clostridium*, *Lactococcus*, and *Streptococcus*, entirely lack this critical general stress response (38). Because of the lack of SigB in anaerobic genera, it has been hypothesized that protection from oxidative stress could be the principal role for this sigma factor.

The relevance of sigma factors for the ability of foodborne pathogens to survive adverse conditions has been studied extensively, and one of the most important approaches to demonstrate their role has been through the use of mutant strains. In particular, the role of σ^S has been comprehensively investigated in *E. coli* O157:H7 and *Salmonella* (40, 41). Cheville et al. reported one of the first observations in which specific mutants were more sensitive to heat, salt, and acid than the wild-type strain (42). An *E. coli* O157:H7 *rpoS* mutant also had a diminished ability to colonize the gastrointestinal tract of cattle (43). Sigma factors may also be involved in virulence factor activation. In *L. monocytogenes*, σ^B regulates acid resistance proteins, but it is not involved in pathogenicity. In contrast, σ^S has an important role in regulating some virulence factors in *Salmonella enterica* serovar Typhimurium (7). In *Vibrio vulnificus*, σ^S was required for inducing an oxidative response, but heat tolerance was not influenced by *rpoS* (44). With the advent of genomic techniques, there is the opportunity to reevaluate the role of *rpoS* in survival of foodborne pathogens.

Other Global Stress Responses

Bacteria have multiple metabolic systems that sense their environment to enable them to respond to adverse conditions, and they use an array of sensory systems to monitor their environment in order to adapt to stressful conditions. Guanosine tetraphosphate and guanosine pentaphosphate (ppGpp and pppGpp) are nucleotidebased secondary messengers referred as alarmones (45, 46). Under adverse conditions, such as lack of amino acids and high temperature, the (p)ppGpp concentration increases, which causes an overall inhibition of all transcription and RNA synthesis. This general response is referred as the stringent response (18, 45). The stringent response has been extensively characterized since it was initially observed when bacterial cultures were transferred from rich to minimal media. The stringent response is critical for survival when nutrients are lacking and under stressful conditions, and its activation frequently occurs simultaneously with that of RpoS

during stationary-phase starvation (45). The stringent response is widely distributed among most bacterial phyla, as the enzymes responsible for (p)ppGpp synthesis are highly conserved in nature (47).

The stringent response is triggered by the lack of amino acids that leads to a buildup of "uncharged" tRNA. The (p)ppGpp-synthesizing enzyme, RelA, is then activated after binding to ribosomes containing these uncharged tRNAs (45). By binding to the RNA polymerase, (p)ppGpp then regulates transcription by directing the enzyme to different promoter regions. Despite the fact that the stringent response inhibits RNA synthesis, the absence of specific amino acids causes a selective activation of biosynthetic genes (18). As a global stress response, the stringent response regulates multiple cellular processes in addition to RNA synthesis, inhibiting translation and promoting proteolysis. While starvation is the main condition that activates the stringent response, different kinds of stress and conditions that inhibit growth can also enhance the response.

Many years ago, microbiologists observed that polyphosphate (polyP) accumulated in bacterial cells as "volutin granules," but its function was not clear (48). Nobel Prize winner Arthur Kornberg spent the last years of his career investigating the role of polyP, as he hypothesized that this family of compounds had a vital function in cells. His belief in the importance of polyP was partially based on the fact that over 100 bacterial species have two homologous enzymes, PPK-I and PPK-2, that are involved in polyP synthesis. The role of polyP in stress responses was discovered when *ppk1* mutants of a variety of Gram-positive and -negative bacteria were found to be more sensitive to stress factors than their wild-type counterparts (49, 50). The phosphokinases may also be involved in virulence regulation in addition to stress response, since *ppk1* mutants of *Salmonella*, *Shigella*, and *Vibrio cholerae* have diminished pathogenicity (48, 49)

Several studies have shown that polyP mutants display reduced tolerance to acids, oxidation, heat, and osmotic stresses (50, 51). In *Salmonella* cultures, the presence of a PPK-1 enzyme was critical for growth in the presence of organic acids and the ability to withstand heat in a mutant strain, in contrast to the wild type (51). PPK-1-deficient strains of *Shigella flexneri* and two *Salmonella* serovars were inactivated more rapidly at 55°C (52). In *V. cholerae*, deficiencies in PPK-1 synthesis resulted in acid-and oxidation-sensitive mutants, but toxin production, motility, and the ability to colonize the gastrointestinal tract were not affected (53).

As our understanding of stress response mechanisms has advanced, we have discovered that the individual global regulatory systems are tightly interconnected and interdependent. This regulatory stress response network spans many components of metabolism, including DNA replication, RNA transcription, protein translation, proteolysis, and small-molecule turnover. The interconnection of global, general, and specific stress responses allows bacteria to adapt and survive adverse conditions. In recent years, it has been recognized that not only do the regulatory mechanisms involve DNA-protein and protein-protein interactions, but also, transcription control is exerted by small RNA molecules (22). The rest of this chapter covers specific stress responses of relevant food bacteria (Fig. 4.2). However, it should be noted that apparently independent specific stress response mechanisms may in fact be subject to control by other interconnected regulators.

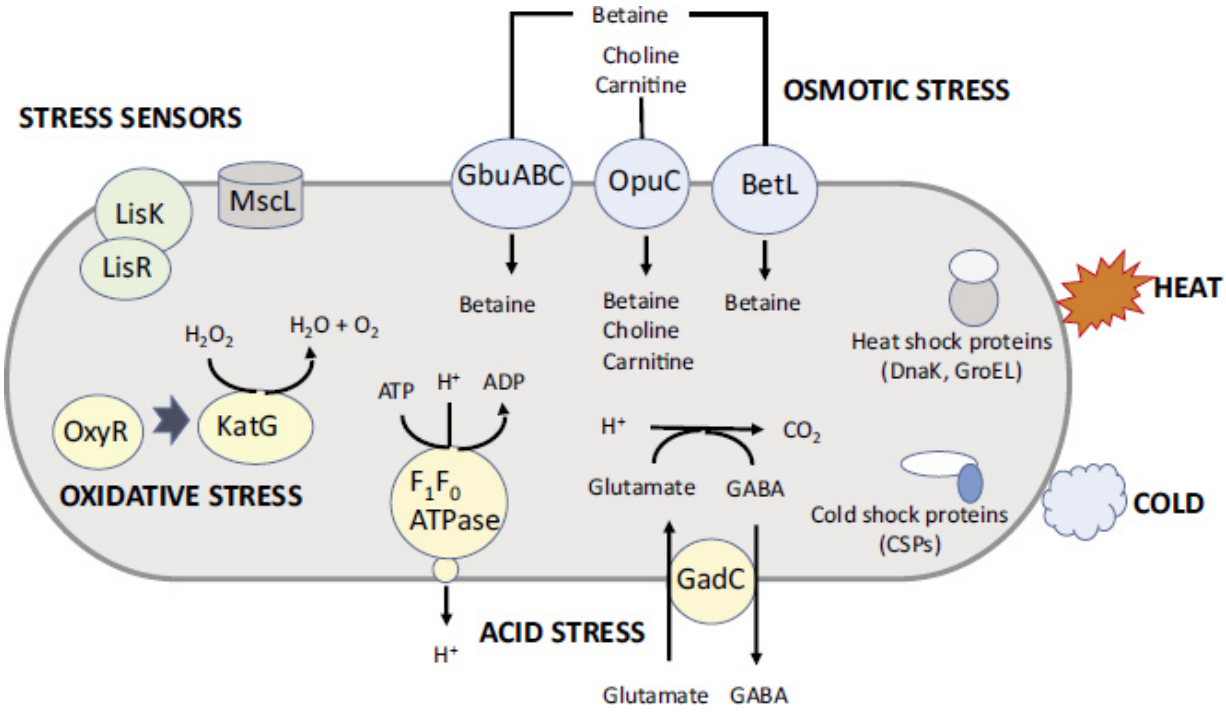


Figure 4.2 Hypothetical depiction of a bacterial cell with some of the most important specific stress response mechanisms reported in foodborne pathogenic bacteria. Illustration ideas were taken partially from reference 12.

ACID STRESS

The intracellular pH of most bacterial cells is tightly controlled within a relatively narrow range close to neutrality. Any changes in the cytoplasmic pH can affect multiple metabolic processes and can potentially cause serious damage to bacterial viability (54). Because an acid threat is a drastic attack, bacteria have evolved a complex and exquisite protective system of genetic and metabolic responses. The extent of success against an acid shock can be influenced by the bacterial species, individual strain differences, type of acid stress, growth phase, and the presence of external small molecules in the environment. The two acid stress response systems most thoroughly characterized among foodborne microorganisms are the acid tolerance responses (ATR) and acid resistance (AR).

Foster studied the ability of *Salmonella* to survive normally lethal acidic conditions when cells are pre-exposed to a mild pH condition and defined this response as the ATR (55). Cells can be adapted to acid when they are grown to stationary phase under mildly acidic conditions, similar to the acidity caused by acid production through fermentative pathways (56). This unique acid stress protection mechanism has been reported to enhance the survival of bacterial cells by a factor of more than 1,000 in comparison with noninduced cultures (10, 57). In addition to increased acid tolerance, cells previously adapted to mild acid have enhanced resistance to other external factors (osmotic, thermal, oxidative, and ethanol) (58). In several organisms, the ATR plays an important role in pathogenicity as a signal to induce virulence factors (56, 59).

While ATR can protect cells at mildly acidic pH values (3.5 to 4.5), this system is not sufficient to promote survival at lower pH levels. The stress response mechanisms that protect cells at extremely low pH values (less than 2.5) are referred to as acid resistance (AR) (10, 60, 61). Most of the AR response systems require the presence of an extracellular substrate, frequently an amino acid, in order to be metabolically active (60). The AR responses are remarkably stable once they have been induced and activated, since it has been reported that AR cells remain resistant during storage for long periods of time and at low temperatures (4°C) (60, 62).

The ability of pathogenic bacteria to cause disease is heavily dependent on survival under acidic conditions. First, foodborne pathogens may survive acidic and fermented foods for long periods thanks to their ATR and AR systems (63). Their ability to survive acidic conditions becomes even more critical when bacteria are ingested with the contaminated food. The pH of the gastric stomach can be less than 2.5, and this is often the first barrier that protects the human host. Bacterial pathogens with some of the smallest infectious doses have the most effective acid stress responses (60). In contrast, acid-sensitive bacteria often require larger numbers to overcome the loss of viability in order to cause disease. AR systems

allow a pathogen to survive the gastrointestinal tract, eventually colonize the intestine, and develop disease (58, 63).

In the remainder of this section, the acid stress responses of *E. coli*, *L. monocytogenes*, and *Salmonella* spp. are discussed as model systems. Note that, despite the detailed characterization of these systems, it is likely that they are more complicated than our current understanding reveals. In addition, published observations are based on a few strains, whereas a number of publications have suggested that there are variations depending on individual strains and experimental conditions (56).

E. coli

The optimal growth of *E. coli* occurs at near neutral pH, but it has been reported to be capable of growth at pH 4.4. Growth at a pH between 4.5 and 5.5 is a requirement to induce its acid resistance (60, 61). At pH values below 5.5, a complex collection of proteins involved in AR are synthesized (57). The AR stress response that protects *E. coli* has been extensively described. The specific stress responses that protect *E. coli* against acid exposure consist of four separate mechanisms. Three of those systems employ enzymes that decarboxylate specific amino acids: arginine, glutamate, and lysine (10). The fourth system is an oxidative response that does not require the presence of substrates and is repressed by glucose (57, 60). The induction of all four systems requires the onset of stationary phase during pre-exposure to mild acid (10, 64).

The σ^S protein is required to induce the glucoserepressed oxidative system, but it has a limited role in activating the arginine-and lysine-dependent decarboxylase responses (60, 65). The amino acid decarboxylase systems can be effective in maintaining cell viability only if the corresponding amino acid is present in the surrounding medium (54). The core components of the amino acid decarboxylase system are the enzyme catalyzing the decarboxylation and the antiporters that translocate both substrate and product simultaneously (Fig. 4.1). From glutamate, lysine, or arginine, the

decarboxylation reaction consumes a proton by producing CO_2 and gamma-aminobutyric acid, cadaverine, or agmatine, respectively, which are transported out of the cell in exchange for their substrate amino acid (65–67). This cytoplasmic neutralization process is maintained as long as there is a supply of substrate or the intracellular pH is stabilized near neutral (68, 69).

The AR system that protects *E. coli* by arginine decarboxylation involves an arginine decarboxylase and the arginine/agmatine antiporter. The operon that encodes the enzyme and transporter includes *adiA* and *adiC*, respectively (70). In addition, the arginine-dependent system involves *cysB* and *adiY*, regulatory genes that promote their expression (71). The activation mediated by AdiY requires its own overexpression. On the other hand, the activation of *adiAC* has a strict requirement of growth under anaerobic and acidic conditions in complex media (72). When the arginine-dependent acid response was studied in *E. coli* O157:H7, the conditions that elicited the greatest protection were growth of cells in rich medium under anaerobic conditions and a pH of less than 5.0 (10).

The second amino acid-dependent AR system is based on the ability of cells to transform lysine and produce cadaverine via decarboxylation. Similar to arginine, the two-component metabolic system involves lysine decarboxylase (*cadA*) and the lysine-cadaverine antiporter (*cadB*) (73). The full expression of the CadBA operon requires a low extracellular pH and high concentrations of lysine and involves two regulatory genes, *cadC* and *lysP* (73). *cadC* is located upstream of *cadBA*, and its expression is required for activation of *cadBA*. The periplasmic domain of CadC and the permease LysP are responsible for detecting extracellular conditions to induce the operon (74). The synthesis of CadA and CadB can also be repressed by intracellular accumulation of cadaverine, the product of lysine decarboxylation (69).

The third AR system that involves amino acid decarboxylation is the glutamate-dependent (GAD) system. The GAD system has been

extensively characterized and is not dependent on σ^S (10, 67). Similar to the other two decarboxylase AR systems, the GAD system has a transporter, the gamma-amino butyric acid antiporter (GadC), but in this case, two distinct glutamate decarboxylases, GadA and GadB, catalyze glutamate decarboxylation (60, 75). Both GadA and GadB are typically expressed during acid shock, but either one is sufficient to protect the cells at a pH above 2.5. However, when the extracellular pH is less than 2.5, the presence of both enzymes is required for survival (75, 76).

The GAD operon includes genes for three key regulator proteins capable of binding DNA: GadW, GadX, and GadY (77). While GadX is an activator for the entire operon, overexpression of the small RNA GadY causes high concentrations of the GadX transcriptional activator, which lead to induction of the glutamate decarboxylases GadA and GadB (77, 78). The interconnection of the specific GAD response system and the global regulators is illustrated by reports that despite the fact that the AR of *E. coli* is largely independent of σ^S, *gadX* is downregulated if σ^S becomes repressed (77). The GAD system appears to be widespread in multiple Gram-negative and Gram-positive organisms. Similar glutamate decarboxylase enzymes and antiporters have been identified in *L. monocytogenes*, *Bacteroides fragilis*, *Lactococcus* spp., *Lactobacillus lactis*, *Providencia alcalifaciens*, *Brucella abortus*, and *Enterococcus avium*, among others (79).

L. monocytogenes

In contrast to *E. coli*, *L. monocytogenes* is more sensitive to extreme acid exposure (less than 3.5) (56, 80). However, this bacterium has a number of mechanisms that allow it to survive the acid challenges that it encounters in nature, such as in the phagosome and the gastrointestinal tract (81). Among Gram-positive bacteria, *L. monocytogenes* has been reported to have an ATR system for protection against low pH (81). Pre-exposure to pH values of 5.0 to 5.5 has been consistently reported to enhance the survival rate more

than 100-fold and to induce its ATR (82). σ^B has an important role in regulating several of the ATR components, but *L. monocytogenes* also has several independent mechanisms (58).

As many as seven different metabolic components have been associated with the *L. monocytogenes* ATR (80). This complex network of protective mechanisms involves a σ^B-dependent general stress response, the two component system LisRK, a GAD component, the SOS response, changes in membrane fluidity, the FoF1 ATPase proton pump, and an arginine deiminase. The first σ^B dependent system is induced by acidic pH, is initially activated in rapidly growing cells, and is enhanced once they reach stationary phase (58). The two-component system LisRK was identified when it was observed that stationary-phase cultures of Δ*lisK* mutants were more acid resistant than those of wild-type cells (83). *lisRK* encodes a membrane-associated histidine kinase (K) and a cytoplasmic response regulator (R) that affects other genes (80). The role of the global regulator SOS has been evidenced with observations that Δ*recA* mutants were more sensitive to low pH (84). The critical role of the FoF1 ATPase proton pump in the ATR was reported by Cotter et al. (85), who observed that cells treated with the ATPase inhibitor *N,N′*-dicyclohexylcarbodiimide had a markedly reduced ability to survive pH 3.5. New ATR components have recently been identified using proteomic and transcriptomic approaches, but they are beyond the scope of this chapter (80).

The glutamate decarboxylase-based ATR is probably the best-characterized mechanism and the specific system that offers the greatest protection against an acid shock (80, 81). The GAD-based ATR system comprises three glutamate decarboxylase genes, i.e., *gadD1*, *gadD2*, and *gadD3*, and two genes for antiporters, i.e., *gadT1* and *gadT2* (86). In contrast to the *E. coli* genome structure, the *L. monocytogenes* GAD genes are distributed in three distinct operons: *gadD1T1*, *gadD2T2*, and g*adD3* (87). The GAD system appears to be the only ATR system that protects the cells at pHs below 3.5, and because of the common presence of glutamate in foods, it has been

suggested to be particularly essential for its survival in acidic foods (79, 88). *L. monocytogenes* possesses another amino acid-degrading pathway that is involved in acid resistance, the arginine deiminase (ADI) system, which catabolizes arginine to ornithine and consumes protons by ammonia production (89). The ADI metabolic components are arginine deiminase (ArcA), catabolic ornithine carbamoyltransferase (ArcB), carbamate kinase (ArcC), and membrane-bound arginine–ornithine antiporter (ArcD), which are regulated by ArgR, a transcriptional activator. The ADI is maximally expressed at low pH (5.0), in a lack of oxygen, and in the presence of arginine in the medium (80). The ADI system and other ATR mechanisms have also been reported to have a role in virulence factor regulation.

S. enterica serovar Typhimurium

Similar to other members of the family *Enterobacteriaceae*, *Salmonella* is capable of rapidly growing over a pH range of 5 to 9, but there have been reports that it can still grow at a pH of 4 (60, 90). The ATR of *S.* Typhimurium is typically activated at pH values between 4 and 5 (62). Exposure of *Salmonella* cells to a pH of 4.5 triggers the production of as many as 52 proteins (91). The protection of *Salmonella* cells against acid stress is conferred by at least three ATR systems, with different regulatory mechanisms (60, 92). The first ATR system comprises the general σ^S-dependent stress response and two pH-induced growth phase-dependent mechanisms (60). The pH-induced stationary-phase ATR and the pH-induced log-phase ATR are completely independent, and they have no common regulators (93). The logphase-dependent ATR is activated in exponentially growing cells when they are subjected to a low-pH environment, protecting them from a rapid change in pH. As many as 50 distinct proteins have been reported to be induced related to the log-phase mechanism (90). The two key regulatory components for the log-phase mechanism are encoded by *rpoS* and *fur* (90). The involvement of Fur in regulating ATR is independent of its role in the iron regulation network. A decrease in external pH

leads to increased Fur expression, which in turn activates the synthesis of unique acid shock proteins that do not require RpoS (90, 94).

A major difference between the stationary-phasedependent ATR and the log-phase-dependent ATR is that the former requires a longer exposure to low pH to be fully expressed (60, 93, 95). The stationary-phasedependent ATR is the predominant protection system under extremely acidic conditions (95). The stationaryphase ATR involves only 15 proteins, and it provides protection at pH 4 or less (55, 95). The stationary-phase ATR is not influenced by σ^S and Fur but is regulated by Omp. In response to an acidic environment, the regulator OmpR is synthesized, triggering the activation of genes that encode part of the OmpR-EnvZ regulatory system (94). OmpR is also involved in pathogenicity by upregulating *ssrAB*, encoding a virulence factor stimulated by acidic conditions. The general stress response and the stationary-phase ATR are largely responsible for protecting stationary-phase cells (90).

In addition to the general stress response and the growth-phase-specific systems, *S. enterica* serovars also have amino acid decarboxylase ATR systems that contribute to its survival at acidic pH (96). Similar to *E. coli*, *Salmonella* has arginine and lysine decarboxylase AR systems that protect cells from acid shock at about pH of 2.5 as long as there is a supply of the corresponding amino acid in the medium (97, 98). Unlike *E. coli*, *Salmonella* has no GAD system, but it has an ornithine decarboxylase ATR system (98). The inducible arginine, lysine, and ornithine decarboxylases are encoded by *adiA*, *cadA*, and *speF*, respectively. All three enzymes require anaerobic conditions for full expression, and they have different roles in protecting against acid and allowing growth at low pH (98). AdiA is known to be able to promote survival at pH as low as 2.3 but has no role at pH 4.5. CadA plays a role in both acid survival and growth promotion. SpeF is not involved in ATR but stimulates growth at moderately acidic pHs. These combinations of protection clearly show a relatively complex ATR response in *Salmonella*.

The advent of transcriptomics and proteomics approaches offers multiple opportunities to identify novel ATR and AR mechanisms not only in organisms that have been extensively characterized but also in bacteria that are not as economically relevant as those described above. Acidic foods will always be part of human diet, and it is critical to continue to understand the molecular basis for bacterial survival and growth under those conditions. Comparative genomics and metabolomics will likely contribute to elucidation of novel stress response networks.

OSMOTIC STRESS

All living cells, including bacterial cells, are surrounded by a semipermeable lipid bilayer membrane that contains all cellular contents and limits the exchange of compounds into and out of the cytoplasm (99). The environment surrounding bacteria exposes the cells to constantly changing chemical conditions that lead to changes in the amount of nutrients, water, and waste compounds in the cytoplasm. When the concentration of solutes in the extracellular environment changes drastically, it causes an abrupt movement of water and small-molecule solutes across the membrane. The rapid gain or loss of water and solutes leading to an osmotic shock is a life-threatening event for bacteria. Similar to other stress responses, bacteria have evolved and acquired multiple molecular mechanisms that protect them from potentially damaging osmotic changes (100).

Damage to DNA replication and cell division caused by an osmotic shock explains the loss of viability in susceptible bacterial cells (101). Bacteria have a system of sensors that detect osmolarity changes and trigger the activation and synthesis of enzymes and transport proteins. For the most part, many bacteria are protected from osmotic stress by global regulatory systems, but in some cases, species possess unique mechanisms, such as the accumulation of glutamate by an *S.* Typhimurium strain (102). Some variations of osmotic stress tolerance seem to be dependent on levels of osmotic

shock and temperature (103). This section describes the most relevant osmotic stress responses that have been described for key bacterial pathogens.

Signals and Primary Responses of Osmotic Stress

Changes in water content and cytoplasm solute concentrations are the signals that bacteria respond to and lead to shifts in cell volume (104). Regulation of cytoplasmic osmotic conditions is triggered by detecting drastic changes in osmotic pressure. The concentration changes that have been reported to trigger an osmotic stress response include those related to membrane damage, concentration of a given compound, lack of potassium, osmolality, and turgor pressure (100, 104). Transmembrane chemoreceptors in *E. coli* sense changes in solute concentrations and provide the first osmoprotective signal (105, 106). The transmembrane chemoreceptors in volve CheY, a response regulator phosphorylated by cytoplasmic kinase CheA. The flagellar motility complex is then affected by CheY, and this binding promotes the movement of bacterial cells away from high solute concentrations (106).

For over 20 years, the role of mechanosensitive (MS) channels in osmotic shock response has been observed in *E. coli* as well as in *L. monocytogenes* (107, 108). The MS channels MscL and MscS in *E. coli* have been described to be involved in sensing and responding to different osmotic conditions. In *Listeria*, the equivalent MS channel is encoded by *lmo2064*, which encodes a protein that is 43% identical to MscL (108). MscL and MscS are almost ubiquitous in most bacterial species, but MscL has also been reported in many eukaryotic organisms (109). These MS proteins appear to have a much wider role, since mutant strains have a diminished ability to grow (107). Recent evidence suggests that MS proteins play a very important role as “emergency release valves” that expel intracellular solutes rapidly (109). The discovery of novel bacterial sensing and signaling systems may offer new insights into stress responses.

Osmoprotectants are small-molecule compounds that are critical in the bacterial response to an osmotic shock. The activation of osmoprotectant transporters is one of the first metabolic changes triggered by osmotic stress. Adjusting the concentration of osmoprotectant solutes counteracts the increase in osmotic pressure and minimizes water efflux caused by simple osmosis (102, 110). Such osmoprotectants include trehalose, carnitine, glutamate, choline, glycine betaine, and proline, and they function by stabilizing the cytoplasm and minimizing the loss of enzymatic activity due to loss of protein tertiary structures (101, 102, 104). Glycine betaine is particularly protective for many bacterial species because it optimizes the amount of available water inside the cells (101, 110). The appropriate concentration of osmoprotectants is dependent on the induction of active transport systems.

Osmotic Response of Gram-Negative *Enterobacteriaceae*

E. coli and *S.* Typhimurium share the same osmotic response mechanism, which involves two steps. In the first step, an increase in the concentration of amino acids and potassium ions is attained by the activation of transport systems (111). The second step in their osmoregulation also involves the activation of transport systems for solutes that will balance the turgor pressure in the cell.

The transport of potassium ions is critical for the cell homeostasis of *E. coli* and *S.* Typhimurium, as it is involved in multiple cellular pathways. For osmoprotection, K^+ transport is largely regulated by Kdp, Trk, and Kup (104). The activity of Kdp is triggered by turgor pressure and the concentration of potassium responding to an osmotic shock (112). The presence of glycine betaine transporters (ProU and ProP) in *E. coli* is critical for its osmotic response. These transporters also have a dual specificity for proline (104). ProU is activated after the concentration increases and is vital during osmoadaptation, and *proU* is transcribed after an osmotic stress (104, 111). Conditions of high osmolarity cause full induction of the

proU operon. A common regulatory component may be responsible for also inducing both *kdp* and *proU* (111).

An increase in external solute concentration triggers the synthesis of a handful of proteins in *E. coli* (111). Genes involved in osmotic stress response are also regulated by RpoS (113, 114). Because osmotic shock can cause bacterial cells to slow their growth and enter stationary phase, the osmotic stress genes are regulated by σ^S (115). RpoS regulates the genes involved in trehalose synthesis, *otsA* and *otsB* (112, 114). The betaine transporters are regulated by several effectors. RpoS, catabolite repression protein, and Fis control ProP transcription, and synthesis of ProU is modulated by TopA, GyrAB, H-NS, and HU (114, 115). At the same time, RpoS is also responsible for releasing the repression of H-NS, a histone-like protein, during hyperosmotic conditions (113).

Another important stress response system is the two-component system OmpR-EnvZ in *Enterobacteriaceae* (112, 116). OmpR and EnvZ regulate the expression of the outer membrane porin proteins OmpC and OmpF, and they have been reported in multiple Gram-negative bacteria (117). EnvZ is a sensor protein kinase responsible for phosphorylating OmpR. Phosphorylated OmpR binds DNA and induces *ompF* when osmotic pressure is low but represses it at high osmolarity. OmpC synthesis, however, is diminished at low solute concentrations but stimulated with osmotic shocks (112, 116). *Salmonella* appears to regulate *ompC* and *ompF* differently than other *Enterobacteriaceae*, since stationary phase is the sole trigger of their activation (102). OmpR plays a role as a global regulator of other stress response mechanisms, and in its phosphorylated form it represses *flhDC*, the operon responsible for flagellum expression (116).

The complex osmotic response system in *E. coli* and *Salmonella* also involves PutP and PutA, which confer the capability of using proline as the sole carbon and nitrogen source (118, 119). PutP is a symporter that carries out the simultaneous uptake of sodium ions and proline, and PutA mediates the oxidation of proline (111). The

proteins encoded by the *bet* regulon (BetTIBA) of *E. coli* transport choline and catalyze its transformation to glycine betaine. BetI and ArcA are the regulators of the *bet* regulon.

Osmotic Stress Response of Gram-Positive Bacteria

Among foodborne pathogens, *L. monocytogenes* is particularly tolerant to high concentrations of solutes and salts. In *L. monocytogenes*, several proteins that are expressed as a result of high osmolarity have been reported (108, 120). The uptake of osmoprotectants is the stress response used by *L. monocytogenes*. The most relevant osmotic response genes are *betL*, *proBA*, *relA*, *clpC*, *clpP*, and *sigB*. $v^{s.}$ plays a particularly important role in surviving high-osmolarity conditions, since it induces the expression of *betL* and *opuC*. After stationary phase has been reached, σ^B-dependent induction allows the transport of osmoprotectants (120). σ^Balso induces the ABC carnitine transporter, peptidases, and antiosmotic stress transport systems (121).

In *L. monocytogenes*, BetL, a sodium-dependent transporter that is activated immediately after osmotic shock, mediates betaine uptake (108). When the osmotic stress is more prolonged, another transporter, GbuABC, that mediates betaine uptake is synthesized. Another osmoprotectant, carnitine, is transported into the cells by OpuC, which is also involved in virulence. The *proBA* operon encodes enzymes functionally similar to the glutamyl kinase and glutamylphosphate reductase that catalyze the synthesis of proline (122). Sleator et al. (108) reported that *proBA* mutants had a diminished ability to survive high concentrations of salt.

Gardan et al. (120) reported a unique protein, Ctc, that was induced by osmotic stress and by stationary phase. In the absence of glycine betaine and carnitine, Ctc became activated in protecting cells against osmotic shock. Ctc seems to also be involved in growth. Additional proteins protecting against osmotic stress have been reported in *Listeria* (108). These osmoregulated proteins include

OpuB, a carnitine transporter, and OppA, a protein that mediates the accumulation of peptides triggered by osmotic stress.

S. aureus has the ability to tolerate high concentrations of salt and relatively low levels of moisture for growth and survival. This pathogenic bacterium has been reported to grow at water activity levels of 0.9 and sodium chloride concentrations greater than 10% (101, 123). The osmotic tolerance of *S. aureus* may be due to increased levels of a pyruvate dehydrogenase during an osmotic shock (124). *S. aureus* is also protected from osmotic stress by two glycine betaine transporters with different affinities (125). The transporter with low affinity for glycine betaine may be more important for stress response, as it is induced by osmotic stress. In contrast, glycine betaine concentration is the inducer of the high affinity transporter.

Future Considerations

Food is a rich source of compatible solutes for bacteria, and therefore, knowing the solute-specific characteristics of a food is essential for choosing a method by which the available water will be limited in a food (121). Keeping this in mind, processors should take into consideration the rate at which organisms are able to recover after drastic internal pH changes by looking at solid and liquid media separately, as all foods should not be treated equally (31). Understanding the intricacies of the osmotic stress response can aid in the design of protocols and effective control measures which act to prevent the growth and survival of pathogens in foodstuffs with low water activity (119).

The challenge lies in the fact that the osmotic stress response of bacteria is a diverse system involving many external signals and coinciding mechanisms which, in large part, have yet to be elucidated (119). At the molecular level, the mechanisms of compatible solute function and their transport in various bacterial species, as well as the possible role of osmoprotectants in bacterial pathogenicity, are not fully understood (43, 119, 126). Additionally,

there is minimal information regarding possible osmoregulation of porins in Gram-negative species other than the enteric organisms (21). On a more holistic scale it is unknown whether osmoregulated operons, such as the OmpR-EnvZ and ProU operons of *E. coli*, possess common regulatory elements that might control these operons at a higher level (21). The facet of osmotic stress response and regulation will remain an area of interesting, and necessary, development for years to come.

OXIDATIVE STRESS

During evolution, successful life forms had to develop protective metabolism against oxygen, one of the most reactive elements in nature (127). It is largely believed that the earliest forms of life developed primitive photosynthetic capabilities which allowed them to transform light energy into chemical energy and at the same time produce oxygen as a by-product (1). As the earth's atmosphere changed to contain large amounts of oxygen, nonphotosynthetic living forms were forced to develop mechanisms to counteract highly reactive oxygen forms. As an ancient form of life, bacteria evolved multiple strategies to allow them to survive oxidative stresses.

ROS are the forms of oxygen that are most toxic for biological metabolism. The predominant forms of ROS are superoxide anion and hydrogen peroxide (33, 127). In order to dissipate the toxic effects of ROS, the key enzymes that bacteria developed are superoxide dismutases, catalases, and hydroperoxide reductases (128). Reacting ROS with cysteine residues provides one effective detection mechanism for their presence. *E. coli* responded to hydrogen peroxide exposure after 30 min by expressing several dozen proteins (129).

In *Enterobacteriaceae*, the OxyR regulon is one of the critical components for detoxifying hydrogen peroxide (128, 129). In Gram-negative bacteria, such as *E. coli*, sensing and detoxification of

hydrogen peroxide are controlled by the OxyR regulon (123, 130). The function of OxyR is not only regulatory; it also serves as the sensor for the presence of hydrogen peroxide. OxyR is activated when a cysteine residue (Cys-199) of one of its subunits is oxidized (127). After this initial oxidation step, Cys-199 reacts with Cys-208 from another subunit. Disulfide-containing OxyR then binds to promoters that will induce specific oxidative stress response genes. OxyR is extremely sensitive to hydrogen peroxide, since just nanomolar concentrations are sufficient for full activation. The OxyR basal state is restored to its inactive form by reduction steps catalyzed by glutathione reductase (GorA) and glutaredoxin 1 (GrxA) (129).

OxyR regulates a wide array of stress response proteins involved in the synthesis of reducing agents, hydrogen peroxide hydrolysis proteins, and other regulatory proteins (129). In *E. coli*, the detoxification of hydrogen peroxide and its free-radical by-products is catalyzed by catalase (KatG) and alkyl hydroxyperoxide reductases (AhpCF) (128). Other specific response components regulated by OxyR include thioredoxin 2 (*trxC*), Dps, and OxyS (22, 128). OxyR is also critical for iron uptake and metabolism. The ferric uptake repressor, Fur, is induced by OxyR, and the Fur regulon includes several proteins involved in iron, zinc, and hydrogen peroxide sensing (127). OxyR also regulates the Ohr regulon, which encodes proteins that mediate organic hydroperoxide detoxification.

In Gram-positive bacteria, such as *L. monocytogenes*, PerR, a stress response regulator equivalent to OxyR, has been reported (131). PerR-defective mutants had a reduced ability to tolerate hydrogen peroxide. PerR in Gram-positive organisms is a metalloregulatory protein with two metal-binding domains. Similar to OxyR, multiple oxidative stress response genes are also regulated by PerR (132). The expression of catalase (*kat*) was upregulated in Δ*perR* mutants, and as in *E. coli*, PerR was also involved in controlling the expression of Fur.

HIGH-TEMPERATURE STRESS

Changes in temperature are a threat that bacteria experience continually in the environment, and temperatures greater than room temperature often present the possibility of serious damage to cells. Microorganisms have evolved a series of protective pathways to allow them to survive high temperatures. The production of heat shock proteins (HSPs) is the most widely distributed heat stress response in bacteria. HSPs protect the cell components involved in protein synthesis, transport, folding, and degradation and can be synthesized at any temperature (126). A sudden increase in temperature enhances the expression of HSPs, and after about 10 min, the production of general HSPs ceases and growth stops (126, 133). At this point, bacteria upregulate several specific heat stress response genes.

Heat can cause severe and extensive damage to bacterial cells that can potentially lead to complete inactivation and death. Heating can cause the loss of tertiary protein structure, protein aggregation, disruption of the entire metabolism, enzyme inactivation, changes in protein phosphorylation, and disruption of cell and outer membranes (11, 126, 134). If bacteria are subjected to sublethal levels of heating, their ability to withstand higher temperatures is enhanced thanks to thermotolerance mechanisms (134). The thermotolerance mechanisms of several foodborne bacteria have been extensively studied, but this subsection is dedicated to two organisms whose mechanisms have been characterized more completely, *E. coli* and *Bacillus* spp. These two models represent not only Gram-negative and Gram-positive genera but also nonsporeformers and sporeformers.

Gram-Negative Bacteria

Several heat response systems have been reported in *E. coli* (135, 136). These systems have also been described in other Gram-negative organisms, and it appears that they are highly conserved. Approximately 400 genes are activated by increasing the incubation

temperature from 37 to 43°C (137). In *E. coli*, a large network of HSPs in combination with two sigma-dependent regulatory systems have been identified, the σ^E and σ^H heat stress regulators. Depending on the location and accumulation of misfolded proteins, activation of one or more of these systems is the primary response. The σ^E regulon is typically activated by damaged proteins in the cell's outer layers, and the transcription of σ^H is upregulated when unfolded proteins accumulate in the cytoplasm (135, 138, 139)

σ^E Regulon

Induction of the σ^E regulatory operon takes place only above 40°C (139). *rpoE* encodes σ^E, and it regulates the expression of at least 10 proteins and the transcription of over 43 genes. The *rpoE* operon includes *rseA* and *rseB*, which encode proteins that integrate into the cytoplasmic membrane and periplasm, respectively (135). Refolding and degradation of proteins are mediated by two proteins encoded by *htrA* and *fkpA* within this regulon (139). Several outer membrane proteins are activated after thermal shock, and their role is to repair DNA and membrane lipids. These outer membrane proteins also have a role in regulating the transcription of σ^E.σ^E is responsible for transcribing *rpoH*, which encodes the transcription factor σ^H (σ^{32}), the other major component of the heat response (126).

σ^H Regulon

The majority of the heat stress response genes are activated by σ^H (140). Transcription of *rpoH* is controlled by four promoters, referred to as P1, P3, P4, and P5, but when the temperature is more than 50°C, the only active promoter is P3 (139, 140). RpoH is inhibited by several heat shock proteins that include DnaK, DnaJ, and GrpE (140). The role of the latter proteins as well as GroEL is to function as chaperones, which are molecules that prevent the accumulation of misfolded or denatured proteins by proteolysis

(141, 142). Additional proteolytic proteins regulated by RpoH are Lon and the Clp proteins (ClpB, ClpP, and ClpX) (133, 135). In this regulon, the heat shock genes *htrC* and *htpY* are located upstream of the DnaK operon. The transcription of *htpY* induces only the *htrA* promoter in the σ^E regulon but activates both σ^H -and σ^E -dependent heat shock regulons, while *htrC* negatively regulates the response (135).

Table 4.1 Classes of heat shock genes in Gram-positive bacteria[a]

Class I	Class II		Class III	Class I/III	Class IV[b]
hrcA	*bmrU*	*trxA*	*ctsR*	*dnaK* (*S. aureus*)	*ahpF*
grpE	*bmr*	*yacH*	*yacH*	*clpP* (*Streptococcus*	*clpX*
dnaK	*bmrR*	*yacI*	*yacI*	*salivarius*)	*ftsH*
dnaJ	*bofC*	*yacL*	*clpC*		*htpG*
yqeT	*csbA*	*ycdF*	*sms*		*htrA*
yqeU	*csbB*	*ycdG*	*yacK*		*lonA*
yqeV	*yfhO*	*ydaP*	*clpP*		*yvtA*
groEL	*csbC* (*yxcC*)	*ydaD*	*clpE*		*yvtB*
groES	*csbD* (*ywmG*)	*ydaE*	*clpC*		*ywcG*
	csbX	*ydaF*			*ywcH*
	ctc	*ydaG*			
	dps	*yfkM*			
	gsiB	*yflT*			
	gspA	*yhdF*			
	gtaB	*yhdG*			
	katB	*yhdN*			
	katX	*yjbC*			
	opuE	*yjbD*			
	rsbR	*ykzA*			
	rsbS	*yocK*			
	rsbT	*ysdB*			
	rsbU	*ytkL*			
	rsbV	*ytxG*			
	rsbW	*ytxH*			
	sigB	*ytxJ*			

	rsbX	*yvyD*			
		yxkO			

[a] Compiled from reference 130.

[b] Unidentified.

Gram-Positive Bacteria

The heat shock response of *Bacillus* has some similarities to that of *E. coli*, but in this case, most heat stressrelated genes are classified into four major groups (Table 4.1) (38, 133, 143). Each heat stress response class is distinguished by the regulatory systems that control expression of HSPs.

Class I

The *hrcA* and *groEL* operons are the main components of class I. The role of HrcA is to repress transcription of heat shock genes by binding to the controlling inverted repeat of chaperone expression *cis*-active sequence (130, 133, 144). The housekeeping sigma factor σ^A regulates the transcription of *groEL* (145).

Class II

The class II regulon is controlled by the σ^B stress factor and it is also referred to as the SigB regulon. More than 200 genes regulated by σ^B are upregulated within 3 minutes following heat exposure. The deactivation of these SigB-dependent genes occurs after 10 minutes, while the rest of the heat response genes remain expressed (130). The genes in this class may also be induced by other stresses, such as osmotic, low-oxygen, and high-pressure conditions (130, 133).

Class III

The class III heat stress response regulon has been reported in multiple low-G+C Gram-positive bacteria (133). The key regulator of this class is CtsR (class III stress gene repressor), which can be

regulated by σ^A and σ^B (127, 145). ClpC, ClpP, and ClpX (ClpX is in class IV) are stationary-phase proteins that are required for motility, sporulation, and growth at high temperatures (144). *Streptococcus* spp., *Clostridium* spp., and *Enterococcus* spp. have their own CtsR regulon, but their specific characterization has yet to be completed (133).

Class IV

Several stress response genes are placed in class IV because they are not regulated by CtsR or by σ^B (130, 133). The majority of these genes are components of transporter systems (130). In contrast to *B. subtilis*, *S. aureus* has only class I and III regulatory systems.

Although the regulation mechanisms of the heat stress response have been studied thoroughly in Gram-positive and Gram-negative bacteria via the model organisms *Bacillus* and *Escherichia*, respectively, additional research is recommended in order to fully understand the complex series of systems and responses that protect against heat stress. In many cases, it is still not fully understood how the bacterial cell initially senses a change in environmental temperature and how signals are transported into the cell to induce specific regulatory mechanisms (138, 141). In other foodborne pathogens, such as *Campylobacter*, the major regulators of the heat stress response have yet to be elucidated or confirmed.

LOW-TEMPERATURE STRESS

For many centuries, humans have relied on the use of low temperature for food preservation. Currently, cold storage and handling of foods continue to be one of the most effective technological approaches to minimize microbial growth and spoilage. Refrigeration is the use of low temperature above the freezing point of water (typically 0 to 7°C) to store foods, and freezing storage keeps foods at less than 0°C, normally at

approximately –15°C. Some bacteria associated with foods are capable of growing at refrigeration temperatures, but freezing completely inhibits allmicrobial growth. Low-temperature exposure of bacteria cells can cause serious damage to their metabolism and can lead to loss of survival ability.

The ability of bacteria to survive adverse low-temperature conditions depends on the presence of a response metabolism that counteracts cold shock. To complement the previous section, in which the stress responses to higher temperatures were discussed, this subsection covers the relevant molecular mechanisms that protect cells from low temperatures.

Drastic temperature changes from the mesophilic range (30 to 40°C) to less than 10°C affect bacterial cells by altering structure and metabolic functions as well as disturbing membrane fluidity and integrity (146). A sudden reduction in temperature inhibits the activity of most proteins and enzymes, and at the same time, it induces the synthesis of several cold shock proteins (CSPs). Low-temperature exposure of *E. coli* cells results in the synthesis of more than 25 proteins (146, 147). After cells are exposed to approximately 1 hour of low temperature, nine homologous CSPs, CspA to -I, are activated. In addition, several DNA-associated factors are also activated. Among the CSPs expressed are DNA gyrase (GyrA), RNA helicase (DeaD), a transcription factor (NusA), and a translation factor (InfB) (146).

CSPs have been characterized extensively, and they are some of the first stress responses for which molecular regulation was described. In general, CSPs are highly conserved among multiple bacteria, and they are small proteins, approximately 7.4 kDa (146). CSPs can bind to only single-stranded DNA or RNA, not doublestranded DNA. Among CSPs, CspC and CspE are also induced by changes to higher temperatures. Stationary phase and starvation can also be signals to induce CspD (146, 147). It was reported that two of the multiple CSPs of *L. monocytogenes*, Csp1 and Csp2, were upregulated after treatment with high-hydrostatic-pressure (HHP) processing (148).

The regulation of transcription is the main protective role of CSPs against low temperatures, because the chromatin-associated protein H-NS is associated with the cold shock response (147). CspA stimulates the expression of H-NS as a transcription activator binding the promoter region of *h-ns*. Similarly, GyrA synthesis is also stimulated by CspA. The majority of CSPs also protect the configuration of RNA tertiary and secondary structures. In addition to their roles as transcriptional activators, CSPs also have a regulatory function during translation, which is critical for cell survival (146). The first sign of CSP regulation is stabilization of mRNA, which includes its own RNA and rRNA (147). Because the low temperature increases the stability of bonds between DNA strands, the role in translation is to facilitate the continuous RNA synthesis of many household genes and minimize misfolded mRNA. Other cold-induced proteins, such as DeaD, are helicases involved in unwinding double-stranded nucleic acids.

HHP SURVIVAL

HHP is an effective technology for inactivating pathogens in multiple food types (149, 150). The damage inflicted by HHP on microorganisms is dependent on different variables related to treatment. The most important factors are the amount of pressure and the temperature. In addition, the type of food, the treatment time, and the bacterial characteristics influence the extent of inactivation. When mild pressure levels are used, the cell membrane is disrupted and metabolism is inhibited by increased permeability. The use of extremely high pressure intended to sterilize food stuffs causes irreversible damage to ribosomes, DNA, RNA, cell walls, and cell membranes (151). The growth of most microorganisms becomes inhibited at 20 to 130 MPa (152). Depending on the species, vegetative-cell death usually occurs between 130 to 800 MPa, but the inactivation of bacterial endospores often requires pressures above 1,200 MPa (151, 153). In general, stationary-phase cells are more resistant to HHP than

exponentially growing cells (149, 154). Overall, the effects of pressure on bacterial physiology and the genetic response elicited by organisms for survival to this stress are not well characterized. More studies are necessary in order to shed light on this facet of microbial stress response.

Exposure to pressure leads to expression of proteins in bacteria, and these proteins are the same as those induced by other stresses such as temperature shifts (155, 156). Similarly, when cells are exposed to thermal stress, acidity, or other stresses, they are often more tolerant of high pressure (149). The *E. coli* pressure stress response has been reported to be regulated by RpoS (157). Of the genes upregulated during stationary phase, as many as 55 are part of the protective mechanism against pressure. Several of these proteins were some of the key components of the temperature shift responses, but approximately 40 were found only in pressure-treated cells (158). High-pressure shocks have also been reported to induce the general stress response SOS, which is independent of RpoS (155). This SOS-dependent pressure response is regulated by RecA and LexA. These activators induce multiple genes, including *sulA* and *uvrA*. The reduction in outer membrane proteins (OmpC, OmpF, and OmpX) may also be due to SOS regulation (155). In *L. monocytogenes*, the general stress response, σ^B, is activated and two CSPs (Csp1 and Csp2) were transcribed as a result of high pressure (148, 159). Additional studies may be necessary to determine the cross-protective stress response to pressure and temperature.

STRESS RESPONSES AND VIRULENCE

The association between bacterial stress responses and pathogenicity has been extensively investigated in multiple foodborne pathogens (160). The main concern stemming from this possible linkage is whether exposure to sublethal stresses during food processing can lead to the presence of microorganisms with enhanced virulence. The hypothesis of this potential relationship was advanced originally by the realization that global regulators

such as sigma factors had such widespread influence on multiple regulons throughout the bacterial genome (12). In addition to the rationales that support the potential coregulation of stress responses and virulence factors, the environment of foods resembles to a certain extent the host's cellular components and defense mechanism.

One of the first connections between stress response and virulence regulation in a foodborne pathogen was reported by Fang et al. (161), when they showed that RpoS (KatF) mutant strains of *S.* Typhimurium had a reduced ability to cause infection in mice. The role of RpoS in systemic infection has also been demonstrated by observations that it is required for the persistence of *Salmonella* in liver and spleen (30). A direct link between RpoS and virulence is the expression of a hemolysin network in *Salmonella* (12). In *V. cholerae*, deficiencies in σ^S expression led to increased sensitivity to starvation and oxidative conditions, and it was found that it was required for efficient gastrointestinal tract colonization (162). Similarly, in *L. monocytogenes*, SigB mutants had a decreased ability to infect mice and guinea pigs, and this effect appeared to be mediated by the control of PrfA, a general virulence regulator, by SigB (12).

In addition to shared global regulators of stress response and virulence, some organisms have specific stress responses that are involved in pathogenicity. The link between osmotic response and virulence was observed when it was found that *opuC*-defective strains had a diminished ability to colonize the gastrointestinal tract of mice and cause systemic infection (12). OpuC is an osmoprotectant transporter in *Listeria.* Oxidative and acid response stress systems are two frequently studied systems with a clear link to the pathogenicity of foodborne bacteria. The low pH and strongly oxidative conditions that invasive pathogens encounter in phagosomes are the barriers that they need to overcome in order to cause infection. In *Salmonella*, OmpR-EnvZ is activated by acid shock, and it also induces the type III secretion system that enhances cellular replication (160). A recent report study linked the

requirements for the type III secretion system, *sopD*, and *sseD* to the ability of *Salmonella* to survive during desiccation (163). The acid resistance of *L. monocytogenes* mediated by arginine deimidase has also been reported to play an important role in promoting infection in a murine model (89). Recent bioinformatics and genomic advances are helping to elucidate the complex metabolic regulatory systems in bacterial pathogens (164). For two recent reviews on the relevance of stress responses and virulence of foodborne pathogens, see references 12 and 160.

SUMMARY

An advance in our knowledge of the molecular strategies for bacterial survival in foods is critical to understand the underlying principles that allow their presence and have deleterious effects, including spoilage and foodborne disease. A deeper understanding of the key components of each of these systems is essential to develop future strategies of control. Organisms such as *E. coli*, *Salmonella*, and *B. subtilis* have served as the model organisms for Gram-negative and Gram-positive bacteria to shed light on the intricacies of cellular responses to threatening conditions. Many of those systems have also been identified in other bacterial species, but many more species-specific stress regulators remain to be discovered. The advent of genomics-, proteomics-, and metabolomics-based techniques has accelerated our knowledge of the components involved in novel stress responses. In addition, the availability of an increasing number of fully sequenced bacterial genomes will also contribute to further advances in the field of bacterial stress responses.

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II
Microbial Spoilage and Public Health Concerns

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5
Milk and Dairy Products

Milk-associated microbes have long been of interest because of their impact on food safety and quality and their necessity for the production of fermented dairy products. The microbial ecology of milk and dairy products has primarily been studied by traditional, mainly culturedependent, microbiological approaches. These techniques have provided tremendous fundamental insights into the genetics, physiology, and ecology of microorganisms in dairy systems, including lactic acid bacteria, spoilage bacteria and fungi, and foodborne pathogens. This field has continued to evolve thanks to the rapid advancement in "-omics" technologies since 2000 (1–3). Genomics application in dairy products was marked by the genome sequence published for *Lactococcus* (*Lc.*) *lactis* IL1403, a dairy-associated strain most commonly used as a starter culture for cheese production (4). *Lc. lactis* IL1403 is also one of the first bacterial strains for which the genome sequence was available. Another significant genomics advancement was the discovery of clustered regularly interspaced short palindromic repeats (CRISPRs) and CRISPR-associated sequences (Cas) and their roles in immunity against bacteriophages of *Streptococcus thermophilus*, an organism routinely used in dairy fermentations (5). The development of high-throughput DNA sequencing methods provided metagenomics studies with unprecedented sampling depth, and these methods are currently pervasively used in exploring the microbial biodiversity of milk and dairy products, among other food commodities (6).

This chapter emphasizes current knowledge on dairy food microbiology from the perspective of cultureindependent microbiome research (Fig. 5.1). Because the majority of studies have relied on sequencing the DNA of selected marker genes, such as the variable regions of 16S rRNA genes in bacteria and internal transcribed spacer regions in fungi, this methodological approach is emphasized (Fig. 5.1). However, when possible, knowledge from metagenomics and other more comprehensive techniques is also discussed (Fig. 5.1). Findings from this emerging metagenomics field are complementary to and build upon prior dairy microbiology research (7, 8).

MILK MICROBIAL DIVERSITY AND ABUNDANCE

Bovine Milk

Around 180 million metric tons of bovine (*Bos taurus*) milk is consumed annually worldwide (9). Although bovine milk has long been studied by culture-dependent microbiological approaches, modern microbiological methods have provided new perspectives on its microbial composition and variation prior to and after pasteurization. It is now understood that bovine milk has a highly diverse microbial composition that fluctuates around a core microbiota consisting of a few key taxa.

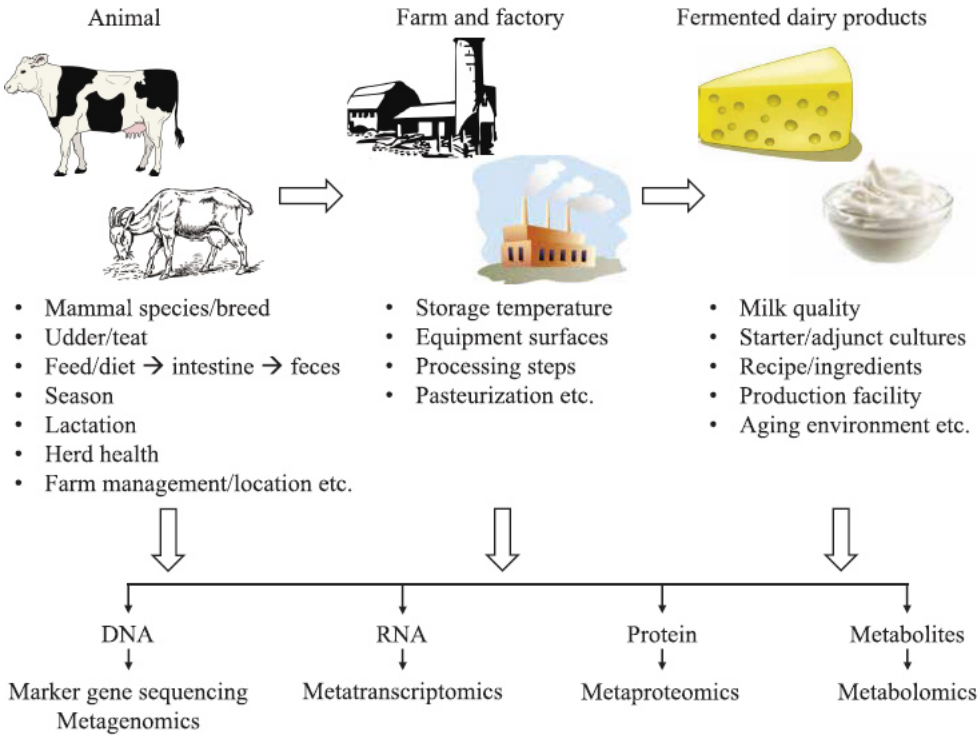


Figure 5.1 Sources and factors influencing milk and dairy product microbial composition, as well as genomics-based methods for determining microbiological variation in dairy products.

Bacteria in Unpasteurized Bovine Milk

The microbiota in milk originates from a variety of sources, such as the cow udder and teat, farm air, grass, dust, dirt, bedding, feed, feces, and equipment (Table 5.1) (10–15). The most direct passage of bacteria into milk is by the cow udder and teat. Verdier-Metz et al. surveyed microorganisms on teat skin and identified various bacterial taxa, including *Enterobacteriaceae*, *Enterococcus*, and *Staphylococcus*, which are also commonly found in raw milk (16). Unprepared teats can result in milk with significantly lower bacterial alpha diversity, which is likely due to the predominance of one or a few bacterial contaminants from the teat surface (17). Regardless of teat surface contamination, milk from the udders of healthy cows is not sterile. Transport of bacteria from the maternal large intestine to mammary glands by leukocytes in bloodstream might occur through an endogenous entero-mammary pathway (18, 19). Comparisons of 16S rRNA gene sequences from the milk, white blood cells, and feces of healthy cows resulted in the identification of operational taxonomic units assigned to *Ruminococcus*, *Bifidobacterium*, and *Peptostreptococcaceae* in all three sample types (20). Conversely, bacterial DNA was not recovered in white blood cells from cows that were the same age but not lactating or pregnant (20).

Bacteria found in milk are also subject to variation depending on cow breed, lactation stage, bovine health, cow, diet, farm location and practices, and season. The milk microbiota was found to differ according to the cow breed, thereby indicating a genetic component to milk microbiota composition (21). Bacterial composition was also found to show lactation stage dependency. Milk from early lactation harbored the highest proportions of *Lactobacillus* compared to milk from later lactation stages (22). *Bacteroides*, *Faecalibacterium*, *Campylobacter*, and *Rhodanobacter* were more abundant in raw milk during mid-lactation, whereas *Corynebacterium*, *Micrococcus*, *Arthrobacter*, and *Clostridium* were more prevalent in milk from late lactation (23). Milk from late lactation was also found to contain higher total numbers of bacteria as well as higher bacterial alpha diversities (22–24).

When a lactating cow is affected by mastitis (i.e., bacterial infection of the mammary gland), it has the potential to shed as many as 107 bacteria per ml into the milk, with increased proportions of

Acinetobacter, *Brevundimonas*, *Burkholderia*, *Dermacoccus*, *Enterococcaceae*, *Lactobacillus*, *Lactococcus*, *Pseudomonas*, *Ruminococcaceae*, *Sphingobacterium*, *Sphingomonas*, and *Stenotrophomonas* (21, 25–30). The enriched taxa can include potentially opportunistic pathogens as well as bacteria known to cause bovine mastitis, such as *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, and certain *Mycoplasma*, *Corynebacterium*, and *Enterobacteriaceae* species (21, 25). It is notable that the overall bacterial alpha diversity in milk was lower in mastitic than healthy cows (21, 25–30). The milk microbiota can also differ between teats on individual animals, depending on the extent of infection (25).

Cow diets influence milk quality and the associated microbiota. In a crossover feeding experiment comparing high-forage and high-grain diets, Zhang et al. showed that the high-grain diet, which is prone to induce subacute ruminal acidosis, increased the relative abundances of *Enterococcus*, *Brevundimonas*, *Lactococcus*, and *Stenotrophomonas*, while it reduced the proportions of *Clostridiales*, *Streptococcus*, *Sphingomonas*, and *Prevotella* and conferred a slight decrease in overall bacterial alpha diversity (31).

Farm location and practices are associated with differences in the bovine milk microbiota (21). Commensal and environmental microorganisms can be introduced and spread by handling personnel, bedding, and milking equipment. Additionally, animal housing conditions (indoor versus outdoor) can also result in variation in the raw milk microbiota. Cows raised in outdoor, pasturebased farms produced milk that harbored a more diverse microbiota (17).

Multiple studies have shown that the microbiota of raw milk varies depending on the season (17, 32, 33). In general, total bacterial cell numbers were estimated to be higher in the winter and spring than other seasons (24, 32). The diversity of bacteria was also greater in the spring (24, 32). *Corynebacterium* and *Acinetobacter* were more abundant in the spring; *Streptococcus* and *Staphylococcus* were more abundant in the summer; and *Turicibacter*, *Pseudomonas*, *Lachnospiraceae*, and *Enterococcus* were more abundant in the fall (32). These findings are likely influenced by variations in farming practices and environmental exposures that change depending on the time of year.

After collection, milk is stored under refrigeration (2 to 6°C) on the farm until transport for processing. Storage conditions can have different effects on the milk microbiota composition. When milk is stored at temperatures lower than 2°C, the microbiota composition stays largely unchanged (23). At higher temperatures (4 to 6°C), the total bacterial population increases, and specific psychrotrophic taxa, including *Streptococcus*, *Pseudomonas*, and *Acinetobacter*, have been found in increased proportions, whereas the quantities of *Staphylococcus*, *Rhodanobacter*, and *Ruminococcaceae* decreased (23, 34, 35). *Streptococcus*, *Pseudomonas*, and *Acinetobacter* are frequently associated with dairy product spoilage via either increased growth or the production of extracellular proteases, peptidases, and lipases (36). The milk microbiota can also fluctuate after transport and during storage or holding at dairy processing facilities. In one study, *Lactococcus*, *Streptococcus*, *Acinetobacter*, *Pseudomonas*, and *Mycoplasma* were enriched in milk kept in holding silos after transport from dairy farms (32).

Processed Fluid Milk

Milk is a nutrient-dense substrate for growth of a variety of bacteria, including foodborne pathogens. The U.S. Public Health Service's advocating the pasteurization of raw milk in 1939 was a landmark for public health which resulted in significant reductions in outbreaks of milk-borne illness (37). The current pasteurization treatment (high temperature, short time) required by the U.S. law is 72°C for 15 seconds. This method was previously designed to destroy *Mycobacterium tuberculosis* and is now used to eliminate *Coxiella burnetii*, the most heatresistant milk-borne pathogen (38, 39). Pasteurization also has a significant impact on the composition of milk microbiota. Overall, the bacterial alpha diversity of pasteurized milk is lower than that of raw milk (40). When specific members of bovine milk are compared pre-and postpasteurization, the relative abundances of *Enterobacteriaceae*, *Moraxellaceae*, *Pseudomonadaceae*, *Corynebacteriaceae*, and *Staphylococcus* are significantly decreased in

pasteurized milk, while the proportions of thermoduric bacteria, such as *Thermus*, and *Turicibacter*, are increased (40).

Table 5.1 The most prevalent bacteria in bovine milk and their likely sources and applications

Phylum	Class	Order	Family	Ge
Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Corynebacteriaceae*	*Co*
			Micrococcaceae	Un *Ar*
				Ko Un
		Propionibacteriales	*Propionibacteriaceae*	*Pr* Un
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Bacteroidaceae*	5-' *Ba* Un
			Prevotellaceae	*Pr* Un
Firmicutes	*Bacilli*	*Bacillales*	*Bacillaceae*	*Ae* Un
			PlanococcaceaeStaphylococcaceae	Un *M*
				St
		Lactobacillales	*AerococcaceaeEnterococcaceae*	Un Un *En*
			Lactobacillaceae	Un *La*
			Streptococcaceae	*La*
				St Un
	Clostridia	*Clostridiales*	*Clostridiaceae*	*Cl* Un
			Lachnospiraceae	*Do*
			PeptostreptococcaceaeRuminococcaceae	Un Un *Os* Un
Proteobacteria	*Gamma-proteobacteria*	*Enterobacterales*	*Enterobacteriaceae*	*Es*

Phylum	Class	Order	Family	Ge
		Pseudomonadales	*Moraxellaceae*	*Sa* Un *Ac*
			Pseudomonadaceae	*Ps* Un *Ps*
				Un

The addition of other processing methods further changes the milk microbiota composition. CO_2 addition by either modified atmosphere packaging or direct injection has been developed to lengthen the shelf life of raw and pasteurized milk (41). It was found that carbonized milk contained lowered proportions of *Pseudomonas*, *Serratia*, and *Erwinia* (42). These bacteria were likely inhibited because of their sensitivity to low O2 concentrations and reductions in pH resulting from CO_2 treatment (42).

Goat Milk

Goat (*Capra aegagrus*) milk has long been a source of human nutrition in countries in the Mediterranean, Eastern Europe, the Middle East, and South America (43, 44). Goat milk production constitutes about 2.1% of global milk production and increased by 39.2% from 2000 to 2012 (43, 45)

Table 5.2. is a compilation of abundant bacterial taxa that have been found in goat milk by recent efforts using DNA sequencing approaches. Like bovine milk, goat milk contains diverse bacterial populations that are affected by lactation stage. McInnis et al. showed that the microbiota of goat milk from late lactation was significantly different from that in milk from early lactation and mid-lactation (46). Specifically, *Pseudomonas* was the most abundant taxon during early and mid-lactation (about 50% of bacteria present). The relative abundance of *Pseudomonas* fell to approximately 2% during Late lactation, and the dominant taxon during late lactation was *Micrococcus*, constituting 68% of the bacterial population (46). McInnis et al. also collected raw milk from transgenic goats that can produce human lysozyme, and significant differences were observed only during late lactation, when *Bacillaceae*, *Alicyclobacillaceae*, *Clostridiaceae*, and *Halomonadaceae* were more abundant in the transgenic milk (46).

Milk from Other Mammals

Milk from other mammals, such as buffalo, donkey, water deer, and reindeer, is also consumed by various indigenous cultures and has been measured for microbial content by targeted 16S rRNA marker gene sequencing. Water buffalo (*Bubalus bubalis*) milk is consumed mainly in India, Pakistan, and southern China. It was shown that mastitic buffaloes produced milk with lower bacterial alpha diversities, which continued to decrease during refrigeration (1 to 4°C) (47, 48). In fresh, raw milk collected from healthy buffaloes, the bacterial community was dominated by *Streptococcus* (14%) and *Lactococcus* (8%) (47, 49). After 72 hours of refrigeration (1 to 4°C), *Pseudomonas* (51%) and *Acinetobacter* (11%) were predominant. After pasteurization, the microbiota of buffalo milk showed an abundance of *Lactococcus* (18%) and *Streptococcus* (7%). The pasteurized milk was stable for over 21 days at 1 to 4°C, after which the milk spoiled and was enriched in *Paenibacillus* (85%) (47, 49). Other abundant taxa (>1%) in buffalo milk include *Aerococcus*, *Corynebacterium*, *Clostridium*, *Enhydrobacter*, *Propionibacterium*, *Psychrobacter*, *Turicibacter*, and *Staphylococcus* (47, 48).

Table 5.2 The most prevalent bacteria in goat milk[a]

Phylum	Class	Order	Family	Genus
Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Corynebacteriaceae* *Micrococcaceae*	*Corynebacterium* *Micrococcus*
Firmicutes	*Bacilli*	*Bacillales* *Lactobacillales*	*Bacillaceae* *Staphylococcaceae* *Streptococcaceae*	*Bacillus* *Staphylococcus* *Lactococcus* *Streptococcus*
Proteobacteria	*Gammaproteobacteria*	*Enterobacterales*	*Enterobacteriaceae* *Yersiniaceae*	*Escherichia Serratia*
		Pseudomonadales *Oceanospirillales*	*Moraxellaceae* *Pseudomonadaceae* *Halomonadaceae*	*Acinetobacter* *Pseudomonas* *Halomonas*
	Alpha proteobacteria	*Rhizobiales*	*Hyphomicrobiaceae*	Unidentified

[a] Data adapted from references 46 and 53.

Donkey (*Equus asinus*) milk has progressively gained more interest for use as a replacement of bovine milk for children with cow milk protein allergies. Milk from donkeys also more closely resembles human milk in regard to essential amino acid and lactose concentrations (50, 51). The use of DNA sequencing to identify the bacteria in donkey milk demonstrated that *Pseudomonas*, *Sphingobacterium*, *Ralstonia*, *Acinetobacter*, and *Citrobacter* are the most abundant members of the microbiota (>1% of total relative abundance, in descending order) (52).

In a comparative study of milk from water deer (*Hydropotes inermis*), reindeer (*Rangifer tarandus*), and goats, Li et al. found that milk from water deer contained the highest bacterial alpha diversity, followed by reindeer and then goat milk, which contained the lowest diversity (53). Water deer milk harbored (>1% of total relative abundance, in descending order) *Pseudomonas*, *Acinetobacter*, *Chryseobacterium*, *Corynebacterium*, *Comamonas*, *Rhizobium*, *Rheinheimera*, *Microbacterium*, *Stenotrophomonas*, *Brevundimonas*, *Kocuria*, and *Sphingomonas*. Reindeer milk was found to contain (>1% of total relative abundance, in descending order) in *Hyphomicrobiaceae*, *Cyanobacteria*, *Halomonas*, *Ralstonia*, and *Propionibacterium* (53).

CHEESE

Cheese is an ancient fermented dairy food that has been a part of the human diet from as early as 5400 BCE (54). Cheese fermentations are prehistoric practices to preserve milk, improve flavors and textures, and increase the availability of nutrients for human consumption (54). It is estimated that there are now approximately 1,500 varieties of cheese produced around the world (55). These varieties are derived from milk of different ruminant animals exposed to coagulation agents (e.g., rennet, acid, heat, and/or crystallization), inoculated with ripening cultures (bacteria, yeasts, and molds), and incorporated with other ingredients (salt, spices, vinegar, smoke, coloring, etc.), as well as prepared using various processing and aging methods involving different factors (temperature, moisture, ripening time, etc.). A diagram of the most important steps in cheese manufacturing influencing the microbial populations is presented in Fig. 5.2.

The microbiotas of cheeses have been investigated since the early days of microbiology research (56). Microbes can be introduced into the cheese-making process in many ways, either intentionally or accidentally. However, knowledge on the ecological processes and molecular interactions that occur during cheese fermentation is incomplete. This is particularly evident because of the more recent applications of metagenomics methods to investigate this food. Table 5.3 is an extensive list of studies

that employed high-throughput DNA sequencing methods to elucidate the microbial communities of cheeses and cheese-related environments.

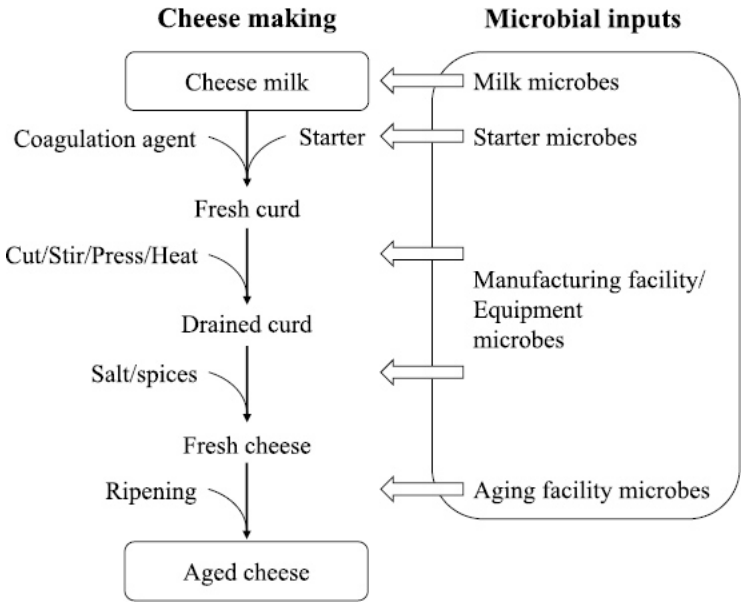


Figure 5.2 Steps in cheese manufacturing with the greatest influence on microbial populations.

Sources of Microorganisms in Cheese

Microbes can be introduced into cheese at nearly every step during processing (Fig. 5.2), leading to differences and variation in the cheese microbiota.

Milk

The process of cheese making typically starts with the standardization of milk to the desired composition in terms of fat, protein, calcium, and pH required for a specific type of cheese (57). Factors that influence the milk microbiota, such as animal species and lactation stage, are also important in cheese fermentations. The factors have implications for cheese quality and safety (22, 40). For example, cheese made from cow's milk contains the highest bacterial diversity, followed by goat's milk and then sheep's milk cheese (40).

The microbiota of raw-milk cheese is measurably different from that of cheese produced with pasteurized milk. Raw-milk cheeses tend to have a more complex and diverse microbiota than pasteurized-milk cheeses (40, 58, 59). Specifically, *Corynebacterium*, *Enterococcus*, *Halomonas*, *Helcococcus*, *Lactobacillus*, *Staphylococcus*, *Streptococcus*, *Tetragenococcus*, and *Vagococcus* are more frequently found in raw milk cheeses, whereas *Clostridium*, *Lactococcus*, *Pseudoalteromonas*, *Psychrobacter*, and *Vibrio* are more abundant in cheeses made using pasteurized milk (40, 58).

Starter and Adjunct Cultures

Starter cultures, derived from either traditional backslopping practices or selected strain(s), are added to milk to facilitate coagulation and jump-start the ripening process. Natural whey cultures are the most commonly used thermophilic starters derived from backslopping practices. These bacteria are in whey collected from a prior fermentation that is incubated at a high temperature (45 to 52°C) for 4 to 18 hours (60). Natural whey cultures contain high numbers of thermophilic lactic acid bacteria (LAB), such as *S. thermophilus*, *Lactobacillus* (*Lb.*) *delbrueckii*, and *Lactobacillus helveticus* (33, 49, 61, 62). In addition to thermophilic starters, mesophilic starter cultures are also widely used in cheese making. Traditional mesophilic starter cultures are naturally derived from self-coagulated milk. Strains of *Lc. lactis* (>90%) and *Leuconostoc* spp. (*Leuconostoc* [*Ln.*] *mesenteroides* and *Ln. pseudomesenteroides*)

with an optimal growth temperature around 30°C are common mesophilic starters (60, 63, 64). The addition of starter cultures reduces the bacterial alpha diversity, resulting in a temporary suppression of growth and activity of other microorganisms present (22, 33, 49, 65–67). For example, Masoud and colleagues found that the growth of pathogenic *Escherichia coli* in cheese was impaired by the reduction in milk pH as a result of starter culture bacterial activities (65).

High-throughput DNA sequencing has been applied in combination with culture-based and other molecular methods to determine the diversity of complex starter cultures in order to fully understand their genetic and functional potential in light of strain and lineage variations (63, 64, 68, 69). Significant intraspecific diversity was observed among lineages of bacterial species in starter cultures. Although a traditional Gouda-style mesophilic starter culture (known as Ur) was found to contain only two bacterial species (*Lc. lactis* and *Ln. mesenteroides*), when it was investigated in more detail using metagenomic methods, five lineages of *Lc. lactis* subsp. *cremoris*, two lineages of *Lc. lactis* subsp. *lactis*, and a single lineage of *Ln. mesenteroides* were identified (63).

In general, mixed-strain starter cultures are more robust and resilient to environmental perturbations than single-strain inoculants (70). Mixed-strain starters have these properties because of strain-specific differences in growth kinetics and metabolic pathways (e.g., citrate utilization and proteolytic activity). Metabolic complementation and cross-feeding between strains enable the bacteria to adapt to the dairy environment more quickly than when they are inoculated individually (63, 64, 71). Mixed-strain starters are also more resilient than singlestrain starters because of metabolic redundancy and compensation following exposure to environmental stress and bacteriophage attack. Because of the limited host range of bacteriophage, the populations of some but not allstrains within the starter culture are affected at any single time (63, 71). The stability of such mixedstrain starters is evidenced by the fact that after repeated back-slopping propagation cycles, species-level composition remains stable (62, 63, 71). To fully preserve the strain-level functionality, industrially important cheese starters are usually carefully maintained to minimize propagation cycles.

Adjunct cultures usually include nonstarter lactic acid bacteria (NSLAB), such as *Lactobacillus* spp., *Lactococcus* spp., and *Enterococcus* spp., as well as molds, such as *Penicillium* spp. (72). Adjunct cultures are vital for flavor development in cheese. The contributions of these microorganisms were shown in a meta-transcriptome study, which found that adjunct NSLAB gene transcripts for proteins required for proteolysis and lipolysis (flavor development) were enriched towards the end of cheese ripening (73). Adjunct cultures can also be added during the cheese-making process as probiotics for human health. Recent animal and human studies using cheese made with probiotic adjunct cultures showed that these bacteria remained viable in cheese and in the gastrointestinal tract (>103 CFU/g of fecal sample) without causing significant changes in the organoleptic properties of cheese (74, 75).

Table 5.3 High-throughput sequencing studies of cheese and cheese-related environments

Milk source	Study focus	Study description	Reference
Cow	Bacterial taxonomy	Danish raw-milk cheese with different starter cultures during ripening	67
		Danish raw-milk cheese and cheese with spiked pathogen during ripening	65
		Store-bought Latin-style cheese from three brands after broth enrichment	165
		Poro cheese and intermediates made in different seasons	33
		Artisanal cheeses and associated production facilities	86
		Fontina cheeses from three lactation phases	22
		Caciocavallo Pugliese cheese during ripening	66
		Herve cheeses from three manufacturers	58
		Pico cheese from three manufacturers during ripening	92
		Continental-type cheese during ripening	91
		Italian cheeses and associated production facilities	88
		Natural whey cultures for pasta filata cheeses	62
		Mozzarella produced with different acidification methods during storage	82
		Reblochon-style cheese during ripening	93
		Caciotta and Caciocavallo Pugliese cheeses and production facilities	89
		Grana-style cheese and intermediates during ripening	61
		Floor drains of an Austrian cheese production facility	90
		Core and rind microbiotas of French cheeses	94
		Dutch-style cheese made with varying protocols during ripening	98
		Caciocavallo Silano cheese ripening at different temperatures	73
		Caciofiore della Sibilla cheese made with animal-and plant-based rennet	85
		Nonstarter LAB in Grana Padano cheese during ripening	96
	Fungal taxonomy	Artisanal cheeses and associated production facilities	86

Milk source	Study focus	Study description	Reference
		Italian cheeses and associated production facilities	88
		Core and rind microbiotas of French cheeses	94
	Functional metagenome	Strain quantification in starter cultures	68
		Lab-made surface-ripened cheese during ripening	99
		Aged Mexican Cotija cheese	97
		Viable microbiota of Gouda cheese during ripening	87
		Dutch-style cheese made with varying protocols during ripening	98
		Gouda cheese starter culture diversity	63
	Functional metatranscriptome	Canadian Camembert-type cheese during ripening	100
		Lab-made surface-ripened cheese during ripening	99
		Reblochon-style cheese during ripening	93
		Caciocavallo Silano cheese ripening at different temperatures	73
Sheep	Bacterial taxonomy	Oscypek cheese during manufacture and ripening stages	101
		Croatian raw-ewe's-milk cheese from two farms during ripening	102
		Canestrato Pugliese cheese during ripening	103
Buffalo	Bacterial taxonomy	Mozzarella cheese and intermediates from two manufacturers	49
Various mammals	Bacterial taxonomy	Irish artisanal cheeses and rinds	40
		Core community in Italian cheese curds and natural whey cultures	166
	Bacterial and fungal taxonomy; functional metagenome	Reproducible cheese rind communities	59

Bacteriophages

Bacteriophages are notorious in milk because of their ubiquitous prevalence and ability to infect starter culture bacteria to result in delayed or even failed fermentations (60). Likely because of the coevolution of bacteria and bacteriophages in milk and dairy products, bacterial starter cultures often possess multiple viral resistance mechanisms. Resistance can occur by modification of phage receptors, resulting in the inhibition of phage adsorption, and also as a result of nucleic acid restriction/modification systems, abortive infection mechanisms (apoptosis), and CRISPR/Cas systems (76, 77). However, these mechanisms are only partially effective. To control phage populations during cheese manufacture, methods such as rotating starter culture use, intentional selection of bacteriophage-insensitive mutants, and mixed-strain starters are commonly used (78, 79).

Bacteriophages might also have important beneficial functions in some cheese fermentations. Cell killing by the viruses helps to prevent strain overgrowth and instead to maintain intra-and interspecies diversity. In this "kill-the-winner" model, phage proliferate in the most active bacterial strain (usually the most abundant strain) and thereby prevent it from using all of the available nutrients (80). In this regard, bacteriophage and prophage (targeting the most abundant bacterial species) sequences are frequently found in metagenomes of complex, functionally stable starter cultures (62, 63, 71, 81). DNA sequencing methods have also been developed for investigating the bacteriophages of dairy products. These methods provide a way to improve starter culture selection and bacteriophage control methods for milk and dairy products (81).

Enzymes and Other Added Ingredients

Citric acid is added to milk to achieve direct and fast acidification for curd formation, particularly for unripened cheese. The addition of citric acid (final pH of curd from 5.8 to 6) instead of starter culture bacteria to Mozzarella cheese processing was shown to result in a higher bacterial alpha diversity and higher proportions of psychrotrophic bacteria, such as *Aeromonas*, *Acinetobacter*, *Pseudomonadaceae*, and *Listeriaceae* (82, 83).

Rennet is often added to milk together with starter cultures. Rennet is traditionally extracted from calf stomach tissue but can also be produced from microbial fermentation and plants, such as fig and thistle (84). Recently, a study comparing commercial animal rennet and thistle rennet revealed that the source of rennet could have an impact on the microbiota of curd and fresh cheese (85). *Lactococcus* was more abundant in cheese made with animal rennet, while *Lactobacillus* and *Leuconostoc* were more abundant in thistle rennet cheese (85).

Physical and Environmental Conditions Used in Cheese Production

After fresh curd coagulates, whey is separated and removed by using different methods, including cutting, stirring, pressing, and cooking. The curd can then be shaped and flavored or brined. Brining of the curd in NaCl results in a strong selection against Gram-negative bacteria, with the exception of those that are halotolerant, such as *Pseudoalteromonas*, *Halomonas*, and *Vibrio casei* (86). The proportions of starter culture bacteria are also changed in response to brining. In Gouda cheese (brined in 19% [wt/vol] NaCl solution), the proportions of different *Lc. lactis* subsp. *cremoris* lineages changed rapidly after brining (87).

Cheese processing facilities and equipment surfaces harbor a diverse collection of microbial species (88) which could also be significant sources of microbes in cheese. These surfaces contain microbes that originate from the environment, personnel, milk, and cheese. The microbes are distributed throughout processing plants, as indicated by the large proportions of bacterial (87%) and fungal (50%) operational taxonomic units that were found to be shared between dairy plant swabs and cheeses (88). Spoilage bacteria, such as *Acinetobacter*, were found in high proportions (33% of the total bacterial proportions) in the vat containing milk to be curdled, and *Pseudomonas* populated the cheese molder and chopper (average, 50%) as well as the curd bench and curd tank (average, 30%) (88). In the same cheese facility, the fungal species *Yamadazyma triangularis* heavily populated (>90% of the fungal population) the Caciocavallo cheese hook, whereas the curd tank, curd bench, and knife were populated by *Trichosporon faecale* (88). In another study that sampled an Italian cheese fermentation site, bacteria known to contribute to cheese making, including *S. thermophilus*, were found in high proportions in the swab samples of the curd knife, brine tank, and drain tables (>35%) (89). NSLAB, including *Lactobacillus plantarum*, *Lactobacillus brevis*, and closely related species, were also found on the surfaces of the curd tank (89). Additionally, swabs from an American artisanal cheese plant showed that the curd-making and brining surfaces were predominantly populated by *Lactococcus* (~50% of the bacterial community), *Lactobacillus* (~10% of the bacterial community), and *Debaryomyces* (~40% of the fungal community) (86).

Surfaces that do not directly contact cheeses, such as floor drains, can also host a complex microbiota that is highly dependent on the products and processes carried out in the room (90). For instance,

Streptococcus and *Lactobacillus* were abundant in the floor drains of culture preparation room, *Pseudomonas* was found at high levels in both the smearing and ripening rooms, and *Psychrilyobacter* was abundant in the ripening room (90).

The accumulation of microbes on facility surfaces throughout the day might also cause quality and safety issues in the final cheese product. Within the same cheesemanufacturing facility, it was found that cheeses produced late in the day had more diverse microbial communities than cheeses made at earlier time points (91). *Acinetobacter*, *Clostridium*, *Lactobacillus*, *Pseudomonas*, and *Streptococcus* were more abundant in cheese made first after equipment cleaning, whereas *Brevibacterium*, *Corynebacterium*, and *Thermus* were more abundant in cheese produced 6 to 8 hours later (91).

Cheese Aging and Associated Environment

Most varieties of cheese undergo ripening, which can last for days to years, during which the microbial communities undergo drastic changes (57). The diversity of bacteria during cheese ripening has been reported to decline towards the end of the aging process (66, 91, 92). However, taxonomic changes are specific to the variety and site on the cheese (e.g., core versus rind). The surfaces of the cheese are more exposed to O_2, dry air, environmental contamination, and variation in temperatures than the centers. Because of these exposures, cheese rinds generally have a higher diversity of microorganisms than the cores (58, 93, 94). *Lactococcus*, *Leuconostoc*, *Lactobacillus*, *Bifidobacterium*, and *Ruminococcaceae* are commonly enriched in the core. *Brevibacterium*, *Brachybacterium*, *Corynebacterium*, *Hafnia/Serratia*, *Halomonas*, *Psychrobacter*, *Pseudomonas*, *Pseudoalteromonas*, *Staphylococcus*, *Streptococcus*, and *Thermus*, as well as yeasts and molds, have been frequently found on cheese rinds (40, 58, 59, 73, 91, 94).

Cheese Varieties

This section discusses recent findings on the examination of the microbial composition of different cheese varieties, emphasizing studies that have pursued systems biology, 16S rRNA gene and internal transcribed spacer marker gene sequencing, and metagenomics methods.

Hard Cheese

The Italian hard cheeses Parmigiano-Reggiano and Grana Padano are made by combining thermophilic natural whey starters, milk that is cooked at high temperatures (~55°C), and long ripening times (>2 years) (95). Bacterial diversity in Grana-style hard cheese was observed to decline during the first 6 months of ripening but then gradually increased again after 8 months (61). *Lactobacillus* species were present throughout the ripening process, and *Acinetobacter*, *Lc. lactis*, *Pseudomonas*, and *S. thermophilus* were also detected sporadically during ripening (61, 96). In Grana-style cheese, assessments of metabolic gene abundance indicated that *Lactobacillus fermentum* was the most active bacterium at the beginning of processing, and then *Lb. casei* and close relatives were the most prevalent, metabolically active group compared with other NSLAB during ripening (96).

Cotija cheese is a handmade artisanal cheese from raw cow's milk without an added starter culture that can be aged from months to years. After 3 months of ripening, over 500 genera were identified in the Cotija cheese metagenome, of which *Lb. plantarum*, *Ln. mesenteroides*, and *Weissella paramesenteroides* were the three most dominant species present, and *Aerococcus*, *Enterococcus*, *Lactococcus*, and *Staphylococcus* were detected in lower proportions (97). Genes coding for enzymes involved in flavor development were detected, including amino acid lyases and transferases as well as carboxylesterases (97). Genes coding for the production of antimicrobial bacteriocins were also detected, indicating the likelihood for the activation of bacterial defense strategies within the cheese (97).

Swiss-type cheeses (hard to semihard) have distinct gas pockets, or "eyes," which develop during ripening as a result of CO_2 production from lactate fermentation by *Propionibacterium freudenreichii* (95). *Lactobacillus* (>70%) and *Streptococcus* (>10%) populations dominated the bacterial community of this cheese type throughout the 2-month ripening period (91). Other bacteria were also present in

lower proportions during ripening, including *Lactococcus*, *Vibrio*, *Pseudomonas*, *Pseudoalteromonas*, and *Psychrobacter* (91). Cheese rinds harbored a more diverse microbiota than the cheese core at the beginning of ripening, but the core microbial diversity surpassed that of the rinds after 10 days (91).

Semihard Cheese

Gouda is a semihard cheese characterized by small eye holes that result from the production of CO_2 from citrate metabolism by *Lactococcus* and *Leuconostoc* (95). At 95% relative abundance, *Lc. lactis* subsp. *cremoris* dominated the Gouda cheese bacterial community during the beginning of ripening (87, 98). Bacterial diversity was then observed to increase during ripening (98). The metagenome of fresh Gouda was enriched in genes associated with bacteriophages, transport systems, and exopolysaccharide production (98). After aging for 3 months, genes for amino acid transport and catabolism, peptidases, monosaccharides, and organic acid utilization were more prevalent (98).

Caciocavallo-type cheese is a medium-aged pasta filata cheese made with raw cow's milk, and *S. thermophilus* or a natural whey starter is the primary starter culture. After 30 days of ripening, the cheese fermentation was driven by NSLAB, and the populations of *Lb. casei* and *Lactobacillus buchneri* increased consistently, whereas starter *S. thermophilus* numbers declined (66, 73). The metagenome of the Caciocavallo cheese also exhibited a spatial distribution. Genes required for amino acid and lipid metabolism were more abundant in the cheese center. The cheese rind was enriched in genes encoding genetic information processing, core cellular processes, and carbohydrate metabolism (73). Among carbohydrate metabolism pathways, metatranscriptome analysis showed that expression levels of lactose and galactose metabolism enzymes gradually declined during ripening (73).

Soft Cheese

Soft cheeses are higher in moisture content than semihard and hard cheeses and are generally aged for shorter periods of time. Mozzarella is one of the most popular soft cheeses consumed globally. Traditionally, it is made with unpasteurized water buffalo milk and natural whey cultures in southern Italy. During the 5 hours of fermentation required to make this cheese, *Lb. delbrueckii* and *Lb. helveticus* numbers increased, whereas *S. thermophilus* associated with the starter culture declined (49).

Pico cheese is a soft cheese traditionally made with unpasteurized cow's milk in the Pico islands of Portugal. A more diverse microbiota was observed in fresh Pico cheeses made with the traditional method than the industrial method, which involves milk pasteurization and the addition of starter bacteria (92). However, after a 3-week ripening period, the microorganisms in cheeses made using the traditional and those made using industrial methods became more similar. Both contained increased proportions of *Lactobacillus* and *Streptococcus* and reductions in *Acinetobacter* and *Stenotrophomonas* (92).

Bacterial Surface-Ripened Cheese

Bacterial surface-ripened cheese probably harbors the most heterogeneous microbiota among all cheese varieties due to its diverse surface bacterial communities (95). For instance, Livarot-style cheese is made by inoculating *Lc. lactis*, *Kluyveromyces lactis*, *Debaryomyces hansenii*, and *Geotrichum candidum* into milk and subsequently smearing (topical application) ripening bacteria (*Corynebacterium casei*, *Brevibacterium aurantiacum*, *Arthrobacter arilaitensis*, *Staphylococcus equorum*, and *Hafnia alvei*) onto the cheese surfaces. Dugat-Bony and colleagues found that, at the early stages of ripening (14 days), *Lc. lactis* (40%), *G. candidum* (30%), and *D. hansenii* (10%) were present in high proportions within the cheese metagenome but were gradually replaced by surface bacteria, primarily *C. casei* (71% of the total microbial metagenome at day 31) (99). Metatranscriptomes of this cheese showed that transcripts for amino acid and carbohydrate transport and metabolism, for protein translation, folding, sorting, and degradation, and for environmental signal transduction were highly diverse and abundant throughout the ripening process, suggesting that numerous metabolic pathways were active within the microbial community (99). It was notable that

the lactate produced by *Lc. lactis* and *K. lactis* was likely rapidly consumed by *D. hansenii* and *G. candidum*, as demonstrated by high levels of lactate dehydrogenase transcripts from these fungi (99). *G. candidum* was also linked to amino acid degradation, especially at the end of ripening, indicating that this species is important for neutralizing the pH and supporting the growth of flavor-enhancing, surface-associated bacteria in mature cheese (99).

Reblochon cheese is a French bacterial surface-ripened cheese. A Reblochon-style cheese made using pasteurized milk was examined for microbial and metatranscriptome composition during ripening. Transcripts in the cheese rind shifted from protein biosynthesis at the beginning of ripening to amino acid and lipid degradation at the time of maturity (93). Among the inoculated surface yeasts, *G. candidum* was the most active yeast at allripening times. *D. hansenii* showed increased activity towards the end of fermentation (93). It was also shown that although *B. aurantiacum* was inoculated as the ripening starter, the abundance and measurable metabolic activities were surprisingly low (2%) throughout the 35-day ripening period (93). Lactic acid starters (*S. thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*), on the other hand, grew at the very beginning (5 days) of ripening but had limited metabolic activities during the rest of the fermentation period (93).

Mold Surface-Ripened Cheese

Camembert type cheese is a typical mold surfaceripened cheese with felt-like, bloomy white rinds. Camembert is traditionally made with unpasteurized milk and surface applications of the ripening culture consisting of *Penicillium camemberti*, *G. candidum*, and sometimes *K. lactis* and *Brevibacterium linens*. Camembert-style cheese is also commercially produced with pasteurized milk, and ripening starter is inoculated into milk directly, especially in North America (95). During ripening, *P. camemberti* and *G. candidum* were highly active in the cheese rinds according to metatranscriptome analysis (100). Transcripts related to energy metabolism from both fungi (glycolysis/gluconeogenesis pathways, pentose phosphate pathways, tricarboxylic acid cycle, and oxidative phosphorylation) were more abundant in the early stage than late stage of ripening. Proteolytic and lipolytic activities were also robust during the first 2 to 3 weeks of ripening, contributing to the flavor and texture development of Camembert cheese (100).

Sheep's Milk Cheese

Cheeses produced using sheep's milk are widely produced and consumed in Europe. In both hard and soft varieties, *Lactococcus* was found to be the dominant genus throughout ripening, and *Enterococcus*, *Lactobacillus*, *Leuconostoc*, *Staphylococcus*, and *Streptococcus* were also detected (40, 85, 101, 102). *Lactococcus* proportions generally declined over time, along with members of the *Enterobacteriaceae* family and *Staphylococcus* genus (85, 102). Canestrato Pugliese sheep's milk cheese was different, however, where *Lactococcus* proportions increased from 17.8% to 87.2% of the bacteria present. *Pseudomonas* and *Carnobacterium* decreased from 44.7% and 29.7%, respectively, to below 1% of the microbial contents during the 90-day ripening period (103).

KEFIR

Kefir is a viscous, acidic, self-carbonated and slightly alcoholic fermented milk beverage produced by adding kefir grains as starters into fresh or pasteurized milk. Kefir typically ferments for 1 day at 20 to 25°C (39, 104). Kefir grains are a white, spongy, symbiotic microbial consortium of LAB, yeasts, and acetic acid bacteria with an appearance similar to that of cottage cheese (104). The consumption of kefir has spread from its origin in the Caucasus Mountains to worldwide, in part due to the putative health benefits associated with this drink (105–108).

Kefir milk and grains studied with culture-based methods were found to be dominated by populations of LAB (*Lactobacillus*, *Lactococcus*, *Leuconostoc*, and *Streptococcus*) as well as lower numbers of acetic acid bacteria (*Acetobacter*) and yeasts (*Candida*, *Cryptococcus*, *Saccharomyces*, *Kluyveromyces*, and *Zygosaccharomyces*) (109–113). With the application of DNA sequencing methods, other genera not traditionally associated with kefir were also found, including *Bifidobacterium*, *Bacteroidaceae*, and

Pediococcus as well as the yeast genus *Dekkera* (114–116). Table 5.4 summarizes the abundant taxa (>1%) found in kefir.

Kefir milk and grains were found to have different bacterial and fungal populations (114, 115). The milk beverage bacterial communities are dominated by *Lactococcus* and occasionally *Lactobacillus* depending on the manufacturer (114–121). *Leuconostoc* proportions are also higher in the milk than grains (114, 115). In contrast, *Lactobacillus* species are the most abundant bacteria in the grains (114–121). The microorganisms are also not uniformly distributed throughout grains (115). *Leuconostoc*, *Clostridiaceae*, and *Enterobacteriaceae* were present at higher proportions in the center of the grains, whereas the surface harbored higher numbers of *Bacteroidaceae* (115).

Table 5.4 Abundant[a] microbes identified in kefir with high-throughput DNA sequencing methods

Group and organism	Source	Reference(s)
Lactic acid bacteria		
Lactobacillus	Kefir and grain	114-117, 119-121
Lactococcus	Kefir and grain	114-119
Leuconostoc	Kefir and grain	114-117, 120
Pediococcus	Grain	116
Streptococcus	Grain	117, 118
Acetic acid bacteria		
Acetobacter	Kefir and grain	115, 117-120
Gluconobacter	Kefir and grain	119
Other bacteria		
Clostridiaceae	Kefir and grain	114
Bacteroidaceae	Kefir and grain	114
Bifidobacterium	Kefir and grain	114, 115, 120
Enterobacteriaceae	Kefir and grain	114, 119
Enterococcus	Grain	116
Propionibacterium	Grain	120
Ruminococcaceae	Kefir	114
Tetragenococcus	Grain	116
Yeasts		
Dekkera	Grain	115, 118
Hanseniaspora	Grain	118
Kazachstania/Naumovozyma/	Kefir and grain	115,118-120
Saccharomyces		
Kluyveromyces	Kefir and grain	115, 119

[a] Here, “abundant” is defined as being present in a relative proportion of > 1%.

Kefir and kefir grains collected from various geographic locations contained different microorganisms (115, 118). This difference could be partially explained by manufacturing practices. Korsak et al. found that the quantity of kefir grains used for inoculation led to differentiation in the microbiota of the beverage at the end of fermentation (119).

Throughout the 24-h kefir fermentation period, the microorganisms in kefir milk and grains undergo succession processes. Walsh et al. found that, from 0 to 8 hours of ripening of an Irish kefir, the microbiota of kefir milk shifted from a complex microbiota similar to that found in bovine milk to one

dominated by *Lactobacillus* (120). After 8 hours, *Lactobacillus* populations decreased and *Leuconostoc* and *Acetobacter* abundances increased (120). *Saccharomyces* and *Kazachstania* were the only abundant fungal genera throughout the fermentation of kefir milk (120). Concurrent with microbial community shifts, the kefir milk metagenome changed in a manner consistent with flavor development. Pathways associated with carbohydrate metabolism, carboxylate degradation, and unsaturated fatty acid biosynthesis were most abundant after 8 hours of ripening, and pathways for amino acid metabolism and 2,3-butanediol degradation were enriched at the end of fermentation (116, 120). Other genes found in the metagenome might have functions in the human intestine, including those coding for extracellular polysaccharide biosynthesis, bile salt transporters, adhesion and mucus-binding proteins, and the type III bacteriocins/bacteriolysins helveticin J and enterolysin A (120).

OTHER FERMENTED MILK BEVERAGES

A variety of fermented milk beverages are produced and consumed using traditional back-slopping methods or simply by natural fermentation relying on the microbes present in milk. It has been shown that artisanal fermented milks sourced from different mammals and regions have distinct microbiota, resulting in highly diverse products in terms of their organoleptic and nutritional properties (122–125). This section discusses several fermented milk beverages that have been studied using -omics approaches.

Fermented Cow Milk

Yogurt is the most popular fermented milk beverage around the world, with a long tradition of human consumption. Yogurt first appeared in historical records around 9000 BCE in the Middle East, India, and Northeast Africa, and it was used as a way to preserve milk and improve food safety. Yogurt fermentation is carried out by a starter culture composed of *Lb. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* incubated in milk at 42°C to a final pH around 4.5 (126). Metatranscriptome and metabolome analyses confirmed previous findings on the symbiotic interactions between the yogurt starter bacteria (127). Specifically, *Lb. bulgaricus* was found to supply *S. thermophilus* with peptides and amino acids, and in return, *S. thermophilus* produces formic acid, folic acid, and fatty acids that stimulate the growth of *Lb. bulgaricus* (126, 128–131). Commercial yogurt often contains other added bacteria, including strains of *Bifidobacterium* as well as *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Lactobacillus paracasei*, and *Lb. casei* (126). These bacteria are typically added because of their probiotic properties, but they are also capable of adapting to the yogurt environment. Gene transcript analysis has shown that these bacteria are metabolically active in yogurt and express genes required for energy production and conversion (128).

Nunu is a viscous beverage resulting from the natural fermentation of raw cow's milk. It is usually fermented for 1 day at ambient temperatures and is widely consumed in rural parts of Ghana and other countries in west Africa (132). The bacterial populations of nunu are mostly composed of *Streptococcus infantarius*, *Lc. lactis*, *Enterococcus faecium*, and *Enterobacter cloacae* (>1%) (132). Unexpectedly, Shiga toxin-producing *E. coli* and pathogenic *Klebsiella pneumoniae* were also present in the nunu microbiota (132), indicating that heat treatment such as pasteurization might be necessary to ensure safe consumption.

Tarag is a yogurt-like beverage consumed in Mongolia, Russia, and northwestern China. It is produced by back-slopping fermentation of cow, sheep, or goat milk or a mixture thereof (133). Tarag is subsequently aged for at least 5 days at 10 to 25°C (134). As with other traditionally fermented dairy drinks, geographical variation in the microbiota of tarag was observed (134). However, *Lactobacillus* was consistently found to be the most abundant bacterial genus, followed by *Streptococcus*, *Lactococcus*, *Leuconostoc*, *Acinetobacter*, and *Acetobacter* (>1%) (123, 124, 134). The fungal populations of tarag consisted of mainly *Galactomyces*, *Saccharomyces*, *Trichosporon*, *Kluyveromyces*, *Pichia*, *Candida*, and *Vanderwaltozyma* (>1%) (124, 134).

Similar to tarag, matsoni (or also called matzoon) is also produced by back-slopping practices with mixtures of milk from cows, sheep, goats, and buffalo. This drink is produced in regions of Bulgaria,

Georgia, Armenia, and Russia. It is fermented at 35 to 42°C overnight, and the finished product is described as a mildly sour yogurt with a syrup-like viscosity (104, 125). Matsoni bacterial communities were found to have abundant proportions of *Lactobacillus* and *Streptococcus* with minor populations of *Lactococcus*, *Enterococcus*, *Pseudomonas*, *Rummeliibacillus*, *Enterobacteriaceae*, *Erwinia*, *Wautersiella*, and *Yersinia* (125). Yeast populations in matsoni are more heterogeneous, although *Kluyveromyces marxianus* and *Saccharomyces cerevisiae* were frequently detected (125).

Fermented Horse Milk

Koumiss, also known as airag, is an alcoholic fermented horse milk with a high vitamin content that has been consumed in central Asia since the 5th century BCE (104, 133, 135). Koumiss is fermented with a liquid starter culture and stirred for 1 to 2 hours before it is ripened at 6 to 8°C for around 3 days (104). *Lactobacillus* spp. such as *Lb. helveticus*, *Lactobacillus kefiranofaciens*, and *Lb. buchneri* are abundant in koumiss (>50% of bacteria present), and *Streptococcus*, *Lactococcus*, *Enterococcus*, and *Acetobacter* have also been detected (122, 123, 136). Koumiss metagenomes were found to have high proportions of lactose metabolism genes as well as pathways for amino acid transport and metabolism that are associated with flavor formation (e.g., casein-degrading proteinases, aminopeptidases, aminotransferases, and amino acid lyases) (136).

Fermented Yak Milk

Kurut is made by the spontaneous fermentation of yak milk and is a staple dairy product among nomadic populations inhabiting the Qinghai and Tibet highlands of China. Yak milk fermentation typically lasts for a week at 10 to 20°C (104, 137). The resulting kurut is a viscous, sour, alcoholic, white or ivory-colored beverage (104, 137). Without the addition of starter culture, the microbial composition of kurut is dependent on production practices and differences among yak herds (138). *Lactobacillus* was found to be the most abundant genus in kurut, followed by *Streptococcus* and *Lactococcus* (122, 138). The fungal communities of kurut had abundant *Saccharomyces* and *Galactomyces* populations, with subdominant genera (>1%) including *Vanderwaltozyma*, *Pichia*, and *Gibberella* (138).

CONCLUSIONS

Modern microbiological methods targeting nucleic acids (RNA and DNA), proteins, and metabolites have provided unprecedented levels of knowledge on microorganisms and their composition and functional relevance in dairy products (Fig. 5.1). High-throughput approaches are powerful tools to study dairy product defects (139, 140) and to test processing parameters and recipes that can lead to the development of new products (73). However, at present, -omics methods are inconsistently applied and standards are not yet available for dairy microbiome research. Additionally, there are technical limitations, such as the inability to detect cells and cellular contents that are present in low levels and difficulty with accurate species identification and molecular resolution. We expect that methodological standardization and sufficient technological and bioinformatics advancements will be achieved in the near future. We anticipate that these emerging methods will propel dairy microbiome research in ways that ultimately ensure dairy product safety and quality and provide fundamental knowledge on the ecology and molecular capacities of food-associated bacteria.

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6
Meat and Poultry

Meat and poultry are important commodities in the United States and worldwide. Given the popularity of these commodities and their high consumption rate, it is critical to understand the sources of contamination and the means to prevent cross-contamination to protect public health. Contamination of meat and poultry by microorganisms occurs naturally as a result of procedures necessary to produce foods of animal origin. In general, most foodborne illnesses and outbreaks result due to undercooking or underprocessing of these products, cross-contamination, or improper handling of cooked meat and poultry. Because meat products are highly perishable commodities, particularly fresh meat and poultry, temperature controls are critical to prevent contamination. Given the broad spectrum of variables that can cause foodborne illnesses linked to meat and poultry products, this chapter discusses microbiological issues related to these commodities and their control measures.

FRESH MEAT

Traditional thinking that muscle of healthy animals destined for human consumption is sterile is still valid. However, evidence that some tissues and organs of healthy animals can contain zoonotic pathogens is being established. While the initial microflora of meat comprises microorganisms originating from the animal itself, either from the surfaces of the hide or from the intestinal contents, other sources include the processing environment and the personnel present during processing. A variety of Gram-negative and Gram-positive bacteria can make up the initial flora of meat, regardless of the animal species.

The populations and the composition of the initial microflora depend on the source of contamination, and this can vary from animal to animal or be normalized during processing if there is a risk of cross-contamination during processing. Further, the microbial populations on meat depend on the processing system utilized during slaughter and the specific unit operations used to harvest the animal. For example, the hides of beef cattle are removed subsequent to exsanguination, whereas in pork processing, the hide stays on the animal until after the carcass is chilled. Further, the unit operations specific to pork harvest, such as scalding, dehairing, and singeing, assist in significantly reducing the microbial load on the hide and exterior surface of the carcass. However, the risk of contamination of the carcass by microorganisms from the hide surface is significant in beef processing. In addition, the characteristics of the carcass surface influence the degree of efficacy of the antimicrobial interventions applied to the carcass and hence the type and cell populations of microorganisms on the carcass and, eventually, the meat.

Beef and Pork

General Ecology of Beef and Pork

The microbial types and the cell populations on carcasses during and subsequent to processing of beef and swine can be substantially different. As stated above, the major sources of microbial contamination of beef carcasses are the hide and the gastrointestinal (GI) tract. The types of microbes on the hide of beef cattle are dependent on the environment of the feedlot or the pasture, the microbes in the soil and water, and the beef ecosystem.

The types of microbes in feedlot air include several species of *Bacillus*, *Corynebacterium*, *Paenibacillus*, *Micrococcus*, and the molds *Alternaria*, *Cladosporium*, and *Penicillium* (1), with the populations ranging from 1 to 2 log CFU/liter and 1 to 4 log CFU/liter in upwind and downwind air, respectively, from beef cattle feedlots. These airborne microbes are largely from the soil of the feedlot environment, and the differences in microbial populations between the upwind and downwind air are attributed to the soil of the feedlot, which is the

source of the organisms. The types and populations of the microbes can be dependent on the weather and the season, with winter and rainy seasons usually resulting in higher populations than the dry, hot environment of the summer. In addition, there is evidence that the geographical location (latitude) of the feedlots has a significant role in the ecology of the foodborne pathogens *Escherichia coli* O157:H7 and other Shiga toxin-producing *E. coli* (STEC) types. The risk of hide contamination from soil during warm, dry periods is lower than that in rainy and humid periods due to formation of dung locks and the amount of mud or soil on the hide, because the microbes on the hides of cattle largely originate from the feedlot soil and the manure of the animals. The populations of *Salmonella* spp. and *E. coli* O157:H7 on cattle hides can increase following exposure to a dust cloud and during cattle load-out on trucks for transportation to the slaughter operation (2). The prevalence of *Salmonella* is usually greater in the wet season than in the dry season (3). Hence, the microflora from animal feces and that from the soil make up the microorganisms on cattle hides when cattle are transported to the slaughterhouse.

The GI tract of beef cattle is colonized by microorganisms that influence animal physiology, nutrition, health, and productivity (4). While the cattle intestinal microbiota has been well characterized using culture-based methods, the application of novel, genome-based methods is revealing greater diversity in these populations. The most common microbes in the beef cattle GI tract include the taxa *Bacteroidetes*, *Prevotella*, and *Bacteroides*; the *Firmicutes Faecalibacterium*, *Ruminococcus*, *Roseburia*, and *Clostridium*; and the proteobacterium *Succinivibrio* (5). Also, the type of cattle (dairy versus beef) influences the microbial diversity in the animal gut, with higher populations of *Arthrobacter*, *Asteroleplasma*, *Bifidobacterium*, *Collinsella*, *Delftia*, *Eggerthella*, *Lactobacillus*, *Mitsuokella*, *Olsenella*, and *Propionibacterium* in beef cattle than in dairy cattle. While a diverse microbial community can contaminate beef cattle during slaughter, the microbes that can grow and cause meat spoilage depend on their ability to survive subsequent antimicrobial interventions and grow in the meat during subsequent packaging, storage, and distribution processes.

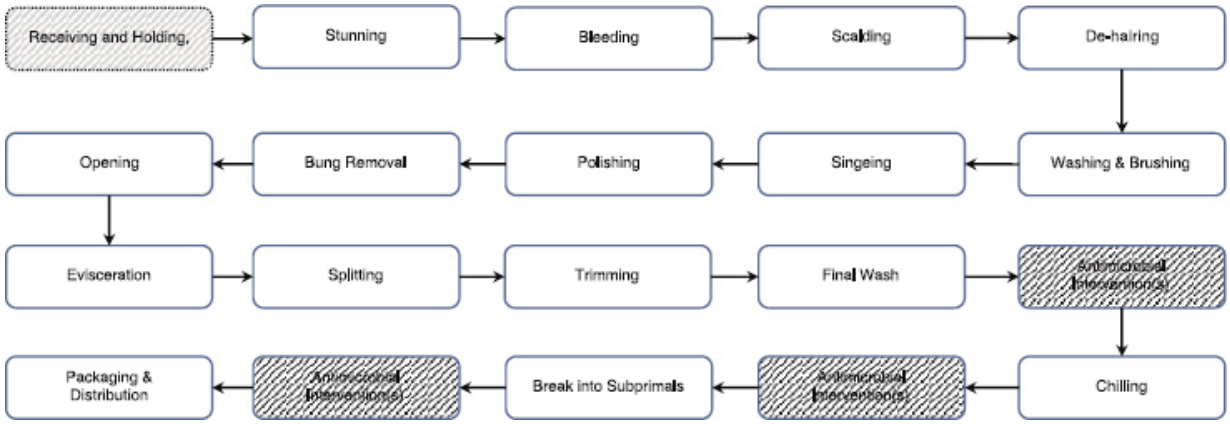


Figure 6.1 Unit operations for pork slaughter.

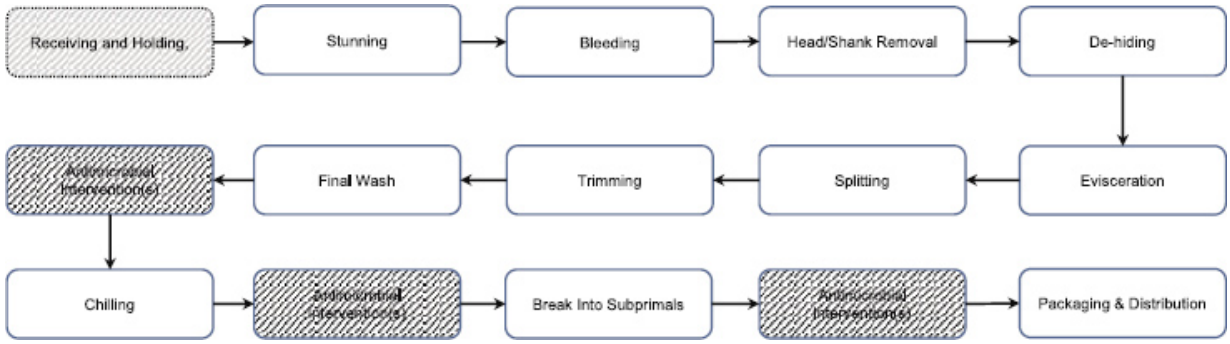


Figure 6.2 Unit operations for beef slaughter.

Beef and Pork Harvest and Slaughter

The main purpose of the cattle and swine harvest processes is to convert the animal to meat. While the major unit operations, such as stunning, exsanguination, bunging, evisceration, splitting, and carcass chilling, are similar between the species, there are significant differences in additional unit operations applied during the harvest process (Fig. 6.1 and 6.2). For example, swine carcasses go through scalding, dehairing, and singeing operations that beef carcasses are not subjected to. Also, characteristics of the hide (presence or absence of hair, mud, and dung balls [for cattle], smoothness of the hide or skin, etc.) and the fact that the hide is left on the carcass in most swine harvest operations until after chilling have a significant influence on the microbial populations of the carcass prior to and subsequent to chilling.

In beef processing, the hide is removed after exsanguination and the hide is pulled while it is being rolled down from the carcass to expose the carcass tissue surface underneath. This processing step can create aerosols if the hide is wet so that microorganisms can be deposited on the equipment or on the freshly dehided carcass itself (Fig. 6.3).

Subsequent to the implementation of the Pathogen Reduction; Hazard Analysis and Critical Control Points rule, beef processors have implemented many antimicrobial interventions at various locations in their processing facilities utilizing a variety of antimicrobial agents and treatments based on processing plant experience and in-plant microbiological evaluations. These antimicrobial interventions are discussed further below.

Contamination of Beef and Pork during Harvest and Slaughter

Sources of contamination

The primary sources of microbial contamination of carcasses, regardless of the species, are the hide and the GI tract of the animal. The secondary sources of contamination include the environment and personnel that handle the carcasses, as these harvest and slaughter operations are labor-intensive. The microbial load on the hide for beef cattle can be 8 to 10 log CFU/100 cm^2 for total plate counts, 6 to 8 log CFU/100 cm^2 for total coliform counts, and 5.0 to 7.5 log CFU/cm^2 for *E. coli* counts (6). These numbers can vary substantially depending on the processing operation, source of the cattle, season of harvest, geographical location of the feedlot or pasture, and the transportation and lairage conditions at the processing operation. While the source of contamination can be the same for swine as well, hogs are purchased through long-term contracts and are reared indoors in large barns for most large swine-processing operations. As such, the hogs are not exposed to the environment and soil, although they are exposed to manure at the growing facility, during transportation, and at the lairage of the processing facility. Regardless, most swine are not dehided prior to evisceration, and hence, the unit operations have the capability of substantially reducing the microbial populations on the hide prior to evisceration; thus, the main source of contamination

remains the GI contents and the feces during or immediately before evisceration.

The harvest process influences which of the two sources—hide or the GI contents and feces—constitutes the predominant source of the organisms and consequently the proportions of the microbes on the carcass surface.

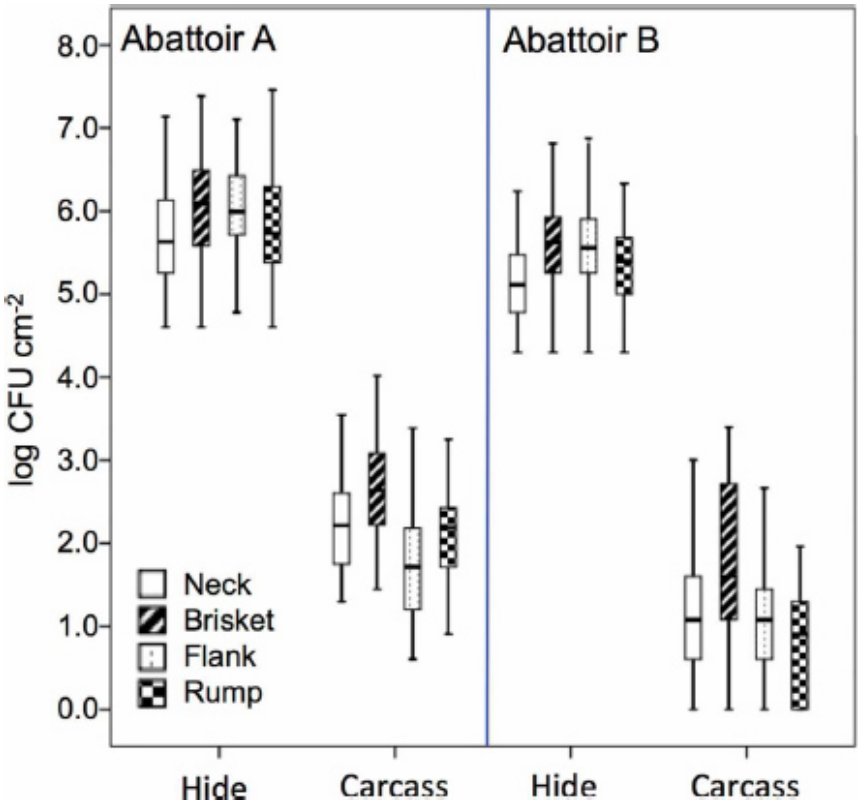


Figure 6.3 APCs on beef cattle hides and freshly dehided carcasses. Reprinted from reference 303.

Microbial loads on carcasses at different stages of slaughter

Using swine slaughter as the example, hogs are moved from lairage to processing chutes and undergo stunning either by electrical means or by a controlled atmosphere and cut by incision in the jugular vein to allow bleeding. After the bleeding, the carcasses go through scalding and singeing steps, which can cause some reductions in microbial populations on the hides of the carcasses (Table 6.1). The scalding process involves immersion of swine carcasses in hot water (61°C) for 8 to 10 min after bleeding and before the dehairing process. In some

instances, the scald water is infused with surfactants to reduce the surface tension of the water and/or pH-modulating agents to enhance loosening of the hair follicles to increase the ease of hair removal. Often, these agents result in higher pH values (>10) in the scald water and, with relatively higher temperatures and the use of surfactants, the scalding process can substantially reduce the microbial load on the skin of swine carcasses, often by more than 3.0 log CFU/cm^2 (7). The subsequent process of dehairing allows recontamination of the carcass from residual hair in the dehairing equipment which has lodged throughout the day of production, with an increase of the microbial load during the production day, often an increase of 2.0 to 3.0 log CFU/cm^2. The subsequent process, singeing, involves flaming (1,200°C for 15 s) the surface of the skin to remove any residual hair on the carcass. This process can achieve substantial reductions of microbial populations on the carcass hide, which is still intact. Following singeing, the carcasses are sprayed with water while brushes remove any singed hair from the hide surface (polishing), which may result in an increase in microbial populations, as the brushes are not exposed to any antimicrobials throughout the processing day. Subsequent to polishing, the carcasses go through several steps during the evisceration process, and carcass contamination can occur at this stage from the fecal material during the bung (distal colon and rectum) removal process or from the GI contents in subsequent steps. Carcass chilling can have a major impact on the microbial load of a carcass, as the carcasses are placed in a chiller for 18 to 24 h, depending on slaughter volume, carcass size, and cooler capacity. With expanding production volumes and minimal increases in cooler and refrigeration capacities, swine carcasses are frequently placed in the cooler in close proximity, often touching each other. This can be detrimental to the microbial quality of the carcass, as cooling of the carcass surfaces that touch each other as well as the exterior hide surface and the proximate internal cavity of the adjacent carcass is slower, resulting in growth of microbes in areas of the carcass that survived the process or recontaminated the carcass during or after evisceration.

Table 6.1 APCs, total coliform counts, and *Salmonella* prevalence on swine carcasses at different stages of harvest[a]

Stage	APC (log CFU/cm²)	TCC (log CFU/cm²)	*Salmonella* prevalence (%)
Bleeding	6.41	6.17	31
Scalding	2.65	2.42	1
Dehairing	4.75	3.64	7
Singeing	2.20	1.03	0
Polishing	3.31	2.58	0
Evisceration	3.05	3.05	7
Chilling	3.19	2.96	ND

[a] Adapted from reference 7. ND, not detected.

Several swine processors have incorporated antimicrobial interventions throughout the slaughter process to reduce *Salmonella* contamination of the carcasses. Examples of these antimicrobial interventions include steam vacuum systems and organic acid sprays applied before and after chilling and at other steps of processing.

The current U.S. Department of Agriculture Food Safety Inspection Service (FSIS) performance standards for swine processing are based on the prevalence of *Salmonella* on carcasses after chilling. In the most recent nationwide survey of microbial contamination of swine processing operations, the FSIS reported the aerobic plate counts (APCs), *Enterobacteriaceae* populations, total coliform numbers, and *E. coli* populations to be 5.81, 3.01, 2.92, and 2.78 log CFU/cm² and 2.02, 076, 0.73, and 0.67 log CFU/cm² after evisceration and chilling, respectively. In addition, the report indicated there was a reduction in *Salmonella* prevalence from 69.6% (at evisceration) to 2.7% (at chilling), indicating that there was a major reduction of *Salmonella* contamination between the evisceration step and carcass chilling resulting from the application of antimicrobial interventions.

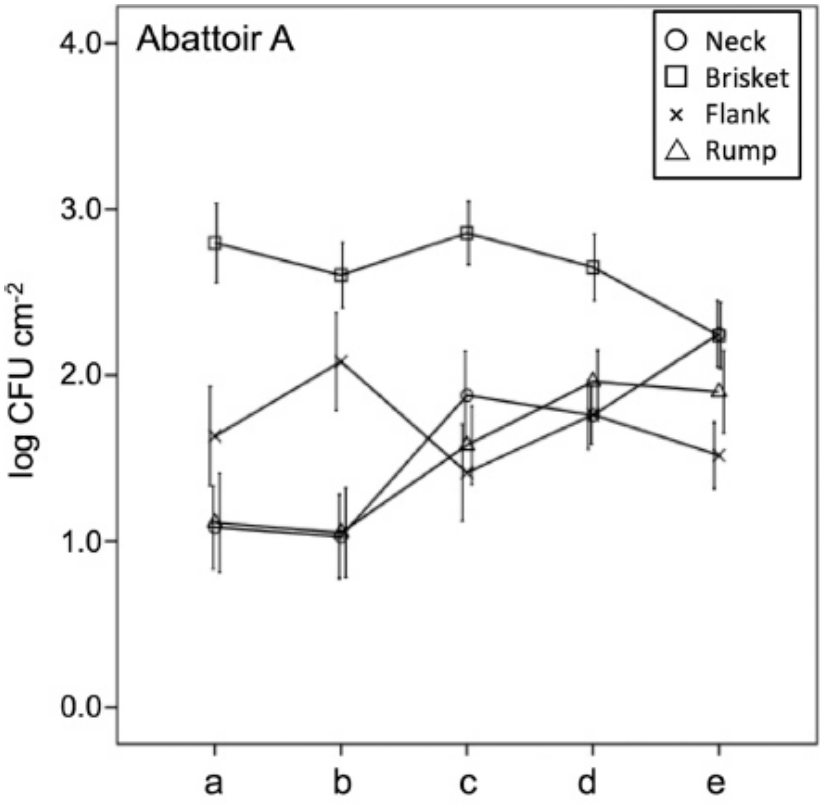
There is minimal information regarding the microbial load on beef carcasses at different stages of the slaughter continuum. However, the

main sources of contamination during the slaughter of beef cattle are the dehiding and evisceration processes. The hide is the primary source of contamination of the beef carcass during slaughter. Microbial populations on hides can vary substantially based on the geographical location of the feedlot, whether the cattle were fed or pasture raised, seasonality, and conditions in the feedlot soil, transportation, and lairage prior to slaughter. Microorganisms on the hides of cattle can be aerosolized in the processing plant, depending on whether mechanized or manual hide removal is practiced, and thereby be deposited on the freshly dehided carcass. Once the microorganisms are deposited, their removal, and more importantly elimination of the foodborne pathogens *E. coli* O157:H7 and other STEC types, is extremely difficult if not impossible. It is reasonable to assume that the carcass surface (interior of the hide prior to dehiding) is sterile or close to being free of microorganisms prior to the dehiding process. Any microbial load on the freshly dehided carcass is most likely the result of contamination through either aerosolization and deposition of the microorganisms on the hide or handling of the carcass by personnel. While the hide can harbor up to 7 log CFU/cm^{2}of microbes, a freshly dehided carcass surface typically has 2.0 log CFU/cm^2 or more microbes, most likely resulting from post-hide-removal contamination (Fig. 6.3).

The evisceration step may further increase the microbial load on the carcass, depending on the hygienic procedures applied. The total microbial populations on the carcass at each stage of the slaughter operation, i.e., on the hide and on carcasses before evisceration, after evisceration, after intervention, and after chilling, were 8.3, 3.8, 3.7, 1.2, and 1.4 log CFU/100 cm^2, respectively, indicating that there was substantial contamination of the carcass from the dehiding step (as represented by the microbial population at the pre-evisceration step) (Fig. 6.4), with minimal change in the cell populations after evisceration; hence, good hygienic practices were in place (8). A substantial reduction in microbial load can be achieved by treatment with effective antimicrobial interventions (in this case, the carcasses were passed through a hot water spray cabinet [90°C] and a peroxyacetic acid [PAA] spray, followed by passage through a steam

pasteurization cabinet). It should be noted that not all beef slaughter operations employ this sequence of antimicrobial interventions; rather, the specific interventions incorporated into the process are plant specific and are based on a variety of factors, such as availability of space on the processing floor, capital investment required, and cost of operation of the antimicrobial treatment, among others.

After the antimicrobial intervention is applied, chilling does not reduce the microbial population. Most beef slaughter operations in the United States chill the beef carcasses in “hot boxes,” whereas some operations in Europe use air blast chilling, in which the carcasses are exposed to a blast of cold air, often at temperatures below 0°C. For swine carcasses, the dynamics of changes in microbial populations on the carcasses during chilling depend on the carcass spacing, ambient temperature, and air movement during the process to enable rapid chilling of the carcass surface. In addition, dehydration of the carcass surface may play a role in minimizing microbial growth during the chilling process. In some beef harvest operations in the United States, the carcasses are spray chilled by applying chilled water to prevent surface drying and to assist in rapid chilling of the carcasses. Many processors are increasingly incorporating antimicrobial chemicals in the water used in spray chill systems to prevent microbial growth on carcass surfaces.


Abattoir A
Neck
Brisket
Flank
Rump
log CFU cm-2
4.0
3.0
2.0
1.0
0.0
a
b
c
d
e

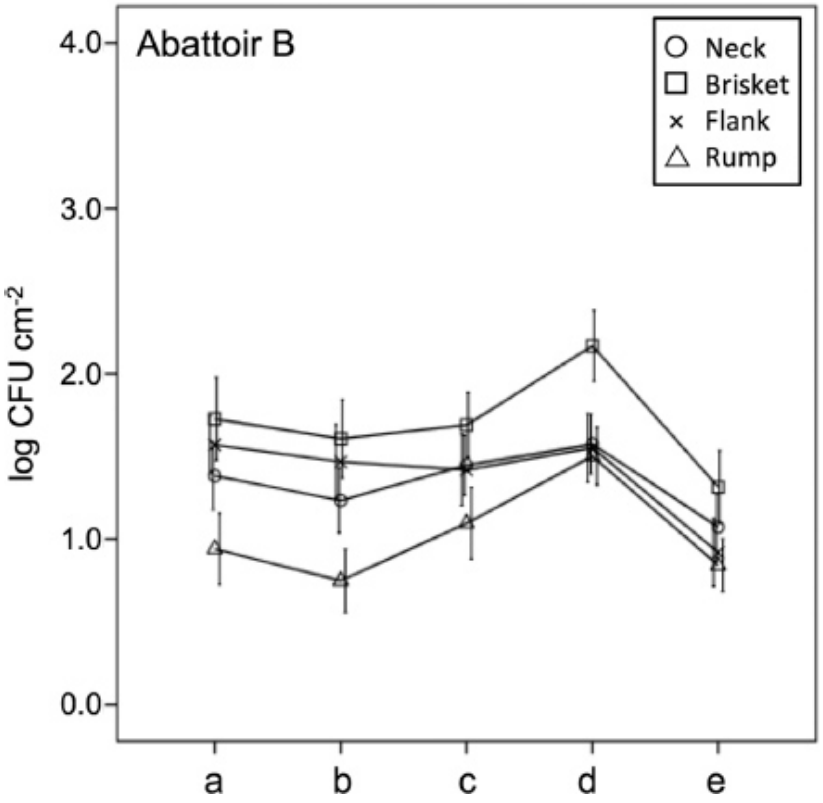

Abattoir B
Neck
Brisket
Flank
Rump
log CFU cm-2
4.0
3.0
2.0
1.0
0.0
a
b
c
d
e

Figure 6.4 APCs on beef carcasses at various locations after dehiding (a), evisceration (b), trimming (c), washing (d), and blast chilling (e) in two beef-processing operations. Reprinted from reference 303.

Intact and nonintact products

Most meat from beef and swine carcasses is consumed as intact cuts, such as steaks, roasts, or chops. However, the palatability of such products can vary greatly, depending on the animal from which the meat is harvested. During beef processing, the carcasses are graded based on several factors, of which intramuscular fat is a major criterion, as it determines the juiciness of the cut. In addition, other factors, including the age of the animal, can play a significant role, with steaks and roasts from older aged animals being chewy and less tender, which can easily be detected by the consumer. Thus, processors or purveyors use a variety of methods to tenderize the sub primals (a portion of meat smaller than a side of a carcass) to achieve consistency in tenderness and eating quality of the cooked steaks and roasts.

Traditionally, food preparers cooked pork to higher temperatures to ensure destruction of the parasite *Trichinella spiralis*, resulting in a loss of moisture and a dry product. To enhance moisture retention, pork processors marinate the meat through mechanical means, such as injection with ingredients such as salt and phosphates, to improve juiciness and palatability of the product. The microbiology and food safety risks of such nonintact meat can be substantially different from those of the intact meat products.

A large proportion of beef products (up to 18%) retailed in the United States have been mechanically tenderized or injected with solutions to enhance tenderness and/or flavor of the meat (9). Beef steaks and roasts available at retail in North America may be blade tenderized at central cutting or retail store facilities (10) and may not be recognized as such at the time of purchase or subsequent cooking.

Nonintact beef products have been involved in recalls (12) and several outbreaks of foodborne illness in the United States (13–16) due to contamination with *E. coli* O157: H7 (17). Most beefsteak purchasers

may not notice that certain beefsteaks have been tenderized, or even though they recognize that steaks are tenderized, they may not know of the potential for internal contamination with *E. coli* O157:H7.

A portion of the U.S. population prefer steaks cooked to rare (cooked to an internal temperature of 60°C) or medium rare (cooked to an internal temperature of 65°C) degrees of doneness; in these cases, it is possible that the internalized foodborne pathogens may survive and cause foodborne illness (18, 19). Due to this increased food safety risk, the FSIS declared that nonintact beef products or intact products that are intended for production of nonintact products (such as mechanical tenderization or grinding) are considered adulterated if they contain *E. coli* O157:H7 (20).

Intact meats. Intact meats or cuts are those prepared from larger pieces of meat, such as subprimals (beef or pork), and have not been subjected to further handling or treatment, which would introduce the surface microflora to the interior of the meat cut. Processes such as mechanical tenderization, injection with marinade solutions, and marination with applied vacuum can translocate the microorganisms from the meat surface to the interior of the meat. In the case of intact meat, the microbes are largely on the surface of the product, and hence cooking, which provides higher temperatures at the surface of the meat and for longer periods of time, results in greater lethality at the meat's surface than in the interior. Therefore, cooking to lower degrees of doneness, such as rare, should achieve a lethality adequate to render the meat (steak or chop) safe for consumption. Thus, cooking of intact meat should achieve adequate inactivation of surface microorganisms and lethality, regardless of the cooking method employed.

Nonintact meats. Nonintact meat products can be broadly classified as (i) those that are visually intact subsequent to the processes such as mechanical tenderization, injection, vacuum tumbling with a marinade, tenderizing using proteolytic enzymes, etc., and (ii) those that are chunked and reformed or restructured, ground, chopped, flaked, minced, etc., and do not retain the appearance of intact products. The second category of products are typically cooked to

higher internal temperatures (such as 71.1°C for ground beef) to achieve adequate lethality for *Salmonella* and *E. coli* O157:H7.

The processes that produce nonintact meat cuts translocate surface microbes regardless of the type of microorganism; however, the food safety risk is primarily due to translocation of foodborne pathogens such as *E. coli* O157:H7, STEC, and *Salmonella*. Sporing (21) and Thippareddi et al. (22) were the first to report extensively on the food safety risks of mechanical tenderization, determining that 3 to 4% of the surface microorganisms (*E. coli* O157:H7) can be translocated to the center of the steak when subprimals are mechanically (blade) tenderized before being portioned into steaks.

Numerous studies have revealed the conditions for inactivating *E. coli* O157:H7, STEC, and *Salmonella* during the cooking of nonintact beef and pork products. Inactivation of these microorganisms in such products is dependent on the surface populations of these microbes, the type and thickness of the meat cut (steak or chop), the internal temperature to which the product is heated, the temperature and relative humidity of cooking, the uniformity of temperature during cooking (e.g., cooking on a heated surface such as a flat grill, two surfaces such as on a clamshell grill, in a kitchen oven, etc.), and other factors. Sporing (21) reported that of three cooking methods (i.e., on a gas grill, in an electric skillet, and under an oven broiler), oven broiling provided the most consistent inactivation of *E. coli* O157:H7 on intact and nonintact steaks, regardless of the thickness of the steaks and the target end point cooking temperature. The reductions of *E. coli* O157:H7 in intact and nonintact steaks upon cooking to internal temperatures of ≥ 60°C were similar, indicating that the integrated lethality achieved was adequate to inactivate microbes that translocated to the geometric center of the steak during blade tenderization of the beef rounds. However, using other cooking apparatus, such as a gas grill or electric skillet, that do not uniformly heat the steak surfaces may result in greater differences in pathogen reduction and hence a greater risk of pathogen survival.

A quantitative microbial risk assessment conducted by the FSIS revealed that the probability of *E. coli* O157:H7 surviving typical

cooking practices in either tenderized (mechanical, or blade, tenderized) or nontenderized steaks is minimal. Further, it was reported that 2.6 of every 10 million servings of steaks that are intact (nontenderized) contain one or more *E. coli* O157:H7 organisms, compared to 3.7 of every 10 million servings of mechanically tenderized steaks (23).

Regardless of the conclusions of the risk assessment, the FSIS considers *E. coli* O157:H7 and other STEC types adulterants in nonintact beef. The industry has applied numerous antimicrobial interventions to subprimals that are destined for nonintact beef products to minimize the risk. The efficacy of these interventions to reduce such risk needs to be evaluated and quantified through a quantitative risk assessment. Other measures adopted by the industry include proper chilling of meat and decontamination of injection solutions during reuse, use of antimicrobials in injection brines, effective sanitation and temperature controls, and effective cooking procedures that are based on product characteristics. In addition, manufacturers should follow industry-recommended best practices (24, 25).

General Spoilage of Beef and Pork

Spoilage of food products, specifically meat and poultry products, refers to chemical, enzymatic, and microbial activities that result in visual or olfactory changes in the product. In most cases, spoilage is caused by microbial activity or growth by nonpathogenic microbes in response to the prevailing extrinsic conditions during processing, distribution, and storage of the products. Spoiled food is considered an economic loss, as it is not necessarily unsafe if foodborne pathogens are absent. A report by the Food and Agricultural Organization of the United Nations on global food losses and food waste revealed that an estimated 3.5%, 1.0%, 5.0%, 4.0%, and 11.0% of food is wasted during production, postharvest handling and storage, processing and packaging, distribution (supermarket and retail), and consumption, respectively, in North America (United States and Canada) and Oceania (Australia and New Zealand) (26). While food losses or waste at these stages in the food chain cannot be thought to be due to

spoilage, most of the losses or waste at distribution and consumption are due to spoilage.

During the harvest and further processing of cattle, swine, or poultry into meat, the inherent barriers, both active (immune responses) and passive (e.g., skin, hide, scales, etc.), break down, and the interior is exposed to the environment, resulting in microbial contamination. The degree of processing and the steps involved dictate the type and number of microorganisms on the product and eventually determine the type of spoilage. The degree of processing required as well as the hygiene employed during processing also affects the type of microorganisms and the cell populations of those microbes and hence determines the types of spoilage observed during distribution and storage at the consumer level. Meat and poultry processors have incorporated many antimicrobial interventions that utilize various antimicrobial mechanisms, such as low or high pH, oxidation, high or low temperatures, antimicrobial chemicals, and packaging atmospheres (modified atmospheres such as CO_2 or $N_2$with high or low oxygen), that dictate the types and the growth rates of spoilage microbes and hence the shelf life of the products. In general, only a few microbial types dominate during storage, based mostly on storage temperature and the gaseous composition of the surroundings (27). Aerobic or facultatively anaerobic Gram-negative bacteria such as pseudomonads dominate under cold aerobic conditions, whereas the dominant microflora consists largely of lactic acid bacteria (LAB) in vacuum-packaged products (27, 28).

Most of the microbes present in meat at the time of packaging are a result of contamination during harvest operations and further processing, with the sources being the hide, GI tract, personnel involved in processing, and the processing environment (e.g., surfaces of equipment and air). The types and numbers and hence the proportions of the microorganisms at packaging determine which microbes grow and predominate at the end of the shelf life of the product (Table 6.2). However, microbial growth and the types of microbes that grow in these products are influenced by intrinsic (pH, water activity [a_w], competitive flora, nutrient composition, oxidation reduction potential, etc.) and extrinsic (temperature of storage and

environmental exposure throughout the cold chain, packaging type, gaseous atmosphere, antimicrobial ingredients, and other meat ingredients) properties of the meat.

With the advances in and availability of molecular methods for detection and identification of microorganisms, it is increasingly possible to identify microbes that have evaded detection by traditional cultural methods for foods. While *Pseudomonas* spp., *Carnobacterium divergens*, *Brochothrix thermosphacta*, *Rahnella* spp., and *Serratia grimesii*, or closely related species, are largely detected in fresh beef, *Photobacterium* spp. are largely detected in meat samples stored in air or in vacuum packages (29). Interestingly, testing by microbial culturing of meat specimens has enabled the detection of microbial species (e.g., several species of *Serratia* or *Rahnella* and *Leuconostoc*) that are not detected by analysis of DNA extracted directly from meat (29). Ercolini et al. (30) used random amplified polymorphic DNA-PCR and 16S rRNA gene sequencing and identified 50 mesophilic and 29 psychrotrophic isolates in beef. The most frequently detected species of both the mesophilic and psychrotrophic populations were *Carnobacterium maltaromaticum* and *C. divergens*, whereas *Acinetobacter baumannii*, *Buttiauxella* spp., and *Serratia* spp. were identified among the mesophiles, and *Pseudomonas* spp. were common among psychrotrophs. Doulgeraki et al. (31) isolated 266 LAB from minced beef stored at 0, 5, 10, and 15°C aerobically or in modified-atmosphere packaging (MAP) with an atmosphere consisting of 40% CO_2–30% O_2–30% N_2in the presence or absence of oregano essential oil. *Leuconostoc* was the predominant bacterium isolated during aerobic storage at 5, 10, and 15°C, as well as during MAP storage at 10 and 15°C, whereas *Lactobacillus sakei* predominated during aerobic storage at 0°C, as well as during MAP storage at 0 and 5°C. Other bacteria, i.e., *Leuconostoc mesenteroides*, *Weissella viridescens*, *Lactobacillus casei*, and *Lactobacillus curvatus*, were isolated sporadically. Hence, different microbial species dominate in different environments and contribute to muscle food spoilage as they release different volatile compounds (28).

Spoilage of Fresh Meat

Even with significant improvements and availability of packaging systems, most intact and nonintact fresh meat packaged at retail is packaged aerobically on a tray pack with a plastic film wrap; however, a small portion is also packaged under anaerobic conditions, such as by vacuum packaging. However, most fresh meat packaged at a processing plant is packaged under a modified atmosphere, with vacuum, high oxygen, or low oxygen (the atmospheric composition mainly consisting of carbon dioxide and/or nitrogen). High-oxygen packaging is mainly used for intact meat as well as ground beef products to enhance the bright red color of oxymyoglobin, the meat pigment responsible for the color.

Based on the packaging system used, the predominant bacteria when fresh meat is stored under refrigeration are either *Pseudomonas* spp. (when meat is packaged under aerobic conditions) or LAB (when meat is packaged under vacuum or other MAP conditions) (28) (Table 6.3). However, when the meat pH is affected by stress on the animal (producing dark, firm, dry [DFD] or pale, soft, exudative [PSE] meat), glycolytic reserves, and the rate of cooling of these carcasses, these parameters dictate the type and rate of microbial growth regardless of the type of packaging system and chilled storage method used. The ultimate pH of the DFD and PSE muscles is typically >6.0 and <5.3, respectively, whereas the pH of normal muscle is 5.5 (32). These are significant differences in pH values, and thus, the rates of microbial growth and spoilage are different in these meats regardless of other storage conditions, such as packaging atmosphere and storage temperature.

In the absence of glucose, higher pH values of meat allow more rapid degradation of amino acids by pseudomonads and detection of spoilage at lower bacterial cell densities (6 log CFU) than in normal meat (33). Under vacuum packaging or other MAP conditions, DFD meat spoils rapidly by developing green discolorations due to growth of *Serratia liquefaciens* and *Shewanella putrefaciens*, which outgrow LAB at the higher pH and in the absence of glucose and glucose-6-phosphate (34). The green discoloration is associated with formation of hydrogen sulfide from cysteine or glutathione by *S. putrefaciens*; hydrogen sulfide reacts with myoglobin to form green sulfmyoglobin.

S. liquefaciens, even at low populations, produces small amounts of hydrogen sulfide that are associated with DFD meat spoilage (34). However, for normal and PSE meats, the spoilage is dictated by the storage temperature and the packaging system used, with pseudomonads (for aerobically stored products) and LAB (for anaerobically stored products) being the predominant spoilage organisms.

Table 6.2 Genera of microorganisms commonly found on meat and poultry[a]

		Presence on meat[c]		
Microorganism	**Gram reaction[b]**	**Fresh**	**Processed**	**Vacuum packaged**
Bacteria				
Achromobacter	–	X		
Acinetobacter	–	XX	X	X
Aeromonas	–	XX	X	X
Alcaligenes	–	X		
Alteromonas	–	X	X	
Arthrobacter	+	X	X	
Bacillus	+	X	X	XX
Brochothrix	+	X	X	
Buttiauxella	–	X		
Campylobacter	–	X		
Carnobacterium	+	X		XX
Chromobacterium	–	X		
Citrobacter	–	X		
Clostridium	+	X		
Corynebacterium	+	X	X	X
Enterobacter	–	X	X	X
Enterococcus	+	XX	X	XX
Escherichia	–	X		
Flavobacterium	–	X		
Hafnia	–	X	X	
Janthinobacterium	–		X	
Klebsiella	–	X		

		Presence on meat[c]		
Microorganism	**Gram reaction[b]**	**Fresh**	**Processed**	**Vacuum packaged**
Kluyvera	–	X		
Kocuria	+	X	X	X
Kurthia	+	X		X
Lactobacillus	+	X	XX	XX
Lactococcus	+	X		
Leuconostoc	+	X	X	X
Listeria	+	X	X	
Microbacterium	+	X	X	X
Micrococcus	+	X	X	X
Moraxella	–	XX		
Morganella	–	X		
Paenibacillus	+	X	X	
Pantoea	–	X		
Proteus	–	X		
Providencia	–	X	X	X
Pseudomonas	–	XX	X	
Rahnella	–	X		
Serratia	–	X		
Shewanella	–	X	X	X
Staphylococcus	+	X	X	X
Streptococcus	+	X	X	
Vibrio	–	X		
Weissella	+	X	X	X
Yersinia	–	X		X
Yeasts				

		Presence on meat[c]		
Microorganism	**Gram reaction[b]**	**Fresh**	**Processed**	**Vacuum packaged**
Candida		XX	X	
Cryptococcus		X		
Debaryomyces		X	XX	
Pichia		X		
Rhodotorula		X		
Saccharomyces			X	
Torulopsis		XX		
Trichosporon		X	X	
Molds				
Alternaria		X	X	
Acremonium		X		
Aspergillus		X	XX	
Aureobasidium		X		
Botrytis			X	
Cladosporium		XX	X	
Chrysosporium		X		
Fusarium		X	X	
Geotrichum		XX	X	
Monascus		X		
Mucor		XX	X	
Neurospora		X		
Penicillium		X	XX	
Rhizopus		XX	X	
Scopulariopsis			X	
Sporotrichum		XX		

		Presence on meat[c]		
Microorganism	**Gram reaction[b]**	**Fresh**	**Processed**	**Vacuum packaged**
Thamnidium		XX	X	

[a] Adapted from reference 28.

[b] +, positive; −, negative.

[c] X, known to occur; XX, most frequently isolated.

Comminuted products, including ground meat, spoil faster than externally contaminated intact meat cuts because of higher levels of initial contamination present in trimmings from beef that had large surface areas exposed to contamination, cross-contamination among trimmings, microbial spreading during grinding, and the release of fluids and nutrients from cells ruptured due to grinding (28, 35, 36). The types of dominating microorganisms and spoilage defects are similar in intact and comminuted products of the same origin. Dominant microflora members in aerobically stored comminuted products include *Pseudomonas*, *Acinetobacter*, and *Moraxella*, whereas LAB dominate in the interior due to oxygen limitation. *Enterobacteriaceae* are more frequently present in comminuted than in intact products. Spoilage microbes dominating in spoiled refrigerated fresh sausage are similar to those present in other fresh meats but may also include *B. thermosphacta* (28).

Meat Spoilage by Psychrotrophic Clostridia

While certain species of clostridia are well recognized as major food safety concerns due to toxin production, others, known in general as psychrotolerant clostridia, cause spoilage in vacuum-packaged meats such as deep tissue or bone taint spoilage. Some clostridia also cause surface spoilage and blown-pack spoilage (37). These unusual types of spoilage of vacuum-packaged refrigerated fresh and cooked meat and poultry products are caused by psychrophilic, psychrotrophic, or psychrotolerant clostridia, such as the generally closely related species *Clostridium laramie*, *C. beijerinckii*, *C. lituseburense*, *C. algidicarnis*, *C. algidixylanolyticum*, *C. estertheticum*, *C. frigidicarnis*, and *C.*

gasigenes, which can germinate, grow, and sporulate at 2°C or lower (0°C) in meat at a normal pH (38, 39). As the meat undergoes proteolysis,

Table 6.3 Microbial species occurring during chilled storage of raw meat in different packaging conditions [a]

	Presence in [b]:			
Microorganism	**Aerobic conditions**	**MAP**	**Vacuum packaging**	**Antimicrobial active packaging [c]**
Achromobacter spp.	X			
Acinetobacter baumannii			X	
Acinetobacter spp.	X	X	X	
Aeromonas spp.	X		X	
Aeromonas salmonicida			X	
Alcaligenes spp.	X	X	X	
Alteromonas spp.	X	X	X	
Arthrobacter spp.	X	X		
Bacillus spp.	X		X	
Bradyrhizobium spp.				X
Brochothrix thermosphacta	X	X	X	X
Buttiauxella agrestis			X	
Buttiauxella gaviniae			X	
Buttiauxella noackiae			X	
Carnobacterium divergens	X	X	X	X
Carnobacterium maltaromaticum	X	X	X	X
Chromobacterium spp.	X			

	Presence in [b]:			
Microorganism	**Aerobic conditions**	**MAP**	**Vacuum packaging**	**Antimicrobial active packaging [c]**
Citrobacter freundii	X	X		
Clostridium spp.			X	
Clostridium algidicarnis			X	
Clostridium estertheticum			X	
Clostridium frigidicarnis			X	
Clostridium gasigenes			X	
Clostridium putrefaciens			X	
Enterobacter cloacae	X			
Enterobacter agglomerans	X	X		
Hafnia alvei	X	X	X	
Klebsiella spp.	X			
Kluyvera spp.	X			
Kocuria spp.	X			
Kurthia spp.	X			
Lactococcus spp.				X
Lactococcus piscium	X	X	X	X
Lactobacillus spp.			X	X
Lactobacillus algidus			X	
Lactobacillus curvatus	X			

	Presence in [b]:			
Microorganism	**Aerobic conditions**	**MAP**	**Vacuum packaging**	**Antimicrobial active packaging [c]**
Lactobacillus sakei	X	X	X	X
Lactobacillus kimchii			X	
Lactobacillus graminis			X	
Lactobacillus oligofermentans		X		
Leuconostoc spp.		X		
Leuconostoc carnosum	X	X		
Leuconostoc gasicomitatum		X		
Leuconostoc gelidum		X	X	
Leuconostoc pseudomesenteroides	X	X		X
Leuconostoc kimchii		X		
Limnobacter spp.				X
Listeria spp.	X	X		
Microbacterium spp.	X	X	X	
Micrococcus spp.	X	X		
Moraxella spp.	X			
Paenibacillus spp.	X			
Pantoea spp.	X		X	
Pantoea agglomerans	X			
Pantoea ananatis		X		
Photobacterium spp.	X		X	

	Presence in [b]:			
Microorganism	**Aerobic conditions**	**MAP**	**Vacuum packaging**	**Antimicrobial active packaging [c]**
Photobacterium kishitanii			X	X
Proteus vulgaris	X	X		
Providencia spp.	X	X	X	
Pseudomonas spp.	X		X	X
Pseudomonas fluorescens	X			
Pseudomonas fragi	X	X	X	X
Pseudomonas lundensis	X			
Pseudomonas migulae	X			
Pseudomonas putida	X	X		
Pseudomonas syringae				
Psychrobacter spp.	X			
Rahnella spp.	X	X	X	X
Rahnella aquatilis	X	X	X	X
Ralstonia spp.			X	X
Rudaea cellulosilytica				X
Serratia spp.		X		
Serratia grimesii	X	X	X	X
Serratia liquefaciens	X	X	X	
Serratia marcescens	X		X	

	Presence in [b]:			
Microorganism	**Aerobic conditions**	**MAP**	**Vacuum packaging**	**Antimicrobial active packaging [c]**
Serratia proteamaculans	X	X	X	X
Shewanella putrefaciens	X			
Staphylococcus spp.	X		X	X
Staphylococcus pasteuri			X	
Staphylococcus saprophyticus	X		X	
Staphylococcus xylosus		X	X	
Stenotrophomonas maltophilia			X	
Streptococcus spp.				X
Streptococcus parauberis			X	
Weissella spp.	X	X	X	
Yersinia spp.	X		X	

[a] Adapted from reference 147.

[b] An "X" indicates the occurrence of the microorganism under the specified storage conditions. A microorganism is considered to occur if, according to the literature, it was identified at least once under the specified conditions regardless of the time of storage.

[c] Nisin-activated antimicrobial packaging (11, 304); in other works on antimicrobial packaging, species identifications were not performed.

the spoilage is characterized by muscle softening, excessive production of hydrogen sulfide ((blown pack, especially by *C. estertheticum* and *C. gasigenes*), associated offensive odors, and release of purge or exudate, while the color of the purge changes from an initial pinkish

red to green (38, 40, 41). Such spoilage has occurred in the United States, Europe, South Africa, and New Zealand. The microbes involved are not considered pathogenic, but the meat is organoleptically unacceptable.

Blown-pack spoilage (where the packaging material of vacuum-packaged meat is distended by massive gas formation during refrigerated storage) has been reported for beef primal cuts, lamb, venison, cooked hot dogs, precooked turkey, roast beef, and sous vide products, resulting in major financial losses, as the meat is unacceptable for consumption. Initially, contaminated product spoils on the surface, with little gas accumulation (surface spoilage), and the bacteria are found in the purge or by swabbing the meat surface. Blown-pack spoilage occurs sporadically and in only a fraction of the product in a consignment. This indicates that contamination with spores is sporadic and that there is a need for specific conditions that allow spore germination and growth (37, 42). While postpackaging heat-shrink treatments of vacuum packaged meats have been reported to accelerate the onset of clostridial blown-pack spoilage (37), this is debatable, because the temperature on the meat surface in contact with the film does not increase significantly, as the meat is at very low temperatures. Prediction or prevention of this type of spoilage is still difficult. Good management practices during meat processing are not always sufficient for its prevention, as some believe that the source of the organism is the feedlot or pasture soil that is carried to the processing operation on the cattle hide. Avoiding higher temperatures (90°C for 3 s or 70°C for 10 s) during heat shrinking of packages, which could stimulate spore germination, and storing vacuum-packaged meats at lower temperatures (e.g., –1.5°C) have been recommended to minimize the risk (40, 41).

Spoilage of Processed Meats

Spoilage defects of processed meats, including frankfurters, bologna, cooked or fermented sausage, and luncheon meats, include slime, souring, and greening. Slime is usually confined to the surface of the product and is associated with growth of yeasts, *Lactobacillus*, *Enterococcus*, and *B. thermosphacta* in the presence of moisture (28).

These microbes metabolize sugars to produce organic acids that result in souring on product surfaces under casings or packaging films. While greening of fresh meats may be due to hydrogen sulfide production, greening of nitrite-cured meat products may develop in the presence of hydrogen peroxide. The reaction product, which is formed during growth of catalase-negative bacteria when surfaces of vacuum-packaged or MAP products are exposed to air, reacts with nitrosohemochrome to produce green choleglobin. This is possible because muscle catalase, which breaks down hydrogen peroxide, is inactivated during cooking. The most common causative agents of such greening are *W. viridescens* and species of *Streptococcus* and *Leuconostoc*, which may be introduced into the product before or after processing (28).

Putrid spoilage of cured meats by Gram-negative psychrotrophic bacteria under refrigeration is inhibited by reduced a_w (43). These products, however, are spoiled by lower-a_w-tolerating lactobacilli or micrococci. While lactobacilli predominate in spoiled vacuum-packaged or MAP products, aerobic micrococci predominate on cured meats stored aerobically. Inclusion of glucose in cured meats allows the formation of a slimy dextran layer by *Leuconostoc* species or other bacteria, including *B. thermosphacta*. Dry-cured products are spoiled mostly by yeasts and molds, because the low a_w, presence of nitrite, and exposure to smoke suppress bacterial growth (44). Growth of spoilage fungi in these products may be inhibited by the addition of antifungal agents. Drycured meats may also be spoiled by microbial growth which occurred before proper and adequate salt penetration, curing, and drying, whereas when properly prepared and stored, dried meat products remain stable. Xerotolerant molds are inhibited at moisture contents of about 15% and below (28).

Foodborne Pathogens

The microbial pathogens of greatest concern in beef and pork meat include *E. coli* O157:H7, non-O157 STEC, *Salmonella*, *Campylobacter*, *Yersinia enterocolitica*, and *Listeria monocytogenes*. While other bacteria, such as *Staphylococcus aureus*, *Clostridium perfringens*,

Bacillus cereus, other *Bacillus* spp., *Clostridium botulinum*, *Aeromonas*, *Arcobacter*, *Brucella*, *Shigella*, *Enterobacter*, *Plesiomonas shigelloides*, *Helicobacter*, and potentially *Mycobacterium* and *Clostridioides* (formerly *Clostridium*) *difficile* (45–47), may be associated with and isolated from meat and meat products, they are not among the majority that have caused foodborne illnesses. Swine can transmit the parasitic agents of trichinosis (*T. spiralis*), sarcocystosis (*Sarcocystis* spp.), and toxoplasmosis (*Toxoplasma gondii*), and beef cattle can transmit tapeworms (*Taenia* spp.), *Sarcocystis* spp., and, especially through fecally contaminated water, *Cryptosporidium parvum* (cryptosporidiosis). Viral agents, such as norovirus, hepatitis A virus, and enteroviruses, cause most foodborne disease cases in the United States, more than bacteria or fungi, but their transmission is mostly associated with poor sanitation, cross-contamination from infected food handlers during preparation and serving, or inadequate cooking (35, 36, 48–50).

According to the U.S. Centers for Disease Control and Prevention (CDC), the pathogens (and some associated foods) responsible for most foodborne illness are *Campylobacter* (poultry), *E. coli* O157:H7 (ground beef, leafy greens, and raw milk), *L. monocytogenes* (deli meats, unpasteurized soft cheeses, and produce), *Salmonella* (eggs, poultry, meat, and produce), *Vibrio* (raw oysters), norovirus (many foods; e.g., sandwiches and salads), and *Toxoplasma* (meats). Viral pathogens are of major concern in food service (largely from contamination by food handlers), whereas bacterial pathogens such as *E. coli* O157:H7 and other STEC types, *Salmonella*, and *Campylobacter* continue to be of importance in the safety of raw meat and poultry, as does *L. monocytogenes* in ready-to-eat (RTE) processed meat and poultry products (www.cdc.gov/foodsafety).

Muscle food safety challenges may be associated with changes in food animal production and product processing and distribution practices, including aquaculture; increased international food trade; changing consumer needs and preference for minimally processed products; increased worldwide muscle food consumption; large numbers of consumers at risk for infection; emerging pathogens and pathogen changes that may be associated with increased virulence and

resistance to control or clinical treatment; advances in microbial detection methods; inadequate food handler and consumer education and training in proper food handling; and increased interest, awareness, and scrutiny by consumers, the news media, and consumer activist groups (50–53).

The presence of biogenic amines, such as histamine, putrescine, spermidine, and spermine, can also be a hazard associated with consumption of stored muscle food products. Amines found in fresh meat and fish products (primarily scombroid species such as tuna, mahi-mahi, and mackerel) in aerobic, vacuum, or MAP storage may lead to scombroid poisoning, a severe, and sometimes fatal, allergic reaction. Biogenic amine formation in some products has been attributed to *Enterobacteriaceae*; however, tyramine can also be formed by some strains of *Lactobacillus* (28). Proper sanitation and storage temperature and time should minimize human health problems associated with biogenic amines in muscle foods (54).

Antimicrobial Interventions

Most beef and pork processing operations in the United States have incorporated, as a result of implementing hazard analysis and critical control point (HACCP) systems, interventions to reduce the prevalence and concentrations of food safety hazards, which in most cases are foodborne pathogens that are "reasonably likely to occur." The microbiological food safety hazards addressed in HACCP plans include *E. coli* O157:H7, STEC, *Salmonella*, and *Campylobacter*. While some antimicrobial interventions may have been in place prior to implementation of an HACCP system, there has been a major increase in adopting antimicrobial interventions, with as many as a series of three to five treatments applied at different stages of production or processing. This is termed a multiple-hurdle approach and includes total or partial external animal washing or hair removal or hideon washes after exsanguination, followed by decontamination techniques applied to carcasses immediately after hide removal but before evisceration, as well as at the end of the dressing process or before and during carcass chilling. Beef processors have incorporated these treatments in spray chilling systems as well as on chilled carcasses

before boning and on meat cuts and trimmings prior to packaging or before grinding or processing into nonintact products (50, 55). These antimicrobial interventions include those applied on the hide (acid, alkaline sprays, or hypobromous acid), those used pre-or postevisceration (e.g., hot water, saturated steam, organic acids, and combinations with mineral acids and oxidizers such as acidified sodium hypochlorite, hypobromous acid, or PAA), and those used on chilled carcasses, subprimals, and/or trimmings destined for grinding into ground or comminuted products (e.g., organic acids such as lactic, citric or some combinations with mineral acids, acidified sodium hypochlorite, PAA, and lauric arginate). While several others, such as chlorine or chlorine dioxide, trisodium phosphate (TSP), hydrogen peroxide, ozone, and lactoferrin, have been evaluated, they are not currently being used.

The effectiveness of these treatments in reducing microbial contamination is affected by factors such as water pressure, temperature, the chemicals and their concentrations used, duration of exposure, method of application, and time or stage of application during processing (55). Decontamination interventions, such as hot water, steam, acetic acid, or lactic acid, reduce bacterial cell populations by 1 to 3 log CFU on carcasses. Importantly, decontamination must be implemented as part of an integrated food hygiene system and should be based on the use of multiple sequential interventions at different points during slaughter (55–62).

According to the FSIS, decontamination agents may be approved for use if they (i) are generally recognized as safe, (ii) do not result in adulterated product, (iii) do not create a need for labeling (i.e., added ingredients), and (iv) can be documented with scientific studies as being effective (55, 63). EU regulations, in principle, allow application of water, including hot water, and chemical solutions for decontamination of carcasses. However, each proposed application needs to be individually approved, and chemical decontamination should be followed by a water treatment to remove residues in order to avoid the need for labeling (35, 64–66). The following are issues that should be addressed when decontaminating agents are being selected, approved, and used: (i) the safety of the application relative

to workers, the environment, and consumers; (ii) potential undesirable effects on product quality; (iii) the potential for spreading and redistributing bacterial cells over the carcass surface or their penetration into the tissue; and (iv) the potential development of stress-resistant pathogenic bacteria (28, 55, 67).

Poultry

Consumption of poultry meat is steadily increasing worldwide, with trends suggesting a shift from whole chickens to further processed (not RTE) products such as parts, i.e., skin-on and skinless products. With further handling of poultry meat, there are increased risks of contamination; thus, such processing requires additional controls to prevent cross-contamination and ensure the microbial safety of poultry meat products.

General Ecology of Poultry

Live poultry naturally harbors microorganisms that are native to their skin, feathers, and feet, as well as other microflora obtained through contact with litter and feces during grow out. The chances for microbial carriage to the slaughterhouse are high, due to cross-contamination in live handling and transportation. This initial microbial load can be concerning for poultry producers, as large numbers of bacteria can decrease the effectiveness of antimicrobial interventions implemented in the processing plant. Most of the microflora present in birds upon slaughter causes meat spoilage and overall quality loss. However, pathogenic bacteria of public health concern are also commonly found in poultry and can persist from farm to processing. The most prevalent foodborne pathogens associated with the consumption of poultry meat are *Salmonella* spp. and *Campylobacter* spp., followed by *S. aureus*, *C. perfringens*, and *L. monocytogenes* (68). The latter is present most frequently in RTE products that have been contaminated during or postprocessing. In recent years, *Aeromonas* spp. have emerged as human pathogenic bacteria isolated from poultry meat products (69).

Poultry Harvest and Slaughter The goals of poultry processing are to ensure the microbiological safety of carcasses while maximizing product yield and nutritional and sensory quality. Poultry processing is an extremely complex and fast-paced operation, where production efficiencies are complemented by good manufacturing practices (GMPs) and other food safety programs to ensure safety and quality of processed poultry. Figure 6.5 shows the primary steps involved in slaughter of poultry. Spoilage and pathogenic bacteria enter the processing plant mainly through live birds that have been contaminated at the farm or during transport. As birds move through each step of the processing system, every effort is made to prevent cross-contamination with or reduce the prevalence of spoilage and pathogenic microorganisms. The success of eliminating bacteria from carcasses or preventing their transfer from carcass to carcass depends on the specific conditions used in each stage of processing.

Transportation and unloading

Processing begins with capture of live birds at the farm and transport to the plant. Birds are loaded into transport units, such as plastic crates or steel cages, and then loaded onto trucks for hauling to the processing plant. These transport units can be an important source of contamination before arrival at the plant. Containers can quickly become contaminated by fecal matter containing high populations of pathogens, such as *Campylobacter*, *Salmonella*, and *E. coli* (70–73). These pathogens can be spread to other birds held in the same or adjacent crates (74, 75). To prevent such contamination, birds should be transported in clean crates lined with single-use paper liners (76). To ensure cleanliness, crates should be cleaned and disinfected between uses. A two-stage approach that first cleans and then disinfects crates is best. Ramesh et al. (77) determined that manually cleaning crates with a high-pressure jet spray of disinfectant solution alone did not substantially reduce coliform levels. However, subsequent immersion of precleaned crates in hot water (60 or 70°C) or sodium hypochlorite (1,000 ppm) did significantly reduce coliform levels. Similarly, Allen et al. (78) determined that the most effective treatment to remove *Campylobacter* from transport crates was the

combination of soaking, brushing, and washing followed by application of disinfectant. However, studies have revealed that some pathogens can survive cleaning and disinfecting of crates (75, 79–81).

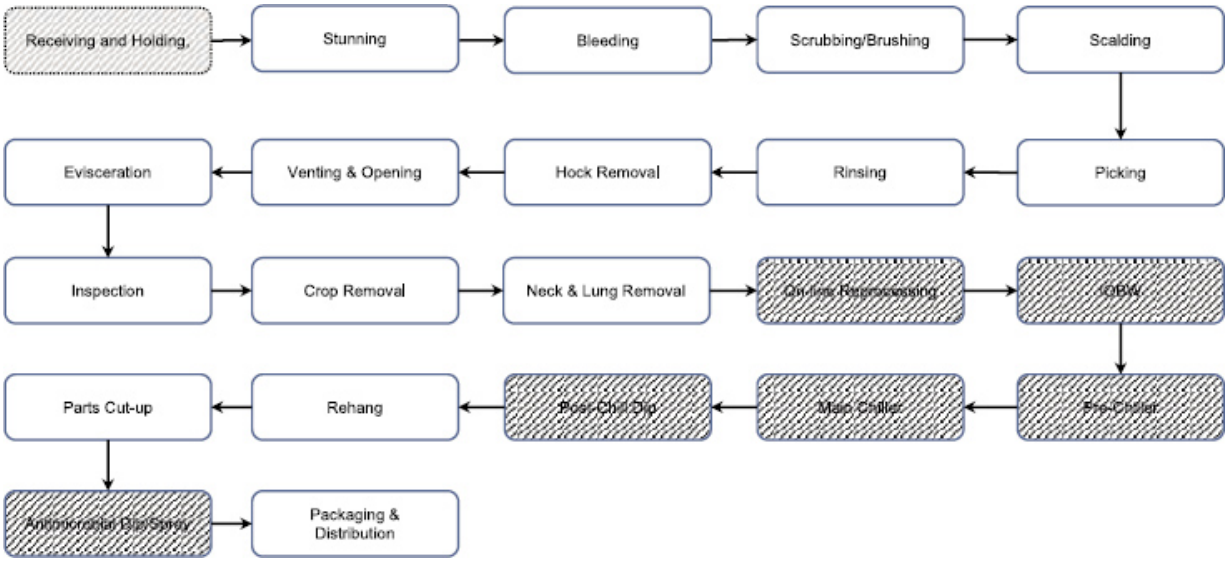


Figure 6.5 Unit operations for primary processing of poultry. IOBW, inside-out bird washer.

Once the birds arrive at the plant, they are unloaded and hung on shackles. High levels of bacteria on birds at this stage of processing can overwhelm plant interventions and be carried to subsequent stages of processing (72, 82). Birds are “dumped” from transport crates onto conveyors where they can become contaminated by aerosols or condensation from equipment or the ceiling (64). Controlling air flow from inside to outside the facility may help avoid condensation as well as prevent cross-contamination (76). Additionally, employee interventions, such as training, changing clothes and boots upon arrival, separate facilities for “dirty” and “clean” employees, and restricting employee movement, can limit cross-contamination (76).

Stunning

Stunning renders birds unconscious prior to killing. The most common method for this is electrical stunning in a water bath containing saline. Electrical stunning renders birds unconscious for 60 to 90 seconds, during which time the neck is cut, allowing them to

bleed out. Other methods of stunning have been developed. One such method is gas stunning, or controlled-atmosphere stunning. These methods lead to a gradual unconsciousness instead of immediate unconsciousness. Different gas combinations are used, including carbon dioxide alone or carbon dioxide in combination with inert gases, i.e., argon, nitrogen, or both. Another method is a low-atmosphere pressure system, which removes air from a sealed chamber gradually, rendering the birds unconscious (83). Wing flapping and quivering can occur during electrical stunning as well as controlled-atmosphere stunning, which may lead to transfer of bacteria from the inside to the outside of birds, to nearby birds, and to equipment.

Scalding

Scalding prepares carcasses for defeathering by opening feather follicles. Carcasses move through one or more scalding tanks filled with hot water. “Soft” scalding occurs at 53.35°C for 120 s, while “hard” scalding occurs at 62 to 64°C for 45 s. This is the first location where birds are exposed to a common bath, which can allow transfer of bacteria from carcass to carcass (84). Due to the high level of organic load in the scalders, including fecal matter, the scalding step can be a major source of cross-contamination, with limited opportunities to use any antimicrobials due to the high temperature of the scalder water.

Defeathering

In the defeathering process, high-speed rotating metal discs with rubber fingers operate to remove the feathers, which have been loosened by scalding. Abrasion by the rubber fingers against the carcass pulls outloose feathers, exposing the feather follicles. Berrang and Dickens (85) reported that this is the only processing step during which *Campylobacter* spp. populations increase; this can be attributed to either cross-contamination from rubber fingers or exposure of the feather follicles.

Evisceration

At the evisceration stage of processing, the edible and inedible viscera are removed from the carcass. This is a highly automated unit operation in modern poultry processing, and its success is strongly dependent upon uniformity of the size of the carcasses. At this stage, if the GI tract is exposed, fecal contamination of the carcasses can occur. Other sources of cross-contamination at this step include the equipment and employees, and the proportion of *Salmonella*-positive carcasses can increase at this stage (86).

Chilling

The goal of the chilling stage is to reduce the temperature of the carcass to below 4.4°C, and this is typically a critical control point in the overall HACCP plans of processing plants. Chilling is the final important step for initial processing of whole-carcass broilers; it is designed to bring the product temperature below 4°C to prevent the growth of microorganisms. The FSIS recommends that the time required to reach 40°F (4.4°C) should not exceed 4 h for birds weighing <4 lb, 6 h for birds weighing 4 to 8 lb, and 8 h for birds weighing >8 lb (87).

Immersion chilling and air chilling are two methods used for this process, with the former being used most commonly in the United States and the latter being commonly used in Europe. The two chilling systems significantly determine the microbial quality and safety of poultry carcasses. Due to the extensive bird-to-bird contact in a water immersion chilling system, there is a greater potential for cross-contamination between carcasses than during air chilling, where carcass-to-carcass contamination is less likely.

General Spoilage of Poultry

Food spoilage is an important economic issue, with an estimated one-third of global food products being wasted annually (88), and it has been estimated that microbial spoilage causes 25% of allpostharvest food loss (89). Given this massive problem, it is important to study the underlying mechanisms of spoilage to facilitate development of novel technologies and techniques for improved food preservation and mitigation of postharvest losses. Poultry meat, especially parts

containing skin, is known to have a higher microbial contamination rate than beef and pork, thus making poultry meat highly perishable, even under refrigeration (90).

Meat spoilage is a natural process of decomposition that is caused by endogenous enzymatic activity in the muscle and by growing microorganisms that metabolize organic molecules in the muscle tissue (91). This process of food degradation results in the production of organic molecules, such as amines, sulfides, alcohols, aldehydes, ketones, and organic acids, which impart undesirable organoleptic characteristics (89, 92). Examples of characteristics that are affected by spoilage include color, odor, texture, and flavor, as well as any other feature that contributes to consumer acceptability of a food product (89). Although spoilage generally encompasses these characteristics, consumer acceptability is influenced by cultural and individual tastes and by economic factors; thus, the definition of spoilage is subjective to some degree (92).

Mechanisms of Meat Spoilage

Microbes that cause spoilage can be introduced to the product from many different sources, but the main source is the animals themselves at the time of slaughter. The intestines, skin, and feathers of birds harbor spoilage bacteria, which are readily introduced into the product during processing steps such as scalding, defeathering, crop removal, evisceration, and chilling (93). Minimally processed meat products provide ideal environmental conditions for microorganisms and spoil quickly unless they are modified or stored in a manner to prevent microbial growth (92). Characteristics of fresh meat products that contribute favorably to microbial growth include a high a_w of 0.99 and a pH range of 5.5 to 6.5, and these products contain numerous energy sources, such as sugars, amino acids, and nucleosides, in addition to many vitamins and minerals (91). The most common microbes associated with spoilage include *Pseudomonas* spp., *Enterobacteriaceae*, *B. thermosphacta*, and LAB, which are collectively referred to as ephemeral spoilage organisms (ESO) (29). Oxygen availability is an important factor for ESO metabolism; therefore,

vacuum packaging can be useful for limiting ESO growth and lengthening the shelf life of poultry products (94).

Foodborne Pathogens

Salmonella

Salmonella belongs to the family *Enterobacteriaceae* and is classified as a Gram-negative, facultatively anaerobic, motile rod. Salmonellae are mesophilic and grow optimally at 35 to 37°C but can adapt to diverse niches and environmental conditions (95). The genus *Salmonella* comprises the species *Salmonella enterica* and *Salmonella bongori*. The former, being the species most commonly associated with human disease, has more than 2,500 serovars according to estimations from the CDC (96). Serovars of *S. enterica* can be further classified as typhoidal or nontyphoidal, according to their diseaseinducing nature in humans. The typhoidal *Salmonella* serovars, *S. enterica* serovars Typhi and Paratyphi, cause enteric fever, whereas nontyphoidal serovars are responsible for the gastroenteric disease referred to as salmonellosis. The most common causative agents of nontyphoidal salmonellosis in the United States are *S. enterica* serovars Enteritidis, Typhimurium, Newport, and Heidelberg (97). Surveillance reports from the FSIS highlight the prevalence of *Salmonella* serovars in poultry products, with *S. enterica* serovars Kentucky, Enteritidis, Typhimurium, and Infantis being the most commonly found in broilers (98). Human infections caused by nontyphoidal *Salmonella* result in acute gastroenteritis characterized by nausea, vomiting, abdominal cramps, diarrhea, and fever. An illness onset time ranging from 4 to 72 hours after contaminated food or water is consumed is typical. The illness can last up to 7 days if it is noninvasive, but cases of septicemia in highly susceptible patients have been reported (99, 100). While common in nature, *Salmonella* has its main reservoir in the GI tracts of animals, including poultry.

Thus, consumption of contaminated poultry meat and eggs has continuously been an important route of *Salmonella* transmission to humans and source of salmonellosis outbreaks (101). With the global consumption of poultry and globalization of poultry meat trade

increasing (102), the industry faces major challenges in the control of *Salmonella*. Traditionally, regulatory efforts to reduce the prevalence of *Salmonella* in poultry have been heavily focused on processing; however, a preharvest approach has been implemented in recent years in hopes of reducing the persistence of the pathogen (76, 103).

Campylobacter

Campylobacter is a foodborne pathogen belonging to the family *Enterobacteriaceae*. It is characterized as a Gram-negative, mesophilic, spiral rod that requires microaerophilic conditions to grow. While susceptible to numerous environmental conditions, *Campylobacter* spp. can easily multiply inside their hosts (104). In particular, the GI tract of poultry is considered a major reservoir for *Campylobacter* (65). In 2012, the World Health Organization reported *Campylobacter* to be the leading causative agent of bacterial gastroenteritis worldwide (105). Humans develop campylobacter enteritis through consumption of contaminated foods or water and experience symptoms of disease after 24 to 72 hours (106). The disease is a self-limiting gastroenteritis, characterized by diarrhea, cramps, and fever, with an infectious dose as low as 800 CFU (107). Serious sequelae of *Campylobacter* infections include the autoimmune disorder Guillain-Barré syndrome. An estimated 20 to 40% of Guillain-Barré syndrome cases are derived from *Campylobacter* infections, substantially increasing the total economic burden of *Campylobacter* infections (108).

According to the CDC and the European Food Safety Authority, there is sufficient evidence to consider *Campylobacter* a foodborne safety hazard linked to poultry meat, with the most prevalent species being *Campylobacter jejuni* and *Campylobacter coli* (97, 109). Vertical transmission of *Campylobacter* in poultry is uncommon, with flocks becoming positive after 2 weeks of age. This foodborne pathogen, however, can spread quickly and colonize flocks through horizontal transmission and persist through transport to the processing plant (85, 110). Higher loads of *Campylobacter* in incoming birds increase the risk of cross-contamination during processing; additionally, the known persistence of the pathogen in carcass skin is of concern.

Hence, preharvest interventions and improvement of pathogen reduction interventions during transport and slaughter are needed.

Staphylococcus aureus

S. aureus is a Gram-positive, aerobic coccus that is a commensal bacterium on the skin and mucosal areas of mammals (96). Certain strains of *S. aureus* can, however, cause infections in poultry, such as tenosynovitis and omphalitis (111, 112). In humans, strains of this pathogen can produce an enterotoxin that induces GI illness upon consumption of foods containing the preformed toxin, and some of these strains have been detected in poultry meat (113, 114). Strains prevalent in poultry, however, have a low incidence of foodborne infections in humans (115). The link between *S. aureus* human infections and poultry appears to be the contamination of poultry carcasses during processing due to improper handling practices (116). Recently, concerns have been raised regarding the occurrence of multidrug-resistant *S. aureus* in poultry and meat production (117). Hence, implementation of and compliance with existing standard operating procedures and GMPs regarding sanitation during poultry processing are crucial.

Clostridium perfringens

Much of the attention that *C. perfringens* has received from the poultry industry is due to an increasing incidence of necrotic enteritis associated with antibiotic-free poultry production practices (118). However, *C. perfringens* is also an important cause of foodborne illness and can be transmitted to humans through consumption of contaminated poultry products (96). This pathogen is a Gram-positive, anaerobic, spore-forming bacterium. *C. perfringens* causes a GI toxicoinfection in humans when large numbers of the organism are consumed (>6 log CFU) (119). In the GI tract, spores of this pathogen can revert to their vegetative state and produce an enterotoxin that induces profuse, watery diarrhea (119). It is its spore-forming ability which makes this pathogen a concern for poultry processors. *C. perfringens* spores can survive thermal processing and persist in the final cooked product. In poultry processing, this scenario likely can

occur when large batches of product are insufficiently cooked and cooled. If the cooling rate during postprocessing is low and the product is temperature abused, spores can easily germinate, leading to potentially large numbers of vegetative cells in the final product that can produce the enterotoxin.

Listeria monocytogenes

L. monocytogenes is a widely distributed foodborne pathogen, and as such, it occurs commonly in poultry processing environments (96, 120). Characterized as a Gram-positive, facultative anaerobic, nonsporulating rod-shaped bacterium, *L. monocytogenes* is an opportunistic pathogen affecting mainly immunocompromised individuals. The disease caused by *L. monocytogenes*, i.e., listeriosis, is often manifested as a flu-type illness in healthy individuals. However, children, elderly persons, pregnant women, and other individuals with a weakened immune system can develop a systemic infection that may eventually lead to death (121). As a psychrotroph, *L. monocytogenes* can grow at a wide temperature range, including refrigeration temperatures (4°C). This pathogen is known to persist in cold and damp environments within poultry processing facilities and delis and has been commonly isolated from floor drains, refrigerators, and freezers (122, 123). Therefore, RTE poultry products that have the potential to be contaminated with the pathogen postprocessing are of great concern. To minimize the risk and incidence of disease associated with this pathogen, the USDA has implemented a "zero-tolerance" rule for *L. monocytogenes* in RTE meat and poultry products. The industry applies a zero tolerance, or <1 CFU/25 g of RTE product, for all *Listeria* species, to ensure control of *L. monocytogenes*.

Antimicrobial Interventions

Similar to antimicrobial interventions used for beef and pork processing, poultry-related food safety hazards such as *Salmonella* and *Campylobacter* are largely controlled by implementing HACCP plans and other food safety programs that include chemical interventions. The most widely used chemicals in the poultry industry include PAA,

cetylpyridinium chloride (CPC), chlorine, and to a lesser extent chlorine dioxide, citric and lactic acid, bromine, and TSP.

Chlorine has been used for more than 50 years in the poultry industry as an antimicrobial, and its application is still one of the most popular and effective methods used for pathogen reduction on poultry carcasses. Low cost and availability make chlorine an easy choice as an antimicrobial in immersion chillers and other large tanks. The FSIS acceptable levels of free residual chlorine are 50 ppm in bird washers and incoming potable water to the main chiller and 5 ppm in the reuse water or red water entering the prechiller (124). The efficacy of chlorine is dependent on several factors, including the pH of the water, the organic matter in the water, and the time in contact with the bacterial cell. The higher the amount of free residual chlorine in the water, the more effective the chlorine is as an antimicrobial; however, too high a level of free residual chlorine in the water can produce a hazardous amount of chlorine gas emanating from the immersion tanks. Northcutt and Jones (125) determined that a free available chlorine concentration of 50 ppm had no effect on *Campylobacter* and *Salmonella* levels when used in an inside-outside bird washer with a contact time of 5 seconds. Mead et al. (126) also observed that 10 to 20 ppm of chlorine had little to no effect on carcass contamination when used in a postevisceration spray.

PAA is one of the most widely used antimicrobials in the poultry industry. PAA is typically introduced into the water as a combination of peracetic acid and hydrogen peroxide, creating equilibrium in the water. The mixture works as both an oxidizer and an acid, disrupting the permeability of the cell membrane and altering protein synthesis. At 85 ppm in chiller water, PAA reduced *Salmonella* on carcasses by 92% and *Campylobacter* on carcasses by 43% (127). *Salmonella* on broiler carcasses was reduced by 2.1 log CFU/ml and *C. jejuni* on carcasses was reduced by 2.0 log CFU/ml after exposure to 1,000 ppm of PAA for 20 seconds in a postchilling immersion tank (128).

TSP is a mixture typically used as a spray in online reprocessing or bird rinse treatments. The FSIS has approved the use of a 12% solution of TSP on poultry carcasses; however, there are several negative

aspects of its use. When residual TSP on the poultry carcasses enters the immersion tanks, it raises the pH of the water to more than 11.0, thereby reducing the antimicrobial effectiveness of any other chemicals (such as chlorine) used in conjunction. Additionally, the phosphate concentration of the waste water of a processing facility increases, requiring further treatment to prevent pollution (129).

CPC is a quaternary ammonium compound (typically used in oral antiseptics) recently approved for use to treat the raw surface of poultry carcasses. The antimicrobial effectiveness of CPC has been determined for *Campylobacter* and *Salmonella*. Arritt et al. (130) obtained a 2.89-log CFU/ml reduction of *C. jejuni* on chicken skin when 0.5% CPC was applied. Beers et al. (131) determined that an application of 0.5% CPC to prechilling broiler carcasses reduced *Campylobacter* and *Salmonella* prevalence from 98% and 34% on untreated carcasses to 8% and 9% on CPC-treated carcasses, respectively.

PROCESSED MEAT PRODUCTS

Processing of meat and poultry products originated from the need to preserve the meat during prehistoric times and basically relied on drying to reduce a_w and on cooking to destroy the microorganisms and improve shelf life of the processed products. Other processes, such as fermentation and curing, developed over the centuries in Europe and other parts of the world and spread throughout the world.

The term “processed meat” refers to a variety of meat and poultry products that have diverse product characteristics, such as pH, a_w, and presence of secondary inhibitors; they can further be distinguished by the thermal treatments they receive—mild heat treatment (not fully pasteurized), pasteurization, and commercial sterilization.

The type of meat, ingredients, and processes used determine the types of microorganisms that survive processing and those that can grow and cause spoilage.

Thermally Processed Products

Types of Products

Advances in processing of meat and poultry have been made through the centuries by developing new techniques and technologies (e.g., fermentation or acidification, thermal processing, drying, and refrigeration) to prepare products with specific characteristics based on the raw materials (e.g., meat species and animal part used such as pork jowls, etc.) and ingredients (e.g., spices, smoke application, water addition, other functional ingredients such as phosphates, nitrite, erythorbate, etc.) used. Although major advances have been made in our understanding of the behavior of muscles and muscle proteins, ingredients used in processing, and processing technologies, meat processing, especially thermal processing of meat products, still remains an art form. A partial list of products and the processes used to prepare these products and the process category is provided in Table 6.4.

While most traditional products include thermal processing and repackaging of products subsequent to cooking, over the past 3 decades, the use of sous vide processing for the manufacture of pasteurized products has increased. While most of these products are prepared in restaurants and sold directly to consumers, some products are commercially manufactured and marketed through retail channels. These products present a different microbial challenge, because while sous vide processes can destroy vegetative cells, spores can survive and can cause spoilage and potentially foodborne illnesses.

Processing and Ingredient Effects

The source and type of meat, the ingredients used in preparation of the product, and the process determine the types of microbes that can survive in the product or contaminate it after processing and proliferate in the product under a variety of storage conditions (Table 6.5).

Typically, the ingredients used in these products can be salt, acidulants, spice and spice flavorings, flavor enhancers (monosodium glutamate and hydrolyzed animal or vegetable protein concentrates), antioxidants (synthetic or natural), water binders (phosphates,

starches, collagens, hydrocolloids, etc.), protein substitutes and enhancers (protein fractions from soy, corn, or milk and milk products), curing agents (nitrates and/or nitrites), cure accelerators (erythorbates or ascorbates), humectants and/or sweeteners (corn syrup solids, alcohols of sorbitol, and other sugars), dehydrated meat or poultry products (stocks), and antimicrobial agents (salts of organic acids). Use of these ingredients and the proportions used can vary, and when they are used, the characteristics of the resulting product (pH, a_w, antimicrobial ingredients, etc.) determine the type of microorganisms surviving the process, whereas the processing environment introduces microorganisms to the product by post-processing contamination. These are the two principal contributors to the types and populations of spoilage microorganisms that will be in the product subsequent to storage.

Foodborne Pathogens and Contamination

Thermally processed meat products are typically pasteurized, with the process being designed to destroy vegetative foodborne pathogens of public health concern, including *E. coli* O157:H7, STEC, *Salmonella*, *Campylobacter*, *S. aureus*, and *L. monocytogenes*, among others. The performance standards for lethality for such products are 6.5-and 7.0-log reductions in *Salmonella* spp. for beef and pork products and for poultry products, respectively (Table 6.6). Thus, the process should inactivate the foodborne pathogens and concurrently reduce or eliminate most of the vegetative spoilage Sausage casings Microbial populations in natural casings for sausages production *Vagococcus*, *Clostridium*, *Pediococcus*, *Lactobacillus*, *Acinetobacter*, *Staphylococcus* Contamination tracking

Table 6.4 Processed meat and poultry product categories, processes used for their production, and examples of products within each catgory

Product	**category Product**	**description Products**
Thermally processed, commercially sterile	Thermally processed, commercially sterile	Canned spaghetti with meat balls, canned corned beef hash, canned ham, canned chicken salad, canned soups with meat or poultry
Not heat treated, shelf stable	Not RTE, otherwise processed meat	Bacon, meals/dinners/entrees, pies/pot pies, rendered fats, oils, sandwiches/filled rolls/wraps, sauces, smoked parts, soups
	RTE acidified or fermented meat (without cooking)	Alessandri (dry sausage), apenino (dry sausage), Arles (or d'Arles) (dry sausage), bockwurst (semidry sausage), cacciatore (dry sausage), cervelat, soft cervelat, chorizo, Lebanon bologna, pepperoni, soft salami, salami (Genoa, Italian, German, summer sausage, Thuringer, soft Thuringer)
	RTE dried meat	Basturma, pastirma, basturmi, beef sticks, carne seca, dried beef, dry duck breast, meat/poultry jerky
	RTE salt-cured meat	Capicola, coppa, country ham, dry-cured duck, Parma ham, prosciutto

Product	category Product	description Products
Fully cooked, not shelf stable	RTE meat fully cooked without subsequent exposure to the environment	Meat: Berliner (cooked, smoked sausage), bologna, bratwurst, cooked, braunschweiger/liver sausage, breakfast link sausage or patties, brown-and-serve sausage, burritos, cheese smokies, cheesefurter, cheesewurst/cheddarwurst, chili, chorizo, cooked beef, cooked ham, cooked pork in barbeque sauce, salami cotto, fleischkaese (cured, cooked sausage), frankfurters, gyros, meatloaf, meat salads, frozen meat soups, nem chua (cooked, pickled ham with shredded pork skin), pasta with meat sauce, pastrami, pickled pigs' feet in vinegar, pickled sausages/meat in vinegar, piroshki, pork barbecue, pork sausage patties, ravioli, roast beef, roast pork, souse, stews, white hots, ieners Poultry (includes products containing any amount of poultry): chicken burritos, chicken barbeque, chicken bologna, chicken breast, chicken franks, cooked poultry, cooked poultry rolls, corn chowder with chicken, poultry loaf, poultry patties, poultry rolls, frozen poultry soups, turkey barbeque, turkey franks
Heat treated, not fully cooked, not shelf stable	Not RTE, otherwise processed meat	Bacon, pies/pot pies, sauces, sausage products, smoked parts, soups

Product	category Product	description Products
Product with secondary inhibitors, not shelf stable	Not RTE, otherwise processed meat	Bacon, pies/pot pies, sauces, sausage products, smoked parts, soups

Table 6.5 Microorganisms associated with processed meat and poultry products

Product	Study topic	Most abundant bacterial species involved in the process	Type of process
Pork	Microbiota and metabolome in spoiled, vacuum-packed pork	*Brochothrix*, *Photobacterium*, *Weissella*, *Lactobacillus*	Spoilage
Beef	Spoilage microbiota and metabolite production in beef stored under vacuum or in high-oxygen atmosphere	*Lactobacillus*, *Leuconostoc*, *Photobacterium*, *Lactococcus*	Spoilage
Salami	Microbiota during fermentation of traditional Italian salami produced in 6 local factories	*Staphylococcus saprophyticus*, *Staphylococcus xylosus*, *Staphylococcus equorum*, *Lactobacillus sakei*	Fermentation
White pudding	Bacterial populations involved in spoilage of Belgian white pudding (meat plus oatmeal)	*Carnobacterium maltaromati-cum*, *Lactococcus lactis*, *Lactobacillus fuchuensis*, *Lactobacillus graminis*, *Serratia*	Spoilage

Product	**Study topic**	**Most abundant bacterial species involved in the process**	**Type of process**
Minced meat	Bacterial populations involved in spoilage of minced meat stored under modified atmosphere with the addition of different preservatives	*Lactobacillus algidus*, *Leuconostoc*, *Pseudomonas*, *Propioni-bacterium acnes*, *Photobacterium*	Spoilage
Sausages, environment	Bacterial populations involved in spoilage of vacuum-packaged sausages and contamination in related processing environment	*Leuconostoc*, *Lactobacillus*, *Yersinia*, *Brochothrix*, *Strepto-coccus*	Contamination tracking and spoilage
Sausages	Effect of packaging (vacuum or modified atmosphere) and salt content on bacterial populations involved in pork sausage spoilage and related metabolites	*Lactobacillus sakei*, *Lactococcus piscium*, *Carnobacterium divergens*, *Carnobacterium maltaromaticum*, *Serratia proteamaculans*, *Brochothrix thermosphacta*	Spoilage

Product	**Study topic**	**Most abundant bacterial species involved in the process**	**Type of process**
Ham	Bacterial populations involved in spoilage of vacuum-packed, cooked ham and effect on sensorial properties	*Brochothrix thermosphacta*, *Lactobacillus*, *Micrococcus*, *Pseudomonas*, *Vagococcus*	Spoilage
Sausages	Bacterial populations during Italian salami fermentation and ripening	*Lactobacillus sakei*, *Leuconos-toc carnosum*, *Staphylococcus succinus*, *Lactobacillus curvatus*	Fermentation
Beef	Microbial populations involved in spoilage of beef burgers packaged under vacuum or in nisin-activated packaging	*Photobacterium phosphoreum*, *Lactococcus piscium*, *Lactobacillus sakei*, *Leuconostoc carnosum*	Spoilage

Product	**Study topic**	**Most abundant bacterial species involved in the process**	**Type of process**
Beef	Changes in spoilage-associated microbiota and metabolome according to storage conditions (air, vacuum, active packaging, MAP)	*Pseudomonas*, *Brochothrix*, *Carnobacterium divergens*, *Lactococcus*, *Streptococcus*, *Lactobacillus*, *Ralstonia*	Spoilage
Sausages	Effect of different concentrations of lactate and diacetate in fresh pork sausage spoilage	*Leuconostoc citreum*, *Pseudomonas lini*, *Carnobacterium divergens*, *Lactobacillus graminis*, *Lactobacillus gasseri*, *Serratia*	Spoilage

Beef, bacon, poultry sausages, shrimp, salmon, cod Identification of core and food-specific bacterial populations involved in spoilage of seafood and meta products and their ecological origin *Staphylococcus*, *Pseudomonas*, *Brochothrix*, *Leuconostoc*, *Flavobacterium*, *Chryseobacterium*, *Photobacterium*, *Corynebacterium*, *Propionibacterium*, *Lactobacillus*, *Entero-coccus*, *Clostridium* Contamination tracking and spoilage microorganisms during processing. Some microorganisms that have greater thermal resistance, such as *Micrococcus* and *Enterococcus*, may survive the process depending on the initial populations.

Pasteurization treatments are not adequate to destroy the spores of the spore-forming pathogens *C. perfringens*, *C. botulinum*, and *B.*

cereus. Since the spore populations in raw meats and ingredients such as spices used in the manufacture of such products are usually low, and the vegetative cells of these pathogens can be destroyed during thermal processing, it is adequate to control germination and growth of the spores of these microorganisms. The performance standards during cooling of thermally processed products are designed to minimize the potential germination and outgrowth of *C. perfringens* spores to less than 1-log growth during the cooling process. Control of this organism implies control of other spore-forming microorganisms as well. The compliance guidelines require rapid cooling of cooked meat and poultry products to minimize the germination and outgrowth of *C. perfringens* spores. Further, most of the antimicrobial agents (salts of organic acids and their combinations, from both traditional and natural sources) used in ingredient formulations to reduce the growth of *L. monocytogenes* in fully cooked RTE meat and poultry products can also reduce *C. perfringens* spore germination and outgrowth.

Most of these products are exposed to the processing environment during the slicing and packaging steps which occur subsequent to thermal processing. This environmental exposure can result in postprocessing contamination by microorganisms, specifically, *L. monocytogenes* and spoilage microbes. However, this postprocessing contamination can be minimized through proper sanitation and environmental control. A survey conducted by the World Health Organization (132) revealed that approximately 25% of foodborne outbreaks are attributed to recontamination of processed products. Additional factors include insufficient hygiene (1.6%), cross-contamination (3.6%), inadequate sanitation in processing or storage facilities (4.2%), contaminated equipment (5.7%), and contamination by personnel handling the products (9.2%). Significant sources of recontamination include the environment (processing equipment, air in the processing rooms, and other non-product contact surfaces harboring microorganisms) and personnel.

Thermal processes used for the manufacture of sous vide products are typically 70°C for 100 minutes or 90°C for 10 min. The main food safety concerns associated with these products are the spores of *C.*

botulinum, which can survive the pasteurization process and subsequently germinate, grow out, and produce toxins if the product is temperature abused. LAB are the main cause of microbial spoilage in sous vide-cooked meat products, with the heterofermentative LAB *Lactobacillus*, *Leuconostoc*, *Weissella*, and *Carnobacterium* (133) producing sour off flavors, slimy exudates, and carbon dioxide. However, spoilage resulting from growth of *Pseudomonas* has been reported in sous vide meat-based products.

In the United States, meat products that receive more severe thermal treatments than traditional sous videcooked meat and poultry products have gained popularity. Such products are distributed and stored refrigerated and allow consumers to reheat the product prior to consumption, thus providing convenience. These products are mostly meat and poultry products that contain gravy or sauce and are heated to 80 to 85°C for several hours in a vacuum pouch but do not achieve commercial sterility. However, literature on the adequacy of such a thermal treatment to destroy spores of *C. botulinum* as well as the potential for germination and outgrowth of such spore-forming pathogens is lacking.

Table 6.6 Performance standards for RTE and allpartially heat-treated meat and poultry products

Product category	**Pathogen or toxin**	**Requirement**
Non-thermally processed, commercially sterile products		
Lethality		
All meat products, other than poultry	*Salmonella* spp.	6.5-log reduction or 6.5D process
Poultry products or meat products containing any amount of poultry	*Salmonella* spp.	7D process
All fermented RTE meat and poultry products containing beef	*E. coli* O157:H7 (in addition to *Salmonella* spp. lethality)	5D process
Stabilization		
All meat and poultry products	*C. botulinum*	No multiplication of toxigenic microorganisms or *C. botulinum*
	C. perfringens	< 1.0 log10 multiplication of *C. perfringens*
General requirement		
All RTE meat and poultry products	Other pathogens, toxins, or toxic metabolites	Achieve performance standards for lethality and stabilization throughout product shelf life, under conditions of storage, distribution, and holding

Product category	Pathogen or toxin	Requirement
Postlethality contamination		
All RTE meat and poultry products	*L. monocytogenes*	Either incorporate one or more controls in HACCP plan or test food contact surfaces for *Listeria* spp.
Thermally processed, commercially sterile meat and poultry products		
All meat and poultry products	*C. botulinum* (depending on pH)	Eliminate (12D process) or control *C. botulinum*; product must be packaged in a hermetically sealed container and should be commercially sterile.

Antimicrobial Interventions

The risk of *L. monocytogenes* contamination of thermally processed meat and poultry products subsequent to cooking has prompted the industry to adopt risk mitigation strategies. The populations of *L. monocytogenes* that arise from postprocessing contamination are typically low (most samples of deli meats had a most probable number of ≤0.3/g) (134), and the contribution of products with low populations of *L. monocytogenes* to foodborne illness is low (135). Thus, mitigation strategies can include (i) control of the organism in the environment, (ii) eliminating listeriae in the product subsequent to contamination (in-package treatments), and/or (iii) preventing growth of listeriae using antimicrobial agents or processes. The FSIS has provided three alternatives for RTE meat and poultry processors based on those mitigation strategies.

Most processors of RTE meat and poultry products in the United States apply antimicrobial agents in their product formulations to mitigate growth of *L. monocytogenes* in the product during distribution and storage. Some processors have incorporated lethality treatments as well as antimicrobial agents into their product manufacturing processes. A brief description of each of those processes is provided below.

Physical methods

Methods to reduce *L. monocytogenes* in RTE meat and poultry products include heat treatments, delivered through either steam, hot water sprays, immersion, radiant heat, or high-pressure processing (HPP).

Thermal treatments (pasteurization). Since the *L. monocytogenes* cells from postprocessing contamination are assumed to be on the surface of the product, increasing the surface temperature of the product to achieve adequate lethality would enhance the safety of RTE meat and poultry products. The surface Temperature can be increased by exposing the packaged product to a heating medium, e.g., hot water or steam. In such cases, the product has been chilled, opened, or packaged in vacuum bags and subsequently exposed to the heating medium. The surface characteristics of the product play a significant role in influencing the temperature increase on the product's surface. Also, any purge (liquid expressed from the chilled product) collected at the bottom of the package or between the package (film) and the product surface requires longer heating times, as the temperature of the purge is the same as the product's internal temperature (chilled). Hence, formulating the product to minimize release of the expressible juice (purge) enhances the lethality achieved for a particular exposure time at a specific ambient temperature.

However, an adequate surface temperature treatment on the product will achieve *L. monocytogenes* lethality. In addition to the product surface characteristics, the product configuration can also be a factor. For example, the time required to heat frankfurters that are packaged as a single layer is significantly different from that required for a

double layer, because the cold spot varies with each product configuration, and so the doublelayered product needs to be heated for a longer time.

When thermal treatments are applied, proper microbial challenge studies for each product type or category and in some cases product formulation should be conducted to validate *L. monocytogenes* lethality.

HPP. The use of high pressure to achieve microbial safety and extend shelf life in a variety of food products has been in practice in the food industry for almost a decade. Commercially, pressures of 586 MPa have been used for treatment of RTE meat and poultry products with minimal changes in nutrient content and sensory properties, depending on the fat content. The order of effectiveness (high to low) of HPP is vegetative cells of Gram-negative bacteria, vegetative cells of Gram-positive bacteria, and fungi; viruses and bacterial spores are the most resistant. HPP inactivates microorganisms by cell membrane damage, solute loss, protein denaturation, and enzyme inactivation. Its ability to act on multiple cellular targets allows its use in combination with other treatments for better success through synergism. Heatassisted HPP for the destruction of bacterial spores in a variety of food products is being evaluated. The food is not heated and may not be deformed because HPP is isostatic (i.e., uniform and instant pressure transmission) and adiabatic (i.e., minimal temperature increases within the product; approximately 3°C per 100 MPa).

HPP has been applied to a variety of foods, including meat products such as cooked ham, fish, and precooked meals (136). Its use in RTE meat products, such as ham, for control of postprocessing *L. monocytogenes* contamination has gained industry acceptance as a lethality treatment. It is marketed as a natural alternative to chemical antimicrobials in order to meet regulatory requirements for antimicrobial alternatives applied to products allowing growth of the pathogen before consumption. HPP has also been considered for use as an alternative to mild heat for inactivation of *E. coli* O157:H7 and STEC in fermented sausages (137). Since the association of fermented

sausages with *E. coli* O157:H7 and STEC outbreaks, some countries have established regulations requiring 3-to 5-log-unit reductions of *E. coli* O157:H7 in such products. Alternative treatments to mild heating of dry-fermented sausages to eliminate STEC have been difficult to identify. HPP may have the potential to improve dry-fermented sausage safety without adversely affecting product quality (137).

As with any process, the product characteristics, such as pH, a_w, and processing temperature, influence the amount of microbial reduction achieved by HPP (Table 6.7). Increasing efficacy at lower pH values and higher a_w values in products can be achieved, thus allowing modification of product characteristics to achieve the desired reductions using HPP. Thus, there is a need to validate the microbial destruction achieved for various products, and any changes in product formulations may require evaluation and revalidation of the process to ensure microbiological safety.

While HPP has been accepted and used for ensuring microbiological safety and shelf life of some processed RTE meat and poultry products, it produces detrimental effects on product color and texture when used on fresh meat. HPP denatures myoglobin, thereby affecting its affinity for oxygen and preventing the formation of the cherry red color of freshly processed ground beef.

In the United States, feeding of raw-meat-based diets to pets has increased considerably. The base ingredients for these meat-based diets are either offal or residual meat from bones. Thus, the risk of foodborne pathogen contamination from such ingredients is high, and processors are required to meet the zero tolerance performance standard of RTE prepared products. Most raw pet food processors use HPP to eliminate the foodborne pathogens of principal concern, namely *Salmonella*, *Campylobacter*, *E. coli* O157:H7, and STEC. For such products, meat color is not a major concern as the meat is usually combined with vegetables and further ground and formed.

Chemical methods

Several antimicrobials have been used to mitigate *L. monocytogenes* contamination of RTE meat and poultry products. The efficacy of

these antimicrobials depends on their mechanism of action, their concentration, the amount applied, surface characteristics of the product, temperature, and other factors. Some of the more commonly used antimicrobials that have been evaluated include organic acids (lactic, acetic, or citric), lauric arginate, acidic calcium sulfate, and liquid smoke fractions. A constraint on using oxidizing antimicrobials such as PAA is their detrimental effect on surface color and fat oxidation of the product. Thus, antimicrobial chemicals having minimal effect on product quality characteristics such as color and flavor are used. Furthermore, these treatments, similar to other surface treatments, can reduce only a proportion of the population and do not eliminate listeriae. It is possible that listeriae, even though injured, can recover and grow during subsequent refrigerated storage. Hence, it is prudent to use such postlethality treatments in combination with antimicrobial ingredients that are included in the product formulation to reduce the potential for *L. monocytogenes* growth.

Table 6.7 Comparison of microbial inactivation after HPP processing[a] in spiked meat products with different water activities[b]

	Result in skin vacuum-packed product		
Measurement	Marinated beef	Cooked ham	Dry-cured ham
No. of *S. aureus* (log CFU/g)	>2.67	1.12	0.55
No. of LAB (log CFU/g)	>3.99	4.57	1.58
a_w	0.985	0.978	0.890

[a] Processing at 600 MPa and 31°C for 6 min.

[b] Adapted from reference 185.

Antimicrobials that are routinely used to control or reduce *L. monocytogenes* on RTE meat and poultry products include combinations of organic acid salts—sodium or potassium salts of lactic, acetic, or citric acid. The literature is replete with studies on the

efficacy of organic acid salts or their combinations in RTE meat and poultry product formulations. Although several potential antimicrobials have been evaluated, organic acid salts are extensively used compared to others and are effective. Mbandi and Shelef (138) determined that a combination of sodium lactate and sodium diacetate provides a synergistic effect in inhibiting *L. monocytogenes* growth on the surfaces of RTE meat and poultry products. Numerous subsequent studies have confirmed the synergism, and currently, the industry uses this combination of antimicrobials extensively in RTE meat and poultry products. Other antimicrobials evaluated include antimycotics such as sorbates, benzoates, and propionates (139, 140).

A recent meat industry trend is to produce "all-natural" meat products, which has resulted in processors searching for natural sources of antimicrobials. Ingredient providers have modified the production processes for several antimicrobials with cane sugar and other natural sources for fermentation and production of the lactic and acetic acids, followed by buffering with bases (hydroxides or carbonates) to produce the organic acid salts. In some instances, lemon and lime juice concentrates have been the sources of organic acids (primarily citric) and are buffered using bases to produce "all-natural" ingredients for use in RTE meat and poultry product formulations. Acetates and diacetates are manufactured from vinegar (natural) produced from cane sugar or similar natural sources of easily fermentable carbohydrates. With an increase in consumer health consciousness giving rise to the desire to reduce sodium intake, the industry is formulating products with reduced sodium content. While sodium chloride is a major contributor to the sodium content of RTE meat and poultry products, other ingredients, such as phosphate and organic acid salts, have also been targets for sodium reduction. Hence, these ingredients are being formulated as potassium salts or, to reduce the bitterness of potassiumbased organic acid salts in product formulations, combinations of sodium and potassium salts.

While these antimicrobials are being extensively formulated for inclusion in a variety of RTE meat and poultry products, the inhibition of *L. monocytogenes* observed is dependent on the other product characteristics, such as the pH, water activity, sodium chloride

concentration, and other ingredients in the product. Care should be exercised when the RTE meat and poultry products are being formulated with the antimicrobials through the use of validated computer simulation models, such as the Corbion *Listeria* control model, or through microbial challenge (validation) studies for individual products or products within similar categories with similar formulations and product characteristics.

The additional advantage of formulating organic acid salts for incorporation into RTE meat and poultry products is their ability to control or minimize *C. perfringens* spore germination and outgrowth. Thippareddi et al. (141) determined that adding buffered sodium citrate to roast beef and pork products inhibited *C. perfringens* spore germination and outgrowth during extended chilling times.

Commercially Sterile Products

Commercially sterile products include canned products containing meat or meat products. Such products are subjected to thermal processes to inactivate *C. botulinum* spores (F_0 [sterilization value] = 2.5 min), although more severe thermal treatments (F_0 = 4.0 to 5.0 min) are applied to address potential contaminating sporeformers with greater heat resistance.

Types of Products

Canned meat products include stews, soups, gravies, sauces, chili, pasta-containing products, sloppy joe products, roast beef, and gravy and similar products.

Foodborne Pathogens, Sources, and Contamination

The pathogen of concern in these products is *C. botulinum*. Since these products are usually stored under ambient conditions, and the spores of the proteolytic *C. botulinum* types are more heat resistant than nonproteolytic *C. botulinum* spores, foodborne intoxication is primarily due to survival of the spores of proteolytic types. Foodborne botulinum intoxications from commercially processed canned products have declined substantially since the FSIS implemented

process control measures based on containers and closures used for such products, which included specific thermal processing requirements such as process schedules for specific products and containers, application of critical factors in the process schedule, and specifications for equipment and procedures for heat processing systems.

Fermented and Dried, Salt-Cured, and Dried Meat Products

A variety of RTE meat products, such as acidulated or fermented and dried salami (dried or semidried), saltcured meat products, and dried meat products, make up this category. The a_w of these products is usually low based on the category of the product, and the pH may be low (≤ 5.0), depending on the product.

Types of Products

Meat products in this group are produced by a variety of manufacturing processes, including acidulation (summer sausages), fermentation and drying to varying degrees of pH and a_w (e.g., salami, pepperoni, Thuringer, and chorizo), salt curing (capicola, coppa, country ham, and prosciutto), and drying (basturma, beef sticks, and jerky). The microbiological profiles of these products vary depending on the product and the process or processes used.

Processing Effects

Several of the products in this category are prepared and consumed by methods developed in ancient times. These processes yield products that have extended shelf life and have reduced microbiological food safety risk. The fermentation processes once relied on back-slopping, a traditional method in which fermented meat from a previous batch was used as a starter culture to decrease the pH of the fermenting meat, and on drying processes that involved slow drying of products subsequent to fermentation (for fermented salami) or heat treatments (for dried products). Salt curing of meats has resulted in controlled

dehydration of the product to an a_w below 0.85 to achieve microbial stability under ambient storage conditions.

Foodborne Pathogens

These products historically have been microbiologically safe, with the exception of growth of *S. aureus* and production of toxin, which can occur when the fermentation rate does not progress steadily to achieve a pH of ≤ 5.0. Foodborne illnesses from *E. coli* O157:H7 in fermented dry salami in the United States have resulted in the industry and the FSIS recognizing the acid tolerance of this microorganism and the need for its control in fermented products that contain beef. Currently, the FSIS specifies a performance standard for achieving at least a 5.0-log reduction of *E. coli* O157:H7 in fermented dried products (e.g., salami), and a 6.5-log reduction of *Salmonella* spp. (142).

To address this, the degree-hour concept has been adopted in the United States using a combination of time and temperature to achieve a product pH of 5.3 during fermentation. The degree-hours of a process are calculated as the number of hours at the process (fermentation chamber) temperature multiplied by the degrees above 60°F.

A process is acceptable when it controls the growth of *E. coli* O157:H7 and staphylococci in the product and consistently reaches a pH of 5.3 or less using the following criteria.

1. Fewer than 1,200 degree-hours when the highest chamber temperature is less than 90°F.

2. Fewer than 1,000 degree-hours when the chamber temperature is between 90 and 100°F.

3. Fewer than 900 degree-hours when the chamber temperature is greater than 100°F.

Table 6.8 Time/temperature relationships for constant temperature processes

Degree-hours above 60°F	Chamber temp (°F)	Maximum hours to pH 5.3
1,200	75	80
1,200	80	60
1,200	85	48
1,000	90	33
1,000	95	28
1,000	100	25
900	105	20
900	110	18

For constant-temperature processes, Table 6.8 provides guidance on the maximum numbers of hours at specific chamber (fermentation) temperatures.

Subsequent to the *E. coli* O157:H7 outbreak associated with consumption of fermented salami, the National Cattlemens' Beef Association assembled a blue-ribbon task force and initiated research to develop validated control methods for this microbe in fermented, dried meat products containing beef (143). The report outlined the following five options.

1. Utilize a heat process as listed in federal document 9 CFR 318.17 (145°F for 4 min). The processor must provide documentation of the heat process.

2. Include a validated 5D inactivation treatment (i.e., treatment that reduces the population by 5 log cycles at a given temperature). A processor using a 5D inactivation treatment will have met requirements for validation.

3. Conduct a "hold and test" program for finished product. Testing finished product as the only means of ensuring safety is contrary to the philosophy of HACCP; this option involves finished product

testing, requires no knowledge of raw ingredients or the process, and is expensive.

4. Propose other approaches to assure there is at least a 5D inactivation. A processor could propose any combination that will demonstrate a collective 5D control but must provide precise documentation.

5. Initiate a HACCP system that includes raw-batter testing and a 2D inactivation. A combination of raw-material testing, processing through fermentation, thermal processing and drying, and finished-product testing can be used to demonstrate the adequacy of a given process to ensure the safety of dry and semidry fermented sausage products. The processor could include a HACCP plan combined with GMPs for fermented sausage; the option combines raw-batter testing and documen tation of at least a 2D lethality for *E. coli* O157:H7 between stuffing and shipping. This is a very practical means of ensuring the safety of certain dry fermented sausage products.

With regard to option 5, there are several key points that must be considered.

- The analytical method must be equivalent to the FSIS method in raw-batter testing.
- The sample size and compositing procedure must ensure a detection level of 1 CFU/g. It is recommended that 15 25-g samples be taken across each lot; these 15 samples can then be composited into five 75-g analytical samples.
- The definition of a "lot" must be scientifically statistically sound.
- GMPs must be applied.
- Processors must have data to document the 2D destruction between stuffing and shipping.
- As in the case of options 1 through 4, option 5 must address *Salmonella*, *Trichinella*, and *Staphylococcus*. The FSIS expanded the *S. aureus* monitoring program to include *E. coli* O157:H7.

- The lots of batter which test positive must be subjected to conditions that provide a total 5D process; procedures for dealing with positive lots must be defined in the HACCP plan.

Since fermented, dried, and semidried sausages containing any amount of beef in the United States are subjected to a mild thermal process to achieve lethality requirements for *E. coli* O157:H7, the flavor and aroma characteristics and the tangy taste are different from those of their traditional European counterparts. Research on alternate methods is needed.

Dried Meat Products

Numerous dry or dried foods with low aw have been implicated in foodborne-illness outbreaks, especially with *Salmonella* and, in some cases, STEC. Among the meat and poultry products, a good example of a dried product involved in such outbreaks is jerky, implicated in *Salmonella* Montevideo and *Salmonella* Kiambu outbreaks in New Mexico in 1995 and 2003, respectively (14, 144). The cause of survival of the microorganism was reported to be the drying process under low-humidity conditions (1% relative humidity; 82°C dry bulb, 30°C wet bulb) prior to achieving lethality at higher relative humidity (product surface moisture) conditions.

The traditional manufacturing process (commercial) of jerky consists of preparation of the meat strips, marination, application of antimicrobial interventions, surface preparation, lethality, drying, post drying heating, and handling. Of these, antimicrobial intervention and the lethality step are designed to reduce the microbial load and achieve adequate lethality to assure microbiological safety of these products. The antimicrobial intervention step includes immersion of sliced strips of meat in marinades (typically containing acids and pH-reducing agents and spices) or antimicrobial solutions (acidic calcium sulfate, acidified sodium chlorite, etc.) at appropriate temperatures and/or periods of time. The lethality step is designed to increase product temperature at high humidity for adequate time to achieve destruction of microorganisms of human health significance such as *Salmonella*, *E. coli* O157:H7, and others. The FSIS (142) specifies that

product time-temperature and relative humidity of the environment are critical operational parameters for the process to achieve at least a 5.0-log reduction of *Salmonella* spp. and STEC in products containing beef. In addition, the lethality treatment of meat and poultry jerky should achieve at least a 3.0-log reduction of *L. monocytogenes*.

While jerky is a traditional product and consumed worldwide, other ethnic foods (dried meats), such as biltong, charqui, pemmican, pastrami, tasajo, nikku, and sou gan, are gaining popularity (145). Many of these products have been prepared and consumed with minimal food safety issues over the years, although the microbiological safety of the processes has not been scientifically investigated and reported. Burfoot et al. (145) categorized the reported outbreaks attributed to these dried meat and poultry products as those caused by (i) *Salmonella* and *E. coli* O157:H7, (ii) *Trichinella*, (iii) *S. aureus*, and (iv) *C. botulinum*. While the foodborne illness outbreaks are a result of survival of the microorganisms due to inadequate lethality of the process, spoilage of these products is mostly from molds due to postlethality contamination.

Antimicrobial Interventions

As mentioned above, fermented dry or semidry products in the United States undergo a mild thermal treatment, resulting in an altered flavor profile, combined with a lower target pH for fermentation, which results in a tangy taste. Recent research using HPP has revealed that fermented products that are HPP treated either before or after drying can meet the criteria for reduction of *E. coli* O157:H7 populations if the HPP reduction is combined with the reduction that occurs during fermentation and drying. Porto-Fett et al. (146) reported that HPP treatment of Genoa salami (pH 4.58; a_w, 0.89 or 0.92) at 600 MPa or 483 MPa for 1 to 12 min reduced pathogen populations by an additional (in addition to those reductions achieved by fermentation and drying to target pH and a_w values) 1.6 to ≥ 5.0 (*L. monocytogenes*), 4.7 to ≥ 5.8 (*E. coli* O157:H7), and 1.9 to 2.4 (*Salmonella*) log CFU/g. After storage for 28 days at 4°C, *L. monocytogenes* levels decreased by up to an additional 3.0 log CFU/g,

whereas additional decreases of up to about 1.1 and 1.7 log CFU/g was observed for *E. coli* O157:H7 and *Salmonella*, respectively.

The HPP process can be used by manufacturers of fermented dry and semidry sausage in the United States to achieve microbiological safety of these products with minimal loss in product flavor and quality characteristics.

DETECTION AND ANALYSIS OF SPOILAGE, PATHOGENIC, AND RESIDENT MICROBES IN MEAT AND POULTRY

Meat and poultry products possess chemical and physical properties that allow the colonization and development of a variety of microbes, including bacteria and fungi (147, 148). Microbial growth can cause spoilage or even adulteration, making products no longer appealing and unsuitable for human consumption. While initial microbial loads significantly impact the products' microbial quality and safety, storage conditions as well as competition and interactions between resident microbes and spoilage and pathogenic microbes also contribute significantly to product quality and safety. Organization and reorganization of microbial communities during processing and storage directly determine the persistence of foodborne pathogens and spoilage microorganisms (149, 150). Therefore, it is necessary to quantitate and characterize the resident microbes in addition to the spoilage and pathogenic microbes present in meat and poultry products.

Techniques and technologies used for studying microbial populations have evolved tremendously over the past few decades. As a result, understanding of the composition of resident microbes present in live animals (fecal samples) and the processing environment, as well as in finished meat and poultry products, has improved dramatically. Cultivable bacteria often represent only a small fraction of the entire microbial community, resulting in frequent underestimation of the microbial diversity present in a given environment or sample when traditional plating methods are used (151, 152). It is also well accepted

that many foodborne pathogens, such as *E. coli* O157:H7 and *Salmonella* spp., may enter a viablebut-noncultivable state under certain treatment or storage conditions, making cell counts obtained based on plating methods questionable (153, 154). The ability of these microbes to remain undetectable and unculturable reveals the need for new technologies that can overcome the limitations associated with conventional plating. Next-generation sequencing, such as 16S rRNA sequencing and shotgun meta-genomics sequencing, allows analysis of microbial populations via culture-independent methods. Such culture-independent methods provide high-resolution identification of microorganisms without the limitations or biases associated with the use of selective and/or non-selective culture media.

Analyzing a microbial community using sequencing technologies provides taxonomic information about the microbial populations in the community. In addition, several indices can be used to evaluate and compare the microbial communities. Examples of these indices include Chao1 and abundance-based coverage estimators for the evaluation of species richness and Shannon's index and Simpson's index for the calculation of species diversity (5). Species richness refers to the number of different species in a given ecological system, whereas species diversity takes into consideration both species richness and species evenness (155, 156). In recent years, the terms "alpha diversity" and "beta diversity" also have been used to describe differences between microbial communities. Alpha diversity refers to the diversity within a sample; it reflects species diversity or the richness of the microbial community. Five main indices are used to describe alpha diversity. They are Chao1 (157), phylogenetic diversity, whole tree (158), observed operational taxonomic units (OTUs) (159), and Shannon (160). Beta diversity refers to the diversity between samples; it is the ratio or the difference of species diversity between samples. Beta diversity can be assessed by using distance matrices, including Bray-Curtis distance (161), Euclidean distance (162), unweighted UniFrac, and weighted UniFrac (163).

Here, the discussion about the detection and analysis of spoilage, pathogenic, and resident microbes in meat and poultry products starts

with a brief overview of the microbial populations present in the GI tracts of beef cattle and poultry. The composition of microbial populations and the prevalence and concentration of spoilage and pathogenic microbes present in meat and poultry products are largely determined by the microbial populations present in live animals, e.g., animals' GI tracts, hides, lymph nodes, and other organs (164). Next, the microbial populations present in the processing environment (e.g., air, floors, walls, processing equipment and containers, and hands of handlers), as well as the changes in micro-biomes during various storage conditions, are reviewed. Finally, methods used to detect spoilage and pathogenic microbes are discussed.

Microbiome of the GI Tracts of Beef Cattle and Poultry

Beef Cattle

Diverse factors, including diet, location, and age, determine the microbial populations present in live animals (165). The microbial communities present in the GI tracts of beef cattle can impact both the live animals' health and the quality and safety of products from the animals. Evaluating microbial populations present in beef cattle feces has been a direct method for determining the characteristics of the microbiome present in an animal's GI tract. These fecal samples contain complex bacterial communities. Shanks et al. (166) collected 30 rectal grab fecal samples from six cattle populations residing in four geographic locations (Oconee County, GA; Larimer County, CO; Hamilton County, OH; and Clay County, NE). These cattle were on three different types of diets, including a forage diet (more than 80% of feed consisted of plants such as alfalfa, corn silage, and/or fescue, wheat, and rye grasses), a processed-grain diet (at least 75% of the feed was steam-flaked, dry-rolled, and/or distiller's grain corn), and an unprocessed grain diet (containing more than 75% whole-kernel corn). The analyses of 633,877 high-quality bacterial V6 pyrotags revealed four main bacterial phyla from these fecal samples, including *Firmicutes* (55.2% relative abundance), *Bacteroidetes* (25.4%), *Tenericutes* (2.9%), and *Proteobacteria* (2.5%). These top phyla were followed by *Actinobacteria* (0.73%), *Spirochaetes* (0.54%),

Verrucomicrobia (0.19%), *Cyanobacteria* (0.15%), *Fibrobacteres* (0.02%), and *Lentisphaerae* (0.02%). The relative abundance of the different bacterial phyla correlated differently to the three types of diets. For example, while the abundances of *Bacteroidetes* exhibited a clear difference between the three diets, *Proteobacteria* abundances were not different. Similarly, different abundances were observed at the family and genus levels. For example, abundances of bacterial species belonging to the family *Ruminococcaceae* and the genus *Prevotella* were dramatically different from one diet to another (166).

The impact of diets on the GI tract microbiome was also evaluated by Rice et al. (167), who collected fecal grab samples from 20 steers. These steers were on five different diets, consisting of a traditional diet fed to finishing beef cattle in the southern high plains (steamflaked corn control with no distilled grain [DG]) and four other diets with different concentrations of cornbased DG or sorghum-based DG. The sequencing analysis generated a total of 127,530 operational OTUs, and 24 phyla were identified. The top six phyla were *Firmicutes* (61%), *Bacteroidetes* (28%), *Proteobacteria* (3%), *Tenericutes* (0.15%), *Nitrospirae* (0.11%), and *Fusobacteria* (0.086%) (167). Different phyla responded in different ways. It was found that steers fed diets containing 10% or more of a DG supplement had a higher relative abundance of *Firmicutes* than steers fed the no-DG diet. At the genus level, a total of 973 bacterial species distributed among 446 genera were identified. The DG diet significantly impacted the relative abundance of the top 60 most abundant genera (covering more than 97.5% of total genera observed; $P < 0.05$). Diets significantly ($P < 0.01$) influenced the average abundance of *Clostridium, Ruminococcus, Oscillibacter, Tannerella, Parabacteroides, Hydrogenoanaerobacterium, Pseudoflavonifractor, Acetivibrio, Ethanoligenens, Selenomonas, Desulfonispora,* and *Barnesiella* (167). Both of these reports, together with other reports (5, 168, 169), reveal that diet is a significant factor that impacts the relative abundance of different phyla, genera, and species identified from the GI tracts of cattle.

In addition to diet, the age of cattle is another important factor impacting the composition of microflora in cattle's GI tract. Zhao et al.

(170) collected fecal samples from 110 calves and 92 dams and determined that fecal bacterial diversity increased as cattle age increased. Such changes in the microflora also impact the shedding of pathogenic *E. coli*. It was found in the same study that the dams' STEC shedding levels were lower than the shedding levels of calves (170). Handling and management practices are two other factors that can impact the shedding prevalence of *E. coli* O157:H7 (171). In a survey study conducted in Washington State, *E. coli* O157:H7 was isolated from 10 of 1,412 fecal samples collected from pastured beef cattle, whereas the pathogen was isolated from only 2 of 600 fecal samples collected from feedlot beef cattle (171). The shedding of *E. coli* O157:H7 also has a seasonal pattern, with prevalence peaking during summer months and declining in winter months (172). Elevated temperatures in the summer provide favorable conditions for the bacteria to survive and even multiply to increase their abundance in animals as well as in the environment (173–175).

Weaning is a critical step in the preharvest continuum of beef cattle production (176). During the weaning process, the stresses on young calves caused by physical separation from dams as well as a change in diet can lead to depressed feed intake and immunity as well as loss of body weight (176, 177). As a result, the weaning step provides an increased opportunity for pathogen colonization and shedding (103, 169). In a study conducted by Zhao et al. (169), 60 calves (28 heifers and 32 steers) were weaned and randomly assigned to two groups for different weaning diets. One diet consisted of peanut and soy hull-based dry feed, and the other was a corn silage-based high-moisture diet. Fecal samples were collected from these calves before weaning, 14 days after weaning, and 56 days after weaning. The shedding prevalence of *E. coli* O157:H7 was evaluated by plating fecal samples onto CHROMagar O157 and confirmed by PCR. Significant loss of body weight was observed before and after weaning, regardless of diet, and the shedding prevalence of *E. coli* O157:H7 increased after weaning. Weaning significantly changed the microbial communities present in the fecal samples ($P < 0.05$), although no difference was observed between the two weaning diets (169).

Poultry

Although chickens have smaller intestines than mammals, which result in shorter digestion times, a chicken's digestive system does not appear any less complex (178, 179). Among the different GI tract sections, a chicken's cecum is the most heavily populated with microorganisms. These cecal microbes, together with the microbiome in other parts of the GI tract, are heavily involved in digestion and absorption of nutrients and in energy metabolism (180). Unfortunately, it is also known that this microbiota can be a source of human infections and a reservoir for antibiotic resistance determinants (181). The impact of the intestinal microbiota on the shedding of pathogens, such as *Salmonella* and *Campylobacter* spp., has been investigated by many studies. Since modern hatchery management prevents intimate contact between hatchlings and hens, inoculation of new hatchlings with appropriate microbial species is necessary for the development of a protective intestinal microbiome (182, 183). The formation of the competitive exclusion concept and the development of a series of competitive exclusion products are the result of more than 40 years of research related to the poultry intestinal microbiota and its impact on animal health and pathogen shedding (184). The competitive exclusion concept was originally designed for reducing the prevalence and shedding levels of *Salmonella* in growing chickens; it has now been expanded to the control of other enteropathogens, such as *C. jejuni* and *C. perfringens* (186), as well as to improving feed digestibility and chicken performance (187, 188).

Next-generation DNA sequencing has provided new opportunities to reinvestigate the GI tract microbiome and reexamine some long-standing observations about performance differences associated with animal husbandry practices and environmental factors. It was reported that up to 11 log CFU/g of microbes comprising hundreds of species can be identified from chickens' intestinal content (189, 190). Recent sequencing results revealed that the top phyla present in the cecal microbiome are *Bacteroidetes* (29.7 to 63.5% of the total bacterial population) and *Firmicutes* (30.2 to 59.8% of the total

bacterial population) (191). This observation matches well with previous reports obtained via culture-based methods (192, 193).

Several factors, such as weather, diet, breed and genetics, age, and location, can influence the composition of the GI tract microbiome of poultry. In a recent study, 87 commercial broiler chickens were sampled for the evaluation of the cecal microbiome and its impact on *Campylobacter* shedding (194). DNA was extracted from the cecal contents, and the microbiome was analyzed using 16S rRNA sequencing. The analyses revealed that the taxonomic composition of the microbiome did not have a significant relationship to the *Campylobacter* shedding status (positive or negative) but did correspond significantly to the season ($P = 0.000$). The relative abundance of several genera changed significantly among different seasons. For example, the OTUs that were classified as *Lachnospiraceae* were less abundant in the fall than in other seasons (194). This seasonal variation in the GI tract microbiome might be caused by temperature changes, as the different taxa have different optimal growth temperatures. In addition, host stresses and immune responses may change as the seasonal environment changes, which in turn impacts the microbial community composition.

The impact of the age of chickens on the GI tract microbiome was studied by Awad et al. (195). In this study, 45 1-day-old broiler chickens were obtained from a commercial hatchery. Five were sacrificed immediately to collect the microbiota for sequencing, while the rest were sampled on days 7 and 14. On day 14, 15 birds were infected with *C. jejuni*, and both the infected and uninfected chickens were analyzed at 7 and 14 days postinfection. Of the 8,285 OTUs identified, *Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria*, and *Tenericutes* were the main phyla identified, with *Proteobacteria* and *Firmicutes* being the most abundant phyla. The factor of age had a significant impact on the composition of the microbiome. Compared to the older birds (which were >14 days old), the young birds had more *Proteobacteria* and *Firmicutes*. In contrast, the older birds had a higher abundance of *Tenericutes*. The 28-dayold birds had more diverse bacterial communities than younger birds. Infection by *C.*

jejuni altered the GI tract microbiome, with the infected birds having significantly less *E. coli* (195).

Cecal microorganisms are involved in detoxifying harmful substances, recycling nitrogen, digesting resistant carbohydrates, and absorbing additional nutrients (196). Thus, the composition of the cecal microbiome can also be altered by diet and dietary supplementation (197–200). Corrigan et al. (198) used 16S rRNA sequencing to evaluate the impact of mannan oligosaccharide (MOS) on both culturable and unculturable bacteria.

Firmicutes, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, and *Tenericutes* were the top five phyla in the groups treated with MOS, representing more than 99% of allsequences identified. Similar dominant phyla, including *Firmicutes*, *Bacteroidetes*, and *Proteobacteria*, were reported in the control group (regular corn-soy diet). MOS supplementation altered the bacterial community 7 days after the treatment, and *Bacteroidetes* replaced *Firmicutes* to become the dominant phylum as a result of supplementation (198).

As discussed above, a complex population of microbes is present in the ecosystem of an animal's GI tract (in both cattle and poultry). These microbes not only play significant roles in animal nutrition, animal welfare, and performance but also impact animal products' safety by altering the harboring and shedding of human pathogens. Traditional culturing methods have limitations in analyzing these microbes, because only 1% of all bacteria are culturable (201). New sequencing technologies provide researchers with opportunities to better evaluate the microbial populations present in the GI tracts of animals. These methods, such as comparative metagenomics, enable the comparison of microbial populations obtained from different ecosystems and help with the identification of factors that impact the diversity and abundance of GI tract microbiome and the shedding of pathogens. Another advanced tool for studying microbiomes is functional metagenomics, which brings the study of GI tract microbes into a new era. Information about the functions of identified genes in microbes aids in the identification of new prebiotics, probiotics, and metabolism

for protecting animal health, controlling human pathogens, and improving animal products' quality and safety.

Background Microbes Present in Meat and Poultry Products

Meat

After processing, more than 80% of the total microbial population of fresh ground beef, or 75 to 85% of the microbial population present on chicken parts, consists of chromogenic bacteria, molds, yeasts, and spore-forming microorganisms (202). However, at the time of spoilage (when food has an off odor and/or sliminess), the primary microorganisms are bacteria. Thus, bacterial counts have traditionally been used to evaluate meat spoilage. A microbial load of >7 log CFU/cm^2 is usually associated with the occurrence of off odors. When bacterial counts are as high as 9 log CFU/cm^2, a "fruity" off odor and sliminess may develop due to the metabolism of free amino acids (202). Unfortunately, plating methods are not adequate to provide an understanding of the actual shifts in the microbial populations of raw meat in response to different storage conditions.

To better understand the changes in beef's background microbiome, Olofsson et al. (151) used two different methods to evaluate the composition of the microbiome of beef stored at 4°C for 8 days. One method used sequencing of DNA samples extracted directly from the meat juice to analyze the microbial population, whereas the other method relied on plating to obtain initial bacterial isolates, which were then used for DNA extraction and sequencing. At the end of storage, viable plate counts for the beef samples increased from ~2 log CFU/cm^2 to 7 log CFU/cm^2. Significant differences in microbial composition were observed between the two analysis methods. While direct 16S rRNA sequencing analysis revealed dominant *Bacillus*-like sequences in fresh meat, no such sequences were found in isolates obtained from the plates. Instead, *Chryseobacterium* was the dominant bacterial genus identified by the plating method. The two methods were in agreement when used to analyze the 8-day-old beef

samples. Both methods revealed that *Pseudomonas* was the dominant spoilage microbe present in the beef samples (151). The results revealed that the plating and sequencing methods matched each other better when a dominant bacterial species is present in the samples. As indicated by Nychas and Skandamis (203), spoilage is usually caused by a fraction of microbial species out of the initial entire microbial population; for the 8-day-old beef samples analyzed by Olofsson et al. (151), *Pseudomonas* was the dominant species.

The composition of microbial populations present in meat samples is determined by several factors, including the type of meat or animal, the physiological status of the animal at slaughter, harvesting procedures, the harvesting and handling equipment and harvesting environment, packaging and storage conditions, and storage time. Based on the literature, members of *Enterobacteriaceae*, LAB, *Pseudomonas*, and *Brochothrix* are principal players in meat decay, although their dominance and prevalence can change depending on packaging or storage conditions (28, 147).

The use of molecular methods enabled the discovery of new microbes that are of significant importance to meat safety and quality. Audenaert et al. (204) evaluated the LAB populations present in cooked ham, turkey, and chicken products that were in MAP. These products were sampled and analyzed on their sell-by dates, and DNA samples were extracted for PCR-denaturing gradient gel electrophoresis analysis. The results of this analysis revealed that the same core microbiome was present in the cooked ham, turkey, and chicken products, with *Leuconostoc carnosum* being the most abundant psychrotrophic species. Ercolini et al. (205) evaluated the microbiome present on beefsteaks when the steaks were stored using three types of packaging systems. The packaging systems were air, high-O_2 MAP, and lower-O_2MAP. *Rahnella aquatilis*, *Rahnella* spp., *Pseudomonas* spp., and *C. divergens* were more abundant in samples stored in air, whereas *Pseudomonas* spp. and *L. sakei* were in greater abundance when samples were stored in the high-O_2 MAP, i.e., with 60% $O_2$and 40% CO_2. When the oxygen concentration was reduced to 20%, *Rahnella* spp. and *L. sakei* were more abundant. All of these samples were refrigerated at 5°C throughout the study.

The use of culture-independent high-throughput sequencing also enables researchers to explore potential sources of contamination by spoilage bacteria. De Filippis et al. (206) collected swab samples and meat samples from different locations on beef carcasses (brisket, chuck, and thick flank) 12 hours before slaughtering and after washing but before chilling. Swab samples were also obtained from the meat processing environment and from the hands of operators, slicing knives, bench surfaces, and walls of the cold storage room. DNA was extracted and used for sequencing analyses. Results revealed that the carcass swabs had a very high degree of microbial diversity, and different microbial compositions were observed from the three sampling locations on the beef carcasses. *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, and *Actinobacteria* were the most abundant phyla present on both fresh beefsteaks and carcass swabs. *Moraxellaceae*, *Aerococcaceae*, *Staphylococcaceae*, *Flavobacteriaceae*, *Rhodobacteraceae*, and *Corynebacteriaceae* were the most abundant bacterial families identified from the carcass swabs. Analysis of the environmental swabs and the beefsteaks revealed that although both had highly complex microbial diversity before cold storage, only a few organisms, including *Pseudomonas* sp., *Psychrobacter* sp., and *B. thermosphacta*, dominated after 1 week of aerobic storage at 4°C. Microbes present in the environmental swabs included allof the species found in spoiled beef, indicating that there was microbial adaptation to the processing and storage environments. Skin-associated bacteria identified from the handlers, such as *Propionibacterium acnes*, *Staphylococcus equorum*, and *Staphylococcus* sp., were detected in the beefsteaks only at time zero; they were outcompeted by the dominant spoilage microbiota after storage. By combining observations of the results from beefsteaks and samples collected from the environment and the handlers, the authors concluded that spoilage-associated bacteria originated from the carcasses and were carried to the butchering environment, where they became part of the resident microbiota. These microbes could then be spread to meat products. Microbes that could tolerate cold storage temperatures and effectively metabolize available nutrients were the ones that caused beef spoilage by the end of storage (206).

Stellato et al. (207) conducted a similar study, sampling and analyzing results from 20 butcheries, including 10 small-scale retail distribution facilities and 10 large-scale retail distribution facilities. Results revealed that although the microbial populations present in the environment were complex, there were no significant differences between different butchery types (small scale versus large scale). These results matched those of two previous studies (208, 209). The complexity of the meat microbiome decreased sharply after storage because of the impact of storage temperatures and the type of packaging. Chopping boards had greater microbial diversity and overall microbial counts than knives, suggesting that meat surface contamination is strongly influenced by the surface materials used for chopping boards. The comparison between the meat microbiome and the environmental microbiome indicated a strong correlation between these two microbiomes. *Pseudomonas* spp., *Brochothrix* spp., *Psychrobacter* spp., *Streptococcus* spp., and *Acinetobacter* spp. were identified from both the meat samples and the environmental samples, suggesting that these species were part of the resident microbiome present in the processing facility. These species were identified in previous reports as well and are recognized as undesirable bacteria in the food processing environment (147, 210, 211).

Different processing practices used for different production lots are another factor that can change microbial populations. For example, some meat may be processed directly from fresh cuts (stored for less than 2 days in cold rooms), whereas some lots may be processed from carcasses that have been stored for 3 to 4 days in the cold room. A study conducted by Säde et al. (212) evaluated the potential impact of different lot practices on the microbial populations present in boneless beef shoulder steaks. They collected steak samples from six product lots during a 3-week period and analyzed these samples 2 days after packaging, on the processor-defined use-by date (8 to 10 days of 6°C storage), and 2 days after the use-by date (10 to 12 days of 6°C storage). All samples were packaged in a high-O_2 MAP system. Results revealed that, although early samples had distinct bacterial communities between different lots, after 8 to 12 days of storage at

6°C, the communities were similar and composed of common meat spoilage bacteria, such as *Carnobacterium* spp., *Brochothrix* spp., *Leuconostoc* spp., and *Lactococcus* spp. (212).

Carcass treatment is another processing practice that can differ from one facility to another. In a study conducted by Botta et al. (213), beefsteaks were treated with aqueous ozone (AO) or electrolyzed water (EW) before they were vacuum packaged and stored at 4°C for 15 days. Results from sequencing analysis revealed that *Pseudomonas fragi* was the most frequently isolated species before and after treatment with AO and EW, as well as in the untreated control. As storage time increased, *L. sakei*, *Leuconostoc gasicomitatum*, and *Lactococcus piscium* became dominant in meat samples. The different carcass treatments (EW versus AO) used in this study did not modify the microbial populations present in beef samples.

As discussed above, storage (both storage conditions and storage duration) has a significant influence on the final microbial populations present in meat. Although initial microbial populations present in freshly processed meat can be significantly different, the microbial composition stabilizes towards the end of the storage. Depending on storage conditions and time, one bacterial species becomes dominant at the end. Culture-independent sequencing methods can provide new insight about the microbiome present in meat, abattoirs, and slaughtering facilities, and the microbiome changes during storage. This information is important for the development of new intervention methods to control microbial populations present in meat products.

Poultry

The microbiome present on poultry carcasses is a reflection of the poultry species (turkey versus chicken), the cut (pieces or whole carcasses), the slaughtering and processing environment, and storage and packaging conditions. Total APCs of bacteria sampled from different parts of poultry carcasses vary significantly. Dawson et al. (214) determined that the total APC of skin-on chicken breast meat was approximately 2.5 log CFU/ml (meat rinse), whereas APCs of

skinless products were ca. ~3.5 log CFU/ml. Handley et al. (215) determined that whole chicken carcasses had total APCs of ca. 4.63, 3.21, and 0.89 log CFU/ml when sampled at the rehanging, prechilling, and postchilling stages, respectively. Their *Enterobacteriaceae* counts were approximately 2.99. 1.95, and 0.35 log CFU/ml at the three sampling points. James et al. (216) determined that postchilling carcasses had *Enterobacteriaceae* counts and APCs of ca. 2.51 log CFU/ml and 1.75 log CFU/ml, respectively. The different designs of the processing lines and the use of different antimicrobials and hurdle techniques are reasons for the different APCs and *Enterobacteriaceae* counts reported in these studies.

Dominant microbial species isolated from poultry products are similar to those identified in beef products, including *Acinetobacter*, *Corynebacterium*, *Micrococcus*, *Pseudomonas*, and *Psychrobacter*. The application of next-generation sequencing has provided new opportunities to analyze the microbiome present in different poultry products and enables detailed comparison between different chicken carcass treatments. In a study by Kim et al. (217), 80 chickens were randomly chosen and processed to carcass following the steps of stunner, bleeding tunnel, scalder, depilation, hock cutter, evisceration, and chiller. Two antimicrobial treatments, i.e., PAA and Amplon (a blend of sulfuric acid and sodium sulfate), were applied to the carcasses. Chicken carcass rinsates were collected for microbial and sequencing analyses. Based on the sequencing analysis, the top five phyla identified from the chicken carcasses were *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, and *Cyanobacteria*. In addition, the abundance of proteobacteria decreased as processing progressed. *Bacillales*, *Lactobacillus*, *Ruminococcaceae*, *Clostridiales*, *Lentibacillus*, *Rikenellaceae*, *Pseudomonas*, *Gallibacterium*, *Veillonella*, and *Clostridiaceae* were the top 10 groups identified at the different stages of poultry processing (217). In addition to the general microbiome analysis, all chicken rinsates were plated to determine the presence of *Campylobacter* and *Salmonella*. The level of *Campylobacter* present in the chicken carcass rinsate was ca. 3.75 log CFU/chicken (n = 80). In contrast, *Salmonella* was detected only by qualitative analysis, which included an enrichment step. When the

microbiome present in the *Campylobacter*-or *Salmonella*-positive samples was compared to that in the negative samples, no significant differences in bacterial abundance at the phylum level were observed between *Salmonella*-positive and *Salmonella*-negative samples, although there was a greater abundance of *Actinobacteria* (3.74%) in the *Campylobacter*-positive rinsates than in the *Campylobacter*-negative samples (1.70%). At the genus level, *Salmonella*-positive samples had a lower abundance of *Clostridium* than *Salmonella*-negative samples, whereas a greater abundance of *Clostridium* (8.41%) was observed in *Campylobacter*-negative samples than in positive samples (4.49%) (217).

Mapping out the indigenous microbial communities and their dynamic changes during poultry processing and product storage is the foundation for identifying new indicator bacteria to evaluate product quality and safety and to provide a basis for designing new antimicrobial carcass treatments. Handley et al. (215) evaluated the microbial profiles present on commercial broilers after evisceration and immersion chilling. Upon analysis of the microbial changes, *Pseudomonas*, *Enterobacteriaceae*, and *Chryseobacterium* were identified as potential indicator microorganisms for evaluating the efficacy of different processing steps. Previous studies have revealed that different antimicrobial treatments, such as dips, rinses, sprays, and chill tanks, have different optimal modes of application, and different antimicrobials have their own target microorganisms. For example, it was found that citric acid was more effective against Grampositive bacteria than Gram-negative bacteria (218–220). As discussed above, next-generation sequencing and the information obtained about the background microbes associated with different antimicrobial treatments will ultimately help optimize microbial food quality and safety control.

Fungi Associated with Meat and Poultry Products

Fungi are ubiquitous in their distribution. Air, water, walls, and floors of abattoirs are considered the main sources of fungi that contaminate meat and poultry products (148, 221, 222). A culture-based study revealed that airborne fungi in various animal confinement buildings

ranged from 3 to 6 log CFU/m^3 (223–226). *Penicillium*, *Cladosporium*, and *Aspergillus* were most frequently isolated from the air as well as from the floors of slaughtering facilities (221, 222, 225). In a study conducted by Ismail et al., a total of 34 fungal genera, representing 62 species and one variety, were identified from beef carcasses and the surrounding environment. *Aspergillus*, *Cladosporium*, and *Penicillium* were the top three genera identified from water and air (80 to 90% prevalence in air and 65 to 100% prevalence in water), whereas *Nectria haematococca* was the top species identified from floor and wall samples, and *Aspergillus niger* was the top species identified from beef carcasses (148). Jay et al. (164) provided a comprehensive list of fungi identified from fresh and refrigerated meats and poultry. According to Jay et al. (164), *Cladosporium*, *Geotrichum*, *Mucor*, *Rhizopus*, *Sporotrichum*, and *Thamnidium* are the top genera of mold identified from fresh and refrigerated meats, with *Candida* and *Torulopsis* being the top yeast genera identified. For poultry products, the mold genera *Alternaria*, *Aspergillus*, *Cladosporium*, *Geotrichum*, *Mucor*, *Penicillium*, and *Rhizopus* and the yeast genera *Candida*, *Cryptococcus*, *Debaryomyces*, *Pichia*, *Rhodotorula*, *Saccharomyces*, *Torulopsis*, *Trichosporon*, and *Yarrowia* are the top fungi associated with poultry.

In addition to fresh meats and poultry, the contribution of fungal species to processed meats has also been investigated. Some of them contribute significantly to the ripening process and flavor development of dry-cured meats because of their proteolytic and lipolytic activities. Taking dry-cured Iberian ham as an example, 95% of the yeast population is *Debaryomyces hansenii* (227). However, yeast species such as *Yarrowia lipolytica*, *Rhodotorula* sp., and *Cryptococcus* sp. have been associated with the spoilage of meat, leading to discoloration, sliminess, and off flavors (228, 229). Because of the importance of these yeast species, rapid detection and identification techniques such as PCR, randomly amplified polymorphic DNA PCR, and inter simple sequence repeat PCR, which target the internal transcribed spacer (ITS) regions of these species (e.g., ITS1–5.8S rRNA–ITS2), have been developed (229–231).

For processed meats, fungal species that are involved in product spoilage are determined by product formula, product types, fermentation and processing steps, and storage conditions (232). Different fungal or even bacterial species can be identified as the cause of spoilage, even for the same type of products. As an example, *Carnimonas nigrificans* was identified as the cause of blackspot spoilage of dry-cured ham, while *Pseudomonas fluorescens* was identified as the cause of black spots in spoiled dry-cured Iberian ham (233, 234). In chilled meat, black-spot spoilage can be caused by *Cladosporium cladosporioides*, *Cladosporium herbarum*, *Penicillium hirsutum*, and *Aureobasidium pullulans* (235). Lozano-Ojalvo et al. (232) investigated the molds that were responsible for black-spot spoilage of dry-cured, fermented sausages. Results revealed that *Penicillium* spp. and *Cladosporium oxysporum* were the two main spoilage molds. *Penicillium* spp. have been reported previously as the most commonly found molds in dry-cured meat products and fermented sausages because they can tolerate large concentrations of salt (236, 237). *Cladosporium* species are widely distributed in indoor environments, the air and foods (232, 238). Both genera, *Penicillium* and *Cladosporium*, are also responsible for black-spot spoilage in dry-cured ham (239).

Next-generation sequencing of the ITS region has provided new opportunities to analyze fungal communities present in animal production and processing environments and animal products. Kumari et al. (225) collected aerosol samples in both winter and summer from seven commercial swine farms in South Korea. The composition, abundance, and diversity of airborne fungi were analyzed using culture-independent methods. The ITS1 region of each DNA sample extracted was targeted and amplified using the universal fungal primers ITS1FI2 and ITS2 (240, 241). Among allfungal phyla, *Ascomycota* (representing 75.4% of allsequences), *Basidiomycota* (15.3%), *Zygomycota* (4.2%), and *Glomeromycota* (1.5%) were the top four phyla identified from the aerosol samples. The dominant fungal classes were *Dothideomycetes* and *Sordariomycetes*, which belong to the phylum *Ascomycota*. Statistical analyses revealed that the fungal OTU compositions were largely determined by the swine house units,

the seasons, and the interactions between these. Microclimate variables, relative humidity, particulate matters, ammonia, and stocking density within the swine house unit were also significantly correlated with OTU composition (225).

Fungal populations, as important background microorganisms present in animal production and processing environments, play important roles in product quality. Their involvement in flavor development as well as product spoilage needs further elucidation through research. Traditional fungal analysis methods have several limitations. For example, some fungal species might not grow on regular plates, and taxonomy analysis based on culturing is time-consuming. In addition, taxonomic analysis of macrofungal fruiting bodies requires years of training and expertise in order to accurately identify and classify morphological characteristics (242). Thus, next-generation sequencing methods are new and efficient tools for fungal analysis in meat and poultry as well as in the animal production and processing environment.

ANTIBIOTIC RESISTANCE

Antibiotic-Resistant Pathogens

The discovery of antibiotics, which are substances that kill or inhibit the growth of microorganisms, is considered one of the greatest discoveries of the 20th century (243). Antibiotic resistance is defined as the ability of bacteria to survive exposure to a definite concentration of an antimicrobial agent, and the emergence of antimicrobial-resistant pathogens has become a serious concern worldwide (244, 245). According to the Centers for Disease Control and Prevention, more than 2 million people are sickened every year from infections caused by antimicrobialresistant agents, and 23,000 people may die from these infections (246). From 1973 to 2011, 55 foodborne outbreaks were associated with bacteria that were resistant to at least one antibiotic (247). It is estimated that the cost of antimicrobial-resistant infections exceeds $20 billion every year in the United States (248). In food animals, antimicrobials have been used

for both disease treatment and growth promotion. According to FDA reports, the sale and distribution (domestic and export) of antimicrobials approved for use in food-producing animals increased by 22% from 2009 to 2015. In 2015, 15,576,975 kg of antimicrobials were sold and distributed in the United States, including 9,701,978 kg of drugs important for human medical therapy (249). The formation of antimicrobial-resistant zoonotic pathogens, which can be transferred to humans through contaminated food or direct contact with the animals, emerged and spread as an undesired consequence of antimicrobial use. Antimicrobial-resistant phenotypes have been identified from multiple zoonotic pathogens, such as *Salmonella* Typhimurium DT104, STEC, *C. jejuni*, *L. monocytogenes*, and *Y. enterocolitica* (245). An increasing number of foodborne pathogens are drug resistant (246).

Shiga Toxin-Producing *Escherichia coli*

Among STEC types, *E. coli* O157:H7 is the classic serotype, causing a substantial number of foodborne outbreaks in North America, Europe, and Japan. In addition, it is estimated that at least 70% of non-O157 STEC cases are caused by the other top six serogroups, which are O26, O45, O103, O111, O121, and O145 (250). These six non-O157 STEC serotypes, which are collectively referred to as the “big six,” have been classified as adulterants of raw beef products in the United States since 2011 (251).

Antimicrobial resistance was not commonly identified in early clinical isolates of *E. coli* O157:H7. In a report by Ratnam et al. (252), 97% of the 174 strains tested were susceptible to commonly used antimicrobial agents, including ampicillin, cephalothin, carbenicillin, tetracycline, kanamycin, gentamicin, tobramycin, amikacin, trimethoprim-sulfamethoxazole, sulfisoxazole, nalidixic acid, and nitrofurantoin (252). However, the percentage of O157:H7 isolates that are resistant to antimicrobials has increased in the last few decades. Meng et al. determined the antimicrobial resistance of *E. coli* O157:H7 and O157:NM strains isolated from animals, food, and humans and found that 30 (24%) of the 125 strains of *E. coli* O157:H7 and O157:NM were resistant to at least one antibiotic and 24 (19%)

strains were resistant to three or more antibiotics (253). Two *E. coli* O157:NM strains isolated from cattle were resistant to up to six antibiotics, including ampicillin, kanamycin, sulfisoxazole, streptomycin, tetracycline, and ticarcillin (253). Vali et al. (254) tested 297 O26, 152 O103, and 13 O145 strains isolated from two cohorts of beef cattle from two farms and found that some of the 297 *E. coli* O26 isolates were resistant to ampicillin (0.3%), amoxicillinclavulanic acid (0.3%), neomycin (0.3%), streptomycin (2%), sulfamethoxazole (2.4%), tetracycline (2%), and trimethoprim (1.7%). *E. coli* O103 isolates (n = 152) were resistant to the same antibiotics, but at higher percentages. For example, 37.5% of the O103 isolates were resistant to sulfamethoxazole, compared to the 2.4% found for O26 isolates. In addition, O103 was resistant to cephalexin. Of the 13 O145 isolates, more than half (53.8%) were resistant to sulfamethoxazole. Singh et al. (255) determined the antimicrobial resistance profiles of 274 STEC strains isolated from poultry, cattle, swine, and humans. Thirty-four percent of the strains were resistant to streptomycin, 32% to sulfamethoxazole, 30% to tetracycline, 18% to ampicillin, 11% to cephalothin, 8% to trimethoprim-sulfamethoxazole, 7% to gentamicin, 5% to chloramphenicol, and 4% to cefoxitin. By analyzing both the resistance and the presence of class 1 integrons, the authors concluded that antimicrobial resistance is common in STEC. Class 1 integrons, which are located on mobile plasmids, can facilitate the formation and dissemination of antimicrobial resistance among different STEC strains isolated from humans and food animals.

Nontyphoidal *Salmonella* spp.

The genus *Salmonella* includes more than 2,500 different serotypes. Nontyphoidal *Salmonella* causes approximately 1.2 million illnesses every year in the United States, resulting in $365 million in medical costs annually (256). *Salmonella* is spread from animals to people mostly through food, and according to reports from the CDC, 100,000 cases of *Salmonella* infections are caused by antibiotic-resistant strains (96). Among the nontyphoidal *Salmonella* strains tested, 3% were resistant to ceftriaxone and about 5% were resistant to five or more antibiotics (246). Resistance to tetracycline in *Salmonella*

species increased from 9% (in 1980) to 24% (in 1990), and resistance to ampicillin increased from 10% to 14% during the same 10-year period (257). Over the past decades, the incidence of human infections with multidrug-resistant *S.* Typhimurium DT104 also has increased dramatically (245). In 2018, several outbreaks associated with animal products and attributed to antibioticresistant *Salmonella* serovars were reported. A total of 92 illnesses had been reported from 29 states by 17 October 2018, due to *Salmonella* Infantis-contaminated raw chicken products, with 21 people being hospitalized. This *Salmonella* Infantis isolate was found to be multidrug resistant (258). In the summer of 2018, multidrug-resistant *Salmonella* Reading was identified in a raw-turkey-associated outbreak in which 90 people from 26 states were sickened and 40 people were hospitalized (259).

The use of antibiotics in food animals plays a key role in the formation of antibiotic-resistant *Salmonella*. Multiple resistance to antimicrobials is commonly observed in isolates from food animals (260–262). In the recent National Antimicrobial Resistance Monitoring System report, multidrug-resistant nontyphoidal *Salmonella* was found more frequently in turkey (34%) and ground beef (20%) than in chicken (10%). The prevalence of multidrug-resistant isolates ranged from 26 to 40% among retail foods (263). The increasing prevalence of antimicrobial resistance in foodborne pathogens globally is indeed challenging, and it is important that more be done in veterinary and human medicine to limit its negative effects.

Most antimicrobial agents exert their effects on bacteria by one or more of four basic mechanisms. These mechanisms are interference with cell wall synthesis, inhibition of protein synthesis, interference with nucleic acid synthesis, and inhibition of metabolic pathways (264). Bacterial cells can be intrinsically resistant to certain drugs or become antibiotic resistant by gene mutation or by acquiring resistance from other microorganisms by acquiring the corresponding genes from plasmids, transposons, integrons, bacteriophages, or a combination of the above (265–268). The main defense mechanisms used by bacteria to resist the effects of antibiotics include antibiotic inactivation or modification (e.g., production of β-lactamase), use of efflux pumps to export antibiotics from inside cells, and alteration of

the drug target in bacterial cells. Food animals, companion animals, wild animals and insects, and environments are reservoirs of antibiotic-resistant microbes and antibiotic resistance genes. In order to mitigate antibiotic resistance in foodborne pathogens and reduce the occurrence of antibiotic-resistant pathogens in animal products, it is crucial to better control the use of antibiotics in animal agriculture and in human medication. New strategies that can effectively detect, prevent, and control the formation and spread of antibiotic resistance genes and bacteria are still needed.

PACKAGING SYSTEMS

More and more meat and poultry products are processed in a centralized location and then transported over increasingly long distances. As a result, the industry has utilized different packaging technologies to increase shelf life of meat and poultry products in order to consistently deliver high-quality product to consumers. Packaging plays a critical role in preserving meat throughoutits distribution cycle. Its primary role is as a physical barrier, and it assists in handling of the product, dissemination of nutritional information, and promotion of sales. Fresh and processed meat is packaged to avoid contamination, to delay spoilage, to control enzymatic activity and improve tenderness, to prevent lipid oxidation, discoloration, and dehydration, to reduce weight loss and loss of aroma, and, where applicable, to improve appearance during retail display. Different packaging systems have different attributes and applications, ranging from simple overwrap packaging for shortterm chilled storage for retail display to a diversity of MAP systems for longer-term chilled storage and distribution. Beef and pork produced in North America are predominantly vacuum packaged and stored and distributed under strictly controlled temperatures. At retail, these vacuum-packaged meats are refabricated and repackaged using simple overwrap packaging for display. Thus, meat packaging systems are designed to enhance consumer acceptance by enhancing the bright red color of the meat and prevention of off odors by controlling the exposure of meat to the high O_2concentration in air. Technological

advances in packaging equipment and material have optimized the challenge that oxygen exposure of meat poses, resulting in the development of MAP systems. These MAP systems include low-oxygen permeable shrinkfilm vacuum packaging; MAP with different concentrations of N_2, CO_2, and O_2; and no-O_2 MAP with low CO, CO_2, and N_2.

Aerobic Packaging

Fresh meat and poultry are packaged and stored aerobically by simply overwrapping the meat placed on Styrofoam trays using high-oxygen permeable films made of polyvinyl chloride. These films do very little to contain microbial growth or extend shelf life but instead enhance the attractive red color due to the reaction of myoglobin and hemoglobin in the presence of oxygen to form oxymyoglobin and oxyhemoglobin, respectively. However, prolonged storage under these aerobic conditions results in the formation of metmyoglobin when the iron has oxidized, resulting in browning, which consumers associate with spoilage. This simple overwrap packaging of meat stored under aerobic refrigerated conditions is widespread in retail stores and results in a shelf life of approximately 5 to 7 days at refrigeration temperatures.

Vacuum Packaging

Vacuum packaging is commonly used for fresh, raw meat and poultry and is simply the process of removing air around the product and then sealing it in oxygenimpermeable barrier bags. Once moist air around the meat product is removed and the oxygen-impermeable barrier bag is sealed, O_2levels continue to decrease while CO_2 levels increase due to muscle and microbial metabolism, which utilizes the residual $O_2$to produce CO_2. These low $O_2$and high CO_2 levels significantly inhibit the growth of bacteria, yeasts, and molds that require $O_2$for their growth, thereby enabling a longer shelf life of the vacuum-packaged product. However, the low O_2levels in the vacuum-packaged products preferentially support the growth of psychrophilic and psychotropic anaerobic and facultatively anaerobic bacteria, such as *Brochothrix*

spp., *Lactobacillus* spp., *Leuconostoc* spp., and *Carnobacterium* spp. and other species of LAB. In addition, the low O_2levels also lead to the unattractive but reversible purple color of vacuum-packaged meat due to accumulation of deoxymyoglobin. In spite of this, a predominant amount of meat produced in North America is stored and shipped to retail under vacuumpackaged conditions at temperatures between 0 and −1.5°C to maintain quality and achieve maximum shelf life. At temperatures between 0 and 4°C, the product is expected to have a shelf life of 40 days (269), whereas when held at subzero temperatures (up to −1.5°C), vacuumpackaged beef has a shelf life of 140 days (270).

Modified-Atmosphere Packaging

MAP is a type of packaging in which the atmosphere surrounding the product is different from air and is one that inhibits the growth of spoilage and pathogenic bacteria. It involves enclosing the product in some kind of barrier and then modifying the atmosphere around the product by either drawing a vacuum or flushing and then filling with a gas mix. Vacuum packaging of fresh meats is essentially a form of MAP, because muscle and microbial metabolism utilizes the residual O_2around the product to produce CO_2, whereas MAP introduces large volumes of gases into the headspace of the package and also eliminates the physical pressure on the product created by the barrier bags during vacuum packaging. Thus, during MAP, fresh meat or poultry is placed in special trays or rigid containers, and the air in the headspace is replaced with a gas mixture and sealed using gas-impermeable films. A variety of gases and gas blends in different ratios have been used to meet different product needs. The three most commonly used gases for MAP are CO_2, N_2, and O_2. Although these gases are present in the air removed from the product atmosphere prior to MAP, the ratio of the gases used in MAP applications is considerably different from that in air. MAP systems can be broadly categorized into two groups depending on the gas blend used: high-oxygen MAP and low-oxygen MAP.

High-oxygen MAP systems utilize gas blends that have high levels of O_2 (70 to 80%) combined with 20 to 30% CO_2 as the atmosphere in the headspace of the meat product. They were developed as an alternative to the simple overwrap aerobic packaging to produce packages of fresh meat that is bright red and case ready for retail display. Fresh meats packaged using high-oxygen MAP may last for 10 to 14 days, compared to 3 to 7 days for fresh meat packaged using simple overwrap aerobic packaging. This allows retailers to offer fresh meat products at lower prices, as they can avoid expenses related to in-store packaging. Exposure to high levels of oxygen results in the saturation of meat pigments with O_2 and slows the formation of surface metmyoglobin. However, exposure to such high concentrations of CO_2 results in the accelerated oxidation of lipids and generation of off flavors. In any case, high-oxygen MAP results in fresh meat products that have a shelf life greater than that achieved with aerobic packaging but substantially less than that achieved with vacuum packaging. Unlike fresh red meat, poultry meat has very low myoglobin concentrations and is generally considered white meat. The color of poultry meat is not as significant a consideration as that of beef, and therefore, maintenance of the bright red color is not a priority for poultry products. Storage of poultry meat in a high-oxygen MAP containing 70% O_2 and 30% CO_2 has no additional beneficial effect on its quality and shelf life (271).

Low-oxygen MAP uses a gas blend in which $O_2$is reduced to the minimum level or completely replaced with another gas to inhibit bacterial growth. For example, MAP of fresh meat primals with a gas blend in which 20 to 25% is CO_2 and the remainder is N_2successfully inhibits bacterial growth and extends shelf life (272, 273). Carbon dioxide is most effective against Gram-negative bacteria and increases the lag phase and generation time of affected microorganisms. It has been suggested that the antimicrobial effect of CO_2 is due to its ability to penetrate bacterial membranes and alter cellular pH and metabolic processes (274, 275). Carbon dioxide is highly soluble in meat to form carbonic acid, which reduces the pH and is thought to contribute to its antimicrobial activity. However, when large volumes and high

concentra tions of CO_2 are used in the MAP headspace, the gas concentration in the headspace declines significantly until a product saturation or equilibrium is reached, which could risk package collapse when the amount of CO_2 absorbed by the meat is large enough. Package atmospheres with less than 30% CO_2 are seldom reported to result in package collapse (276). Hence, N_2 is typically used as a filler gas to exclude $O_2$and create the modified atmosphere in the package headspace. Nitrogen has low solubility in fat and water and does not affect microbial growth, but when used to exclude O_2, it prevents growth of aerobic bacteria and indirectly helps to extend the shelf life of fresh meat.

An issue with excluding O_2 during packaging of meat is discoloration of the product. The principal role of O_2 in packaging of meat is the development of the cherryred color that is considered essential to display fresh meat. Carbon monoxide has a very high affinity for hemoglobin in the blood and myoglobin in the muscle that results in a stable, bright red meat color. Very low concentrations (0.3 to 0.5%) of CO are sufficient to produce the stable, bright red meat color, and these concentrations do not pose a human hazard. The FDA has approved the use of 0.4% CO for meat packaging and has categorized CO at these concentrations as generally recognized as safe when it is used as described for MAP (277). Use of CO in low-oxygen MAP at very low levels of 0.4% combined with 20 to 30% CO_2 and the remainder N_2can result in a shelf-ready product that is bright red with low microbial counts and superior quality attributes, such as flavor acceptability and tenderness, compared to aerobic or high-oxygen packaging. Despite these benefits, consumer concern that the use of CO can mask the appearance of spoiled meat and a perceived potential health hazard due to CO toxicity have been hurdles in widespread application of CO in MAP. The color of poultry products such as ground chicken and turkey meat remains more stable in an oxygen-free environment (278), and the best color stability and lowest microbial counts were observed in a 100% CO_2 atmosphere compared to 100% O_2, 100% N_2, or a mixture of 25% CO_2, 66% O_2, and 9% N_2(279, 280). Hence, for poultry products where the appearance of

bright red color is not a requirement, low-O_2 and high-CO_2 atmospheres are preferred to enhance shelf life.

Smart Packaging Systems

Packaging systems that apply technologies that assist in the maintenance or enhancement of the quality and safety of packaged foods without any physical modification to the food are generally referred to as smart packaging systems. Technologies that are in use or being developed are broadly grouped into two categories: active packaging and intelligent packaging systems.

Active packaging refers to the incorporation of additives or changing the environment around the food in a package to extend shelf life and/or to improve safety and sensorial properties, while maintaining the quality of packaged foods. This can involve physical, chemical, or biological actions that alter the interaction between the package or package headspace and the product to reach a desired outcome. Some of these interactions include oxygen scavengers in low-O_2 MAP, CO_2 emitters and scavengers in low-O_2 MAP and fermented foods containing meat, and moisture control to lower aw. Oxygen scavengers are used to absorb the residual oxygen in fresh meat and poultry products packaged using low-O_2 MAP systems or vacuum packaging, thereby enhancing the shelf life of these products by controlling growth of aerobic bacteria and also reducing lipid oxidation, product discoloration, and drip loss. Existing O2-scavenging systems use one or more of these reactions: iron powder oxidation, ascorbic acid oxidation, photosensitive dye oxidation, enzymatic oxidation, unsaturated fatty acid rice extract, or immobilized yeast on a solid substrate. These O2-scavenging reactions can be packaged in a sachet, label film (reactive agents incorporated into the packaging film), card, closure liner, or concentrate. Similarly, CO_2-generating systems are complementary to O_2scavengers. When fresh meat is packaged using vacuum packaging or MAP, CO_2, which is highly soluble in the muscle, is absorbed, resulting in a partial vacuum and pack collapse. In these situations, the simultaneous release of CO_2 from CO_2-generating systems could eliminate the partial vacuum and maintain CO_2 levels

in the packages. A list of commercially available O_2 scavengers, CO_2 emitters and scavengers, and moisture control systems has been published by Wyrwa and Barksa (281).

Microbial contamination and the subsequent growth of microbes on fresh meat and poultry products reduce shelf life and increase the risk of foodborne illness. On fresh meats, because microbial contamination is primarily restricted to the surface, antimicrobial sprays and dips have been used in an attempt to improve safety and shelf life. However, neutralization of the antimicrobials on the surface of the meat limits their antimicrobial efficacy when they are applied as sprays or dips. Similarly, by including the antimicrobial agents in the meat formulation, the meat constituents can partially inactivate the active ingredient or make the antimicrobial inaccessible to the surface-contaminating microflora. Hence, antimicrobial packaging is a promising form of active packaging, especially for meat products. To confer antimicrobial activity, antimicrobial agents are used to coat, incorporated into, immobilized onto, or surface modified onto the packaging material, which, when the packaging comes into contact with the food, results in migration of the antimicrobial agents to the food surface. Although antimicrobial packages have had relatively limited commercial success outside Japan, where Ag-substituted zeolite is the most common antimicrobial agent incorporated into plastic, a large volume of available scientific information has been published on the antimicrobial properties of different classes of antimicrobials in various film structures, synthetic polymers, and edible films. These classes of antimicrobials include bacteriocins, bacteriophages, enzymes, essential oils, organic acids, plant extracts, and polysaccharides. Most of the research on antimicrobial active packaging has been on RTE meats, whereas their application in fresh meat and poultry has been limited.

Antioxidant active packaging is another type of active packaging. Oxidation is a major mechanism of food deterioration. Lipid oxidation in meats results in the development of rancidity and off odors, especially in meats with high fat levels. Many studies have addressed the direct application of antioxidants to meats to control lipid oxidations; however, the use of antioxidants in packaging materials is

rare, but it is of interest. Oregano oil has been evaluated for its ability to inhibit lipid oxidation and extend the shelf life of beefsteaks. Similarly, rosemary extract, tocopherol, ascorbic acid, and many plant extracts have been successfully used in biobased films, and their antioxidant potential to decrease lipid oxidation in meat products has been tested. Publications by Appendini and Hotchkiss (282), Camo et al. (283), Irkin and Esmer (284), and Suppakul et al. (285) provide a comprehensive list of antimicrobial and antioxidant food packaging materials.

Intelligent packaging systems use sensors and indicators to monitor the quality of packaged foods during transport and storage. These sensors and indicators determine whether a product has been subjected to temperature abuse or has been tampered with, thereby resulting in the loss of package integrity. An example of such sensors are gas sensors which measure $O_2$and CO_2 levels in MAP systems. These gas sensors visually indicate O_2 and CO_2 levels by using the principle of luminescence quenching, or absorbance changes caused by direct contact with the analyte. OxySense was the first commercially available fluorescence quenching sensor system for measurement of headspace or dissolved oxygen in transparent or semitransparent sealed packages. Toxin-Guard is a biosensor developed by Toxin Alert (Ontario, Canada) that incorporates antibodies in a polyethylenebased plastic system capable of detecting *Salmonella* spp., *Campylobacter* spp., *E. coli* O157, and *Listeria* spp. (286, 287). Another biosensor system, the Food Sentinel System (SIRA Technologies, Pasadena, CA), is capable of continuous detection of contamination through an immunological reaction wherein a barcode is rendered unreadable in the presence of contaminating bacteria.

Indicators indicate the presence or absence of a substance or the degree of reaction between two or more substances which are visualized by a change in color. Specific types of indicators include gas leak indicators that monitor pack integrity in MAP systems, freshness indicators, and time-temperature indicators. Examples of commercialized $O_2$and CO_2 indicators that are used in different foods include the Ageless Eye, Vitalon, and Samso-Checker. Freshness

indicators react with metabolites associated with food spoilage. These include indicators sensitive to organic acids, ethanol, and biogenic amines, which are metabolites produced by spoilage microbes during the degradation of meat (288–291). Time temperature indicators are used to show a measurable, time-temperature-dependent change that reflects the full or partial temperature history of a food product to which it is attached (292, 293). These indicators are dependent on microbiological, chemical, biochemical, mechanical, and electrochemical changes that result in a visible response, such as a mechanical deformation or color change. Essentially, time-temperature indicators monitor the time-temperature relationships of a product throughout its movement through production, processing, storage, transportation, distribution, and retail display. Finally, radio frequency identification tags are semiconductor tags that are placed on packaged products to assist in traceability, inventory management, labor cost savings, security, and promotion of quality and safety. Although such tags have been available for almost 40 years, their application in the food packaging and distribution arenas is relatively recent.

Many active and intelligent packaging systems are being developed, and their commercialization has been enhanced by the rapid pace of sensor and indicator development.

MEAT TRACEABILITY

Traceability of meat and poultry products is essential to provide transparency and security to consumers. It guarantees the quality and safety of products entering commerce and prevents fraud and unfair competition. In general, traceability is the ability to know where food comes from and where food goes at any given stage of the food production chain. It enables locating products implicated in foodborne illness outbreaks and facilitates their recall when a safety or quality standard is breached (294). A key concept relating to product traceability is that of the “traceable unit.” GS1, an international notfor-profit association that designs and implements global standards and solutions to improve supply chain management, divides traceable

units into batches, trade units, and logistic units (295). While trade units and logistic units are defined as externally traceable units, batch is considered an internally traceable unit (296, 297).

The meat and livestock sector has been at the forefront of traceability policy because of the number of outbreaks and food safety crises (298). Different beef supply chain traceability models can be used based on production methods. The GS1 guideline (299) lists the key traceability elements for various trade items. For example, when livestock is the trade item, the key elements for traceability include individual or mob animal identifiers, cattle production information (e.g., age, region, fattening, and finishing), feed and quality identification through supply documentation for feed supplements, livestock mob movement declarations, and animal treatment. When beef carcasses are considered the trade item, important elements to link the carcasses to individual animals are the application identifier, the global trade item number, the application identifier batch, lot, and/or serial numbers, and the registration number of the slaughterhouse facility issued by the government or authority. Also important are the date, time, batch, and properties that supply the livestock (299). For poultry products, one document for industry to follow is “GS1 global meat and poultry traceability guideline, part 5. Poultry supply chain” (300). As described in this document, key traceability elements for live poultry are similar to those for beef products. These elements include batch and/or lot identification, information about flock production (e.g., age, region, fattening, and finishing), feed and feed supplements, animal treatment, and the poultry batch or lot movement declaration (300).

Technologies and devices used for traceability have continuously improved to ensure implementation. Efficient methods for tracking agro-products include radio frequency identification, near-field communication, isotope analysis and DNA barcoding, and the combination of the use of isotopes with other techniques, such as chromatography, isotope ratio mass spectrometry, inductively coupled plasma mass spectrometry, and near-infrared spectroscopy (301, 302). Overall, traceability systems are important tools to ensure the safety and quality of meat and poultry products. The constant improvement

of technologies for wireless sensing, real-time tracking, and remote monitoring will lead to a more rapid and reliable traceability system for animal products.

CONCLUDING REMARKS

The contamination of raw meat and poultry is natural; however, efforts should be focused on minimizing contamination throughout the farm-to-fork continuum. Control measures should begin on the farm and should continue through the harvest stages until the products are consumed by consumers. Contamination is minimized through the production and processing continuum by regulatory inspection programs and other food safety programs used by the meat and poultry industry, i.e., HACCP systems, to prevent any foodborne illnesses related to meat and poultry products.

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7
Microbiological Issues Associated with Fruits, Vegetables, Nuts, and Grains

This chapter addresses several plant-based food groups, each of which has been extensively investigated. While each of these food groups is easily distinguished from the other, processing operations may produce another set of unique products from each food group that have their own characteristics and associated microbiological issues. Hence, a vast variety of foods are covered in this chapter. The first major section provides a short description of each food group. The second section addresses the major microbial groups associated with these food groups and their products. The remaining sections discuss common themes associated with all these food products, including quality and safety repercussions associated with microbial contamination of these food groups, sources of contamination, detection of contamination, interventions available to reduce microbial contamination, and risk assessment.

PRODUCT TYPES ASSOCIATED WITH EACH FOOD GROUP

Fruits, vegetables, nuts, and grains are classified according to the part of the plant from which they originate. In broad terms, fruits are foods that develop from the flower of a plant and contain seeds, whereas the other parts of the plant (i.e., roots, stems, and leaves) are categorized as vegetables. There are a number of subcategories within the fruit classification scheme that are based on the fruit's morphology (Table 7.1). Similarly, subcategories of vegetables are dependent on the part of the plant that is eaten, but differentiation of vegetables is also often based on where they originate (Table 7.2). Of note in Table 7.1 is that tomatoes, nuts, and grains are categorized as fruits. Mushrooms, on the other hand, are included in Table 7.2 as vegetables but are actually fungi. The lists of crops provided in Tables 7.1 and 7.2 are not comprehensive, as they constitute only about 50% of the produce items listed in USDA Agricultural Handbook 66 (1).

Many fruits and vegetables are consumed in their raw state, as their preparation steps (i.e., peeling, stoning, cutting, shell cracking) are minimal. Other processing steps, such as drying, scalding, blanching, pressing, and

freezing, are more involved and may be conducted in the home or by the food industry. The resulting product may in some cases be consumed shortly thereafter, but it may also be stored. When the product is stored, both the processing and storage will likely influence the microbiological profile associated with the final product.

Although this chapter addresses many crops and plant-based products, these foods may be divided into two categories: high-moisture foods and low-moisture foods. Moisture availability is a critical factor in determining the dominant microflora that will be resident initially and subsequently during storage. The high-moisture category is addressed first, followed by crops in the low-moisture category.

TYPES OF MICROBIAL CONTAMINATION ASSOCIATED WITH EACH FOOD GROUP

Bacteria, molds, yeasts, and viruses are ubiquitous in the environment, but within each crop, there are major types of microbes that dominate shortly after harvest. In many cases, the dominant microbial groups that are initially present do not persist, as the environmental conditions favor other microbes that may be present in small numbers at the outset. This is the situation for undamaged fresh fruits and vegetables. In some cases, microorganisms may also be introduced after the crop is harvested, which is a reflection of the hygiene conditions to which the food item is exposed. Hence, there is a wide diversity of microorganisms that may be present on various crops during their journey to the table (2). Some examples of the microbial contaminants that could be associated with fruits, vegetables, nuts, and grains are provided in Tables 7.3 and 7.4. Most of our knowledge in this area has been obtained from culture-dependent methods; however, results from culture-independent methods (i.e., metagenomics studies) may reveal differences in the diversity and structure of the microbial communities in those food groups as we know them today.

Table 7.1 Fruit classification

Fruit type	Fruit category	Description	Examples
Fleshy (pericarp is soft or fleshy at maturity)	Drupe or stone/pit	A one-seeded simple fruit in which the innermost portion of the ovary wall (endocarp) becomes hard and stony and the outermost part (exocarp) becomes either fleshy or fibrous	Almond, apricot, cherry, mango, olive, peach, plum
	Berry	A simple fruit in which the inner portion of the ovary wall becomes enlarged and juicy but it has no rind	Grape, pepper, tomato
	Hesperidium	A berry in which a leathery rind forms and the interior part of the fruit is divided by septa	Grapefruit, lemon, lime, orange
	Pepo	A berry in which a relatively hard rind is formed but no septa divide the fruit	Banana, cantaloupe, cucumber, papaya, pumpkin, squash, watermelon
	Pome or seed fruit	An accessory fruit formed by a group of carpels more or less firmly united with each other and surrounded by and united to the floral tube or receptacle	Apple, pear
	Aggregate fruit	A fruit formed by the development of multiple pistils from the same flower	Blackberry, raspberry, strawberry
	Multiple fruit	A fruit formed by the coalescence of many flowers crowded together in the same inflorescence, typically surrounding a fleshy stem axis	Fig, pineapple

Fruit type	Fruit category	Description	Examples
Dry (pericarp is dry at maturity)	Legume	A dry fruit developed from one carpel that at maturity splits along both the dorsal and ventral sutures	Bean, chickpea, lima bean, mung bean, peanut, pea, soybean
	Nuts	A dry fruit where the seed is attached to the fruit wall, which is greatly thickened and hardened	Chestnut, hazelnut, hickory
	Grain	A dry fruit where the fruit wall and the seed coat are fused	Barley, corn, oats, rice, rye, wheat

Table 7.2 Vegetable classification

Location	Category	Description	Examples
Underground	Bulbs	Usually consist of layers or clustered segments just below the surface of the ground with a fleshy, leafy shoot above ground	Fennel, garlic, leek, onion, shallot, spring onion
	Roots	Usually a long or round-shaped taproot	Beet, carrot, parsnip, radish, turnip
	Tubers	Grows underground on the root of a plant	Artichoke, potato, yam
Above ground	Flowers	Immature flowers	Artichoke, broccoli, cauliflower
	Fungi	Possesses a stem, cap, and gills	Mushroom
	Leaves	Edible leaves of plants	Brussels sprouts, cabbage, lettuce, spinach
	Stems	Edible stalks of plants	Asparagus, celery, kohlrabi

QUALITY AND SAFETY OF FOOD CROPS ASSOCIATED WITH MICROBIAL CONTAMINATION

Most food crops that are harvested are of optimal or near-optimal quality and ready for consumption or further processing. However, some food crops are harvested in an immature state and then, during storage and transport, continue to metabolically change to their mature state. Since crops are living tissues, it is inevitable that chemical and biochemical changes will continue past maturity and will contribute to quality losses, a state that is termed spoilage. However, in addition to this chemical activity, microbiological contamination and its associated activities will also be a major factor contributing to spoilage. Such spoilage is often caused by a complex mixture of bacteria rather than one microbial species (3), and those activities ultimately lead to changes in the appearance, texture, and flavor of the product. Examples of these types of spoilage for both high-moisture and low-moisture food crops are given in "Spoilage" below.

Another serious consequence of microbiological contamination of crops is that some contaminants cause human illness. These microbial pathogens can be categorized as two types, pathogens of which a low dose (<100 cells) can cause illness (e.g., *Escherichia coli* O157:H7, *Salmonella*, and norovirus) and pathogens requiring growth in the food to large cell populations to cause illness. In the latter case, food becomes unsafe either from consumption of toxins produced during the pathogen's growth (e.g., mycotoxins) or from consumption of the amplified pathogen populations (e.g., $>10^6$ cells) present in the food (e.g., *Listeria monocytogenes*). Examples of the pathogens that may be present in both low-and high-moisture crops are described in "Outbreaks and Prevalence of Pathogens and Toxins" below.

Spoilage

High-Moisture Food Group (Fruits and Vegetables)

Appearance is the main factor influencing consumers' purchase of produce, which includes color, gloss, shape, size, and absence of defects. One of the first noticeable changes in quality is often browning, which arises from the interaction of polyphenol oxidase and phenols that are released during minimal processing. Hence, microorganisms are not directly responsible for this defect, but they may contribute indirectly through their release of enzymes that break down plant constituents to accelerate the interaction of the browning enzyme and its substrate (4). Interestingly, browning is often mistaken for mold growth, such as that which occurs with the brown or black spots on cauliflower.

Another noticeable change in produce quality is the loss of firmness. Contributing to this quality defect are both bacteria (e.g., *Erwinia* and *Pseudomonas*) and yeasts that produce and release pectinases (pectin methylesterase, polygalacturonase, and pectin lyase) and cellulases that are in turn responsible for breaking down pectins and cellulose, respectively. The latter compounds are key components in the middle lamella and plant cell walls, whose breakdown results in tissue maceration, loss of rigidity of plant tissue, and nonreversible cell damage (3). However, prior to this textural breakdown, the pectinolytic activity often results in "soft-rot" spots or other unacceptable visual defects. Bacterial and yeast counts that are associated with these spoilage defects often exceed 8 log and 5 log CFU/g, respectively (3). However, a high microbial count is not always indicative of spoilage, nor does a low microbial count necessarily indicate that spoilage is absent. In the latter case, densities of spoilage bacteria or yeasts may be high near damaged surfaces but could be diluted by less-contaminated Undamaged surfaces when plant tissue is homogenized for determining microbial counts (3).

Table 7.3 Examples of bacterial genera contaminating crops at harvest and processed foods from those crops

Fruits	**Vegetables**	**Nuts**	**Grains, flour, bakery products**
Acidovorax	*Acinetobacter*	*Achromobacter*	*Bacillus*
Alicyclobacillus	*Bacillus*	*Acinetobacter*	*Clostridium*
Bacillus	*Chromobacterium*	*Bacillus*	*Enterobacter*
Buchnera	*Clostridium*	*Brevi bacterium*	*Escherichia*
Erwinia	*Enterobacter*	*Clostridium*	*Klebsiella*
Gluconacetobacter	*Escherichia*	*Corynebacterium*	*Pantoea*
Microbacterium	*Erwinia*	*Enterobacter*	*Salmonella*
Pantoea	*Flavobacterium*	*Escherichia*	*Serratia*
Pectobacterium	*Janthina bacterium*	*Flavobacterium*	*Staphylococcus*
Photobacterium	*Klebsiella*	*Lactobacillus*	
Pseudomonas	*Lactobacillus*	*Leuconostoc*	
Xanthomonas	*Leuconostoc*	*Microbacterium*	
	Listeria	*Micrococcus*	
	Pantoea	*Proteus*	
	Pectobacterium	*Pseudomonas*	
	Pedobacter	*Salmonella*	
	Pseudomonas	*Staphylococcus*	
	Salmonella	*Streptococcus*	
	Xanthomonas	*Xanthomonas*	

Table 7.4 Examples of fungal (yeast and mold) genera contaminating crops at harvest and processed foods from those crops

Fruits	Vegetables	Nuts	Grains, flour, bakery products
Alternaria	*Alternaria*	*Acremonium*	*Alternaria*
Botrytis	*Aspergillus*	*Aspergillus*	*Aspergillus*
Candida	*Botrytis*	*Chaetomium*	*Candida*
Colletotrichum	*Candida*	*Cladosporium*	*Cladosporium*
Debaryomyces	*Cladosporium*	*Eurotium*	*Claviceps*
Fusarium	*Colletotrichum*	*Fusarium*	*Cryptococcus*
Geotrichum	*Cryptococcus*	*Mucor*	*Debaryomyces*
Hanseniaspora	*Debaryomyces*	*Paecilomyces*	*Eurotium*
Issatchenkia	*Fusarium*	*Penicillium*	*Fusarium*
Kluyveromyces	*Geotrichum*	*Phialophora*	*Hanseniaspora*
Monilinia	*Issatchenkia*	*Phomopsis*	*Helminthosporium*
Mucor	*Penicillium*	*Rhizopus*	*Issatchenkia*
Penicillium	*Phoma*	*Tricothecium*	*Kluyveromyces*
Phytophthora	*Phytophthora*	*Trichosporon*	*Mucor*
Pichia	*Pichia*		*Penicillium*
Rhizopus	*Rhizopus*		*Pichia*
Torulaspora	*Rhodotorula*		*Rhizopus*
Zygosaccharomyces	*Saccharomyces*		*Rhodotorula*
	Sclerotinia		*Saccharomyces*
	Trichoderma		*Schizosaccharomyces*
	Trichosporon		*Sporobolomyces*
	Torulaspora		*Torulaspora*
	Yarrowia		*Trichosporon*
	Zygosaccharomyces		*Wallemia*
			Zygosaccharomyces

It is common practice to package many perishable produce items in atmospheres that minimize oxygen exposure to reduce browning and extend shelf life. In addition, modifying the atmosphere shifts the dominant microbial species of produce, such as fresh-cut iceberg lettuce, from *Pseudomonas* spp. to

lactic acid bacteria such as *Leuconostoc* spp. and *Lactococcus* spp., which thereafter proliferate (5, 6). Off odors described as sour (6) or pungent or fermented (5) then become the principal characteristics for rejecting the lettuce as spoiled.

Off odors are also responsible for the rejection of fruit products. For example, immature apples are stored postharvest to accommodate exportation and extension of shelf life; however, if the apples are infected with fungi such as *Penicillium expansum*, the growth of these microorganisms leads to the production of primary and secondary volatile metabolites that adversely affect their odor and acceptability (7). Similarly, in commercial pasteurized fruit juices, the presence of the heat-resistant, acid-tolerant spore-forming bacteria *Alicyclobacillus* spp. leads to the production of medical, phenolic, or antiseptic off odors and off flavors (8).

Low-Moisture Food Group (Nuts and Grains)

Maintaining water activity (a_w, a measure of the water available to microbes) values of less than 0.70 largely mitigates bacterial and most fungal growth in nuts. Under such conditions, spoilage of nuts is primarily due to oxidative rancidity of their fats. When nuts are not kept dry, mold may grow on them and can change the product's appearance, making it unacceptable to the consumer. However, the more serious concern regarding the presence and growth of molds is that the production of mycotoxins makes the product unsafe. Additional discussion on mycotoxins is found in "Low-Moisture Food Groups (Nuts, Grains, and Dried Fruits)" below.

As in nuts, molds are a common contaminant of cereal grains both pre-and postharvest. Damage of the entire crop may occur when grains, particularly wheat and barley, are infected with field fungi and subjected to below-normal temperatures and above-normal precipitation (9). When grain has been separated from the chaff and dried to moisture levels of 12 to 14%, it may be stored for 1 year or longer before being processed (10).

Once grain is processed into flour and subsequently baked products, the mold spores and vegetative microbial cells originating from the grain are eliminated. However, during the relatively long period of product cooling before packaging, the surfaces of baked products are subject to recontamination with airborne mold spores. Hence, mold growth is the predominant cause of baked-product spoilage. In addition, for bakery products with a moist interior, a spoilage condition known as "ropy" formation can develop. In this case, rope formation is the result of extracellular capsular material produced by germinated endospores of *Bacillus subtilis*. These spoiled products are characterized by a

mucilaginous appearance when pulled apart and by the presence of a melon-like odor (10).

Outbreaks and Prevalence of Pathogens and Toxins

High-Moisture Food Group (Fruits and Vegetables)

Although fruits and vegetables are considered part of a healthy diet, foodborne illness associated with the consumption of produce has become a major food safety concern in the past 2 decades. Li et al. (11) compiled and summarized data on produce-related outbreaks and sporadic illnesses that occurred in five industrialized regions or countries (Australia, European Union, Japan, New Zealand, and the United States) from 2010 to 2015. Overall, there were 988 produce-related outbreaks and 45,723 cases of produce-related illness in those regions and countries. As to the magnitude of the food safety risk of this commodity group compared to other groups, Gould et al. (12) reported that produce was responsible for about one-third of the outbreaks and more than 80% of outbreak cases associated with imported foods in the United States during the period of 1996 to 2014.

One of the challenges in assessing the food safety risk of fresh produce is the difficulty of finding enteric pathogens in the products during routine surveys (13, 14). Based on such results, the European Commission estimated that the overall prevalence of foodborne pathogens on fresh vegetables is <1% (15). This low prevalence is influenced by many factors, including the methods used for detection, the sources contributing to contamination, the environmental stresses encountered by the contaminated produce item, and the management strategies and interventions applied both pre-and postharvest to reduce pathogen contamination.

Low-Moisture Food Group (Nuts, Grains, and Dried Fruits)

Until recently, the primary food safety risk associated with low-moisture plant-based foods was the presence of toxigenic and pathogenic fungal species and their exposure to environmental conditions that would allow their growth and production of mycotoxins and other toxic metabolites (e.g., aflatoxins, ochratoxin, fumonisins, zearalenone, patulin, and trichothecanes, including deoxynivalenol). Consumption of mycotoxin-contaminated foods can cause acute or chronic toxicity in humans and animals. In addition to these adverse effects from direct consumption of mycotoxin-contaminated foods, there is also concern regarding the use of these contaminated crops as animal feeds and subsequently the ingestion of animal-derived food products that could contain residues or metabolites of mycotoxin residues or metabolites. Contamination

of plant-based foods by mycotoxins remains a problem despite extensive efforts to minimize their presence (16). A recent estimate reveals that about 25% of the world's harvested crops are contaminated by mycotoxins annually (17). Mycotoxin contamination of low-moisture plant-based foods is particularly pervasive in developing countries where there are high temperatures and humidity levels, which are conducive to mold growth and mycotoxin production. As a result, hundreds of hepatocellular carcinoma cases occur each year due to ingestion of mold-contaminated products (18). Not discounting these acute cases, many more carcinoma cases may be identified as our understanding of the complementary toxicity mechanisms of action that occur when multiple types of mycotoxins are present in a food system increases (19).

Following are examples of studies conducted within the past decade addressing the pervasiveness of molds and their metabolites in nuts. Investigators determined that 100% of walnuts (n = 20), 91% of pine nuts (n = 11), 76% of almonds (n = 17), 6% of pecans (n = 16), 25% of dried apricots (n = 8), 20% of dried cranberries (n = 10), 8% of dried pineapple (n = 12), 60% of dried raisins (n = 10), and 0% of dried papaya (n = 10) sampled in the Washington, DC, area were contaminated with viable fungi (20). *Aspergillus* was identified as the most common mold, being present in 66% of the nut samples and 50% of dried raisins. Analysis of dried fruits (n = 75) and nuts (n = 90) purchased from retail shops in Pakistan revealed that 22.7% and 35.6% of these items, respectively, were contaminated with aflatoxin (21). The presence of low levels of aflatoxins is tolerated; however, this study revealed that 6.7% and 11.1% of the dried fruit and nut samples, respectively, were contaminated at levels that exceeded the limit (4 μ g/kg) set by European Union regulations (22).

Generally, all cereal crops improperly stored for a prolonged time can be subject to mold growth and mycotoxin contamination; however, maize is considered the crop most susceptible to mycotoxin contamination, and rice is the least susceptible (23). The molds most likely to contaminate grain and produce mycotoxins include *Fusarium* species, which infect and grow on crops (e.g., wheat, barley, and corn) in the field, and *Aspergillus* and *Penicillium* species, which grow on these crops during storage (16). Neme and Mohammed (24) recently reviewed studies of the prevalence and levels of mycotoxins that have been found in grains such as maize, peanut/groundnut, sorghum, millet, wheat, and rice. A literature review by Ferrigo et al. (25) addresses *Fusarium* mycotoxins, providing a perspective on the range of the levels of different *Fusarium* mycotoxins in different cereals originating from different geographical areas. These authors suggest several factors that potentially contribute to the variability in type and level of mycotoxins between regions, including the species and strain of *Fusarium*, the environmental conditions

surrounding the crop in the field and during storage, and other endogenous and exogenous factors that can affect mycotoxin production (25).

Another food safety issue of major concern is the contamination of low-moisture food products with enteric pathogens. Outbreaks of foodborne illness associated with the consumption of tree nuts, peanuts, and sesame seeds have occurred frequently within the past decade, and a list of these is available online (26). Most have been attributed to *Salmonella*, a pathogen noted for its resistance to desiccation (27). Hence, the long-term storage of nuts does not preclude them from being contaminated, albeit generally at low cell populations (28). A recent outbreak of salmonellosis associated with Kellogg's Honey Smacks cereal, which has a 1-year "best if used by" date, highlights the concern that this wheat-based product continued to cause illness when consumers failed to heed warnings to discard the product (29). Many other outbreaks have been associated with grains, dried fruits and vegetables, and nuts (30, 31), and such outbreaks are likely to continue, as their low levels of contaminants are typically heterogeneously distributed (28, 30), thereby precluding the testing of final product for *Salmonella* as a realistic safety measure.

ENUMERATION AND DETECTION OF MICROBIAL CONTAMINANTS AND INHERENT LIMITATIONS

For many decades, enumeration of microbial contaminants in produce, nuts, and grains primarily relied on dislodging the microorganism from the food item into a liquid medium using some mechanism of physical force (shaking, stomaching, vortexing, sonicating, or grinding). In the case of bacterial, yeast, and mold counts, this extract was then collected and applied to a culture-based assay for enumeration of the microbes using some form of solid agar medium. The composition of the medium varied and was dependent on the metabolic requirements of the organism being enumerated. Once the agar plate had been held for a specified period of time at a specified temperature, often optimal for the target organism, the colonies were counted. Although it is still widely used, a limitation of this approach is that the plant-based food to which the microbes are attached and the strength of attachment by different microorganisms to the food vary (32). This variability in attachment can affect the number of cells dislodged into the extract, which can represent a large portion of the total number of cells from the food when populations are low, such as often occurs when enteric pathogens are present. A similar limitation may occur when viruses or parasites are extracted from plant-based foods; however, in this

instance, either microscopy or molecular detection would primarily be used for the detection and/or quantification of those microbes.

Although enumeration of the microbial content yields more information on the degree of spoilage of a food, often only the presence or absence of a specific type of microorganism is necessary. Molecular detection methods have become a preferred approach by many laboratories for detecting foodborne pathogens in foods. For molecular detection, either the food item may be homogenized directly with an enrichment medium or an extract from the food item can be mixed with the enrichment medium. An enrichment is needed to enable the growth of the pathogen of interest to sufficient levels for detection (typically 5 to 6 log). After incubation of these enrichment mixtures, portions are removed for DNA or RNA extraction and subjected to a molecular assay, such as PCR (the polymerase chain reaction assay), whereby a gene specific for the target organism is amplified and detected. The shorter time to results is the primary advantage to using molecular assay-based detection rather than culture-based detection. However, two limitations of molecular-based detection have become apparent. One is that when an enrichment medium is used, the medium itself predisposes some strains of a targeted microbe such as *Salmonella* to outcompete others (33). This limitation is especially relevant for epidemiologic investigations, as certain strains may appear to be less significant than they would be under natural conditions. However, such concerns may not have to be considered in the future if metagenomic sequencing is able to overcome this limitation (see chapter 36 of this volume).

A second limitation of molecular assay-based approaches for detecting microbial contaminants in plant-based foods is the inability to distinguish between viable and dead cells of the microbe of interest when quantitative PCR (qPCR) is used. Two approaches (propidium monoazide qPCR [34] and reverse transcriptase qPCR [35]) have been applied with the assumption that they would amplify only RNA associated with viable organisms and not amplify DNA from dead organisms. However, Ju et al. (36) observed that in lettuce, when dead *E. coli* O157:H7 cells were present at 100-fold-higher numbers than viable cells, false-positive enumerated results occurred with propidium monoazide qPCR. These investigators also determined that highly degraded RNA was amplified by reverse transcriptase qPCR, thereby discounting the presumption that mRNA quickly degrades after death of the cell. Given these findings, investigators should be cautious in using these approaches.

As noted previously, enteric pathogens contaminating produce, nuts, and grains are often present at very low levels and heterogeneously distributed. Although mixing and/or pooling of samples or testing more samples or greater quantities can increase the likelihood of detection, there is often an increased analytical

cost that can be disproportionate to the benefit. For those reasons, a common practice used by the industry, environmental agencies, and public health organizations is to screen for indicator organisms instead of microbial pathogens (37). Such indicator organisms (e.g., generic *E. coli*) may serve as markers of potential fecal contamination; however, their validity as predictors of pathogen presence is often not reliable. Generally, the relationships between the presence or levels of an enteric pathogen and an indicator microbe are random, site-specific, or time-specific events. Therefore, the suitability of predicting the presence or cell populations of pathogens through indicator organisms has been challenging to determine and strongly depends on the circumstances (38).

SOURCES OF MICROBIAL CONTAMINATION

Preharvest Sources

During the cultivation of fruits, vegetables, nuts, and grains, the open nature of fields and orchards in which they are grown can expose the crop to multiple sources of contamination. However, for any of these sources, it is often difficult to determine their impact on a crop's microbiological community, as there are competing factors (i.e., biological, chemical, and physical) that could potentially modify that input fairly rapidly. For example, soil could contribute substantially to both the rhizosphere (i.e., below-ground) and phyllosphere (aboveground) microbial communities. However, when the bacterial communities on leaf surfaces and their surrounding soil were compared through pyrosequencing, only a very small fraction (0.5%) of microbial Taxonomic units were found to be shared between the two environments (39). In contrast, Truchado et al. (40) determined that the phyllosphere bacterial community of baby spinach was influenced more by the soil bacterial community than that of the irrigation water.

In addition to field soil contributing to the microbial community of crops grown in that matrix, other sources, both intentional and unintentional, may contribute both beneficial and pathogenic microorganisms to the crops. Two of the intentional sources introduced into crops in the field are manure and soil amendments. Added primarily for their nutrient content, these materials themselves have complex microbiotas and thus could contribute a new array of microbial groups into the soil that would then compete with the existing microflora for space and nutrients. Despite this dynamic interplay of nonpathogenic microbial communities, food safety specialists are more concerned with untreated manure, as domesticated animals' fecal waste is often contaminated with enteric pathogens, but not always predictably. For

example, the prevalence of *E. coli* O157:H7 in cattle feces has ranged from 0% to 57% (41). Due to this contamination risk, good agricultural practices (GAPs) and regulatory guidelines advise that one of the following measures be taken: (i) the manure be treated with some approved intervention to inactivate the enteric pathogens residing within the matrix or (ii) before application to fields, the manure be stored for some predefined interval that is appropriate for the specific region and field conditions in order to allow die-off of the pathogen (42).

Another major potential source for introducing microorganisms to fields and orchards is irrigation water (42, 43). The amount and type of irrigation water will vary with the crop and the aridity of the region. Types of irrigation water include municipal water, groundwater, collected rainwater, surface water (i.e., rivers, lakes, ponds, and reservoirs), and wastewater (43). In terms of microbiological quality, municipal water is considered one of the safest sources, although protozoan parasites may be present, as they are resistant to water treatment systems that use chlorine for disinfection (44). However, the chief disadvantages of using municipal water are its expense and unavailability in many developed countries. Groundwater is the next safest water source, and while it is used quite extensively in some areas, the quality and sustainability of natural groundwater reservoirs are threatened in some regions by excessive removal. In other production areas, surface waters may be used, but the quality of these waters is unpredictable, as discharges of wastewater, storm water runoff, and livestock or wildlife feces may occur in them. The degree to which irrigation water impacts microbial contamination of food crops depends on the method by which it is applied to fields and orchards, with methods that result in direct contact of the water with the phyllosphere tissue (i.e., spray and furrow irrigation) having a greater risk of contamination than those in which the water is applied directly to the soil (i.e., drip or subsurface drip irrigation) (45).

Soil amendments and irrigation water are two sources of contamination that can result in the entire crop being contaminated; hence, they are examples of sources of broadcast contamination (46). Other types of contamination that may occur during preharvest include directional contamination, whereby microorganisms in aerosols or in storm runoff reach the field either from adjacent urban areas or from adjacent land used for domesticated animal production or for manure composting operations (47–49). Under these conditions, there may be a gradient in pathogen populations, with the greatest concentrations at the site(s) of introduction. Another mode of preharvest contamination is pinpoint contamination, which occurs when fecal matter from wildlife is deposited at discrete locations or when pathogens are deposited on

plants from contaminated insects traversing a section of the field (46). This mode of contamination could also be used to describe the contamination of nuts that drop into fecal wastes left by domesticated animals grazing in the orchards. An additional mode of preharvest contamination is designated as cross-contamination, which occurs when biotic (i.e., humans) or abiotic (i.e., harvest machinery) vehicles are contaminated from an isolated contamination site and subsequently spread the pathogens to other plants within the field (46).

Considering all four modes of contamination, it is evident that there is a major difference among them in the extent of contamination that occurs within the crop. Hence, it is reasonable to assume that sampling plans designed to determine whether a field is safe for harvesting in each of these instances would likely have different probabilities for detecting the contamination.

Postharvest Sources

After crops are harvested, they are subjected to a range of operations that varies with the crop type and the desired end product. However, an element common to all of these operations is the introduction of spoilage and pathogenic microorganisms when facilities used to store or process the crop have not been properly sanitized (50).

Another contamination event that can occur during postharvest storage and processing is contact between one batch of product or its residues with another, which transfers portions of one batch's microbial load to the other batch. For example, Holvoet et al. (51) reported that in the absence of sanitizers, *E. coli* was transferred from lettuce to washing water and then from the water to lettuce that had not been contaminated with this organism. Poorly cleaned and maintained equipment may also harbor microorganisms and act as a reservoir for contamination, as it did when contaminated shredding equipment led to an outbreak of salmonellosis in Queensland, Australia (52). Equipment surfaces can have "hot spots" where buildup of food residues and microbial contaminants in fresh ready-to-eat vegetable processing occurs, especially at the conveyor belt and shredder (53). Postharvest operations identified by Castro-Ibáñez et al. (54) where cross-contamination may occur include produce selection, cleaning, washing, trimming, peeling, cutting, sanitizing, and packing. Regarding the packing operation, both spoilage and pathogenic microorganisms have been determined to survive on cardboard and plastic packaging materials (55). Hence, if these packaging materials become contaminated and are not sanitized before use, they may serve as a source of contamination.

FACTORS THAT CONTRIBUTE TO THE FATE OF MICROBIAL CONTAMINANTS

Inherent Properties of Individual Food Groups and Variability between Cultivars

Structural Properties

Agricultural crop plants are composed of many different plant cells, some of which provide protection from microbiological contamination and others of which permit access by microorganisms to inner tissues. When microorganisms are restricted to the crops' surfaces, water and nutrients are often limiting. These conditions, in turn, restrict their growth and direct microbes to shift their metabolism to a survival mode. Restricting entry of microorganisms from the surface to internalized tissues is one of the first lines of defense for food crops. For example, in nuts, seed coats or pellicles develop from tissues originally surrounding the ovule. These seed coats may be as thin as paper (e.g., peanuts) or thick and hard (e.g., coconuts). Another barrier that nuts possess is a relatively rigid outer casing or shell. In addition, some nuts (e.g., almonds, coconuts, pecans, pistachios, and walnuts) possess a hull whose pulpy tissue serves as a protective outer covering for the shell.

In most food crops, one or more layers of epidermal cells form the core outer structural covering and serve as the first line of defense to minimize microbial intrusion. In some crops, such as potatoes, several layers of epidermal cells form a corky layer protecting the underlying cells, whereas in other crops, such as citrus and melons, the epidermal cells form rinds whose thick structures offer a greater resistance to crack formation and thus penetration by spoilage microorganisms. Another common feature of epidermal cells in many crops is synthesis of a cuticle layer that forms on top of the epidermal cells. Composed of a lipid polymer impregnated with waxes, the cuticle can vary in thickness in different crops and hence has varying capacities to prevent contamination of underlying tissues by invading microorganisms. However, the main function of the cuticle is to serve as a barrier to prevent evaporation of water from the epidermal surface. Hence, vegetables from above the ground are more likely to have waxy cuticle skins than root and tuber vegetables.

Interrupting the outer layer of parenchyma cells or cuticle are natural openings known as stomata and lenticels. Stomata are openings that allow gas exchange between the inside and outside of the plant tissues, but the degree of opening is actively controlled by guard cells responding to water pressure within the cell. Lenticels, in contrast, are sunken openings in the outer covering that cannot be

closed. They also tend to be found on storage organs such as sweet potatoes, cucumbers, and other fruits and vegetables. Both types of openings have been suggested as entry points for both pathogenic and nonpathogenic microorganisms that occur at internalized tissue sites (56–59). Flowers are also entry points of pathogens for tomatoes, cucumbers, and wheat heads (60–62). However, these modes of entry are different from those seen in roots, where microorganisms infiltrate and translocate to the edible tissues. Infiltration of microorganisms through roots can occur in hydroponic systems (63) but is less likely in natural soil matrices given that in order for it to occur, either the microbial exposure levels must be extremely high (64, 65) or the roots have to be wounded (63).

Attachment of microbes to nonwounded aerial plant surfaces is not uniform, and one of the major contributing factors is presumed to be differences in localized physical topography. In general, preferential residence of microorganisms on leaves occurs at the base of trichomes, on the outer rims of stomata, and in cell grooves along veins (66), whereas in certain fruits, *E. coli* O157:H7 cells were found entrapped in the grooves or cavities (67). Another study that revealed localization at selected niches determined that *Salmonella* had a higher affinity for older than younger romaine lettuce leaves, as the former was characterized as having greater topographic complexity (68). Once present in these niches, the microorganisms have substantial protection against dehydration (69). Hence, leaf blade roughness correlated positively with the persistence of *E. coli* O157:H7 on different spinach cultivars (70). Similarly, minimization of dehydration explained the preferential location of *Salmonella* on romaine lettuce leaf regions close to the petiole compared to surfaces at the far end of the leaf blade (68).

The observed results of microbial attachment and fate studies on plant tissues are dependent on the method used to introduce the microbes to the plant tissue. For example, many studies submerged the tissue in a suspension of the microorganisms under study. This method is representative of postharvest washing in the absence of a disinfectant but is not typical of a route of preharvest contamination such as spray irrigation. Ultimately, these two modes of contamination (i.e., dipping and spraying) result in different patterns of microbial deposition. For example, bacteria remained at the site of initial contact when administered in small aerosol droplets (average volume of 750 μm^3), but when bacterial cells were applied in large droplets (>5 μl), the microorganisms collected through the forces of gravity at sites of indentation (71). The collection of water and microbes at grooves and indentations also occurs in response to the repellency of the hydrophobic epicuticular waxes

present on plant surfaces, which are constituents that do not favor plant-bacterium interactions (72).

Under the premise of developing more effective practices for preventing microbial contamination of plant surfaces, studies involving microfabrication of plant surface structures and simulation exercises involving a Lagrangian particle tracking model have been conducted with the purpose of systematically varying parameters of interest and avoiding the confounding influences of natural variation (69, 73, 74). As an example, silicon surfaces were microfabricated to study attachment of *E. coli* to trichome-and stoma-like structures and grooves between epidermal cells (73). Results from the study revealed that more bacteria attached at the base of the trichome and at a location 2.5 to 5.0 μm from the stoma opening. However, no localization was observed for *E. coli* at grooves. The investigators provided a completely different perspective by concluding that surface protrusions (i.e., trichomes) decreased attachment, whereas openings (i.e., stomata and grooves) created shear gradients that increased attachment (74). More specifically, the simulation results revealed that trichomes decreased attachment by reducing shear stress, whereas stomata and grooves enhanced attachment by creating small regions of increased shear stress.

Chemical Properties

In addition to surface topography, the fate of attached microorganisms is dependent on the accessibility of the microbe to the crop's water and nutrient content. Fruits and vegetables are characterized as generally having a high water content (>95%). Therefore, when the microorganism is able to gain access to internal tissues where the water is stored, inactivation due to desiccation stress is no longer an issue. Instead, there is the potential for microbial growth, as fruits and vegetables also contain significant amounts of nutrients essential for that activity. However, the type of nutrients available depends on the type of fruit or vegetable and the rate at which it starts to metabolically break down from its optimal state.

For metabolic breakdown, fruits and vegetables are classified according to their respiration rate (75). For example, commodities with a high respiration rate that equates to >20 ml of CO_2/kg/h at 5°C (e.g., strawberries, raspberries, Brussels sprouts, spinach, broccoli, asparagus, and mushrooms) have a shorter shelf life than those with a low respiration rate, i.e., one that equates to <10 ml of CO_2/kg/h at 5°C (e.g., apples, citrus fruit, potatoes, onions, garlic, carrots, cantaloupes, and watermelons). Metabolic breakdown of the fruit or vegetable is important in that many of the structural barriers that had prevented access of microbial contaminants to internal tissues are eroded. Hence, microbial

degradation of the product becomes an issue of concern. However, in the case of fruit, initiation of this metabolic breakdown is also influenced by the maturity status at which it is harvested. For example, nonclimacteric fruit (e.g., strawberries, grapes, and cherries) are picked at their horticultural maturity, when consumers may immediately consume them. In contrast, climacteric fruits (e.g., apples, tomatoes, avocados, and bananas) are harvested at an immature state. Then, during storage, ethylene is produced through respiration, and that, in turn, leads to horticultural maturity (76). However, once horticultural maturity is achieved for either fruit type, deterioration of the product (i.e., senescence) ensues, making the fruit more susceptible to microbial invasion.

Although agricultural crops are susceptible to microbial invasion and deterioration, plants have at their disposal variable levels of plant defenses that help prevent a microbe's residence and proliferation. These plant defenses can be divided into those that are constitutive (i.e., components that are present in the plant prior to its being exposed to the microorganism) and those that are activated (i.e., components that are generated when the plant senses that a microbe is in its vicinity). Constitutive defenses include the structural barriers discussed in "Structural Properties" above as well as the presence of antimicrobials (e.g., phenols, tannins, glucosinolates, etc.). An example in which such antimicrobials were suggested to have contributed to reduced survival of *Salmonella* was a study in pine nuts, hazelnuts, and pecans (77). In this study, it was noted that *Salmonella* was in contact with the polyphenolic-containing skin of hazelnuts and pecans, thereby increasing the rate of *Salmonella* inactivation, whereas pine nut kernels do not have this skin, and *Salmonella* survived in them for a longer period of time. However, demonstrating the effects of antimicrobials in plant resistance has been difficult because of the heterogeneous distribution of these compounds in plant tissues and therefore the difficulties in correlating their levels to the degree of inhibitory activity for invading microorganisms (78).

In contrast, activated plant defenses are expressed through a process known as microbe-or pathogen-associated molecular pattern (MAMP/PAMP)-triggered immunity and effector-triggered immunity. MAMP/PAMPs are common proteins found on invading microorganisms that are recognized by receptors in the plants. Once recognized, a cascade of reactions is initiated to limit the invasion (e.g., stomatal closure) and kill the microorganisms (e.g., generation of reactive oxygen species). For more information about the nature of these plant defensive reactions, see the study by Peng et al. (79). However, the level at which the plant's immune system routinely participates in thwarting microbial invasions in field environments has not been fully elucidated. Still,

these defensive reactions are likely constantly being stimulated to minimize microbial threats but are effective only against threats involving small numbers of invading microorganisms. Hence, when plants are exposed to large numbers of microbes (>3 log), as often occurs in an experimental study, the results are often not an accurate representation of what is likely occurring in the field.

Processing and Mechanical Damage

Once crops are harvested, they are often subjected to one or more operations that will provide a product that is distributed for institutional and consumer preparation and consumption. These operations, as described below, directly affect the fate of microbes contaminating the product. However, physiological processes are also often accelerated by these operations, and their activities indirectly affect microbial activities and the fate of those microbes. Hence, physiological and microbial activities are often intertwined, and their respective roles are difficult to separate. Given the widely differing set of operations and physiological activities associated with each of the crop types addressed in this chapter, the fruits and vegetables, the nuts, and the grains are discussed separately below.

Fruits and Vegetables

Processing of fruits and vegetables involves a series of operations. For products that are considered minimally processed and will be consumed in the raw state, operations are primarily restricted to washing and cutting or removal of outer barriers (i.e., destruction or removal of surface cells). At this stage, many types of fruits and vegetables undergo additional processing operations, some of which involve subjecting the product to heat or extreme cold (e.g., blanching, canning, steaming, freezing, etc.) either in the processing plant, in an institutional setting, or in the home. Such operations generally delay or halt any microbial activity that may be present at the time the operation is performed. The product, if properly stored under recommended conditions, then has a defined quality state and is safe to consume. Hence, microbial contamination is of greater concern in minimally processed fruits and vegetables that do not receive a treatment to kill pathogens and many spoilage microbes and are to be consumed raw.

Washing fruits and vegetables in chlorinated water is one of the more common operations applied to harvested items in the field and in minimal processing plants. Its primary function is to remove soil and other debris from the product; however, it also removes some, but not all, of the microbial load on the surface (80). In particular, substantial numbers of microorganisms remain in hollows at the junctions of epidermal cells and in folds in the epidermis.

Although any reduction in microbial loads may be beneficial from the standpoint of extending shelf life or reducing risk associated with consumption of pathogen-contaminated stored product, these advantages are manifested only when the wash water contains a disinfectant that is capable of destroying the microorganism before cross-contamination of previously noncontaminated product occurs. Further discussion as to the types of disinfectants used in wash water to kill dislodged microorganisms is addressed in “Oxidizing treatments” below.

Additionally, microbial dislodgement from produce surfaces may be improved by applying a suitable mechanical washing process in combination with a selected surfactant that enhances the wettability of the surfaces. This concept is supported by research using a benchtopscale washing device that revealed detachment of *E. coli* O157:H7 from inoculated leaf samples increased with increasing shear stress and the addition of a surfactant to the washing solution (81).

Another concern associated with fruits and vegetables is the potential for internalization of microorganisms during washing or cooling of the product with nondisinfected water (i.e., hydrocooling or vacuum cooling). For example, enhanced internalization can occur through the stem scar of numerous fruits (e.g., apples, avocadoes, mangoes, oranges, and tomatoes) when the product is washed with water colder than the product temperature (64, 82, 83). Under these conditions, a negative pressure differential is produced as air spaces within the product are contracted, allowing water to seep in. In the case of vacuum cooling applied to crops (e.g., lettuce, spinach, and celery), cooling occurs when the applied vacuum evaporates water at the surface; however, water is sprayed on the product to replace this moisture loss. A study by Li et al. (84) revealed that this method of cooling is risky, as there was greater infiltration of pathogens from contaminated water sprayed on vacuum-cooled lettuce than when the product was not cooled by vacuum. Similarly, increased internalization occurred when produce having exposed internal tissue was washed in contaminated water (85). These results from internalization studies highlight the importance of maintaining effective levels of sanitizing agents in produce wash or cooling waters.

Even under processing conditions in which disinfectants are used in wash and cooling waters, operations that adversely affect the integrity of the fruit or vegetable also change the dynamics of the food system and the extent to which microbial contaminants can infiltrate tissue and proliferate. More specifically, unit operations of cutting, peeling, slicing, shredding, and removing outer skins and rinds inflict damage to the product. In turn, this damage can accelerate the respiration rate of the product and associated deterioration, promote the

release of nutrients or antimicrobials from plant cells, and increase the surface area on which microbes may reside and proliferate (86). A study has revealed that increased microbial growth occurred on carrots cut with blunt machine blades as opposed to sharp machine blades or razor blades, presumably through an increased release of exudates from the carrots (87). Similarly, mechanical abrasion peeling of carrots resulted in greater microbial contamination and more rapid microbial growth than hand peeling.

Nuts

Both labor-intensive and highly mechanized processing operations are available worldwide to harvest and process nuts; however, the methods that are applied vary significantly among the various types of nuts. This section focuses on different types of nuts for which at least one harvest or processing operation is distinctly different from the others.

Two distinct methods of harvesting nuts are available. For pistachios, the nuts are harvested directly from the trees by mechanically shaking the trees and collecting the nuts on a V-shaped catch frame. From there, the harvested nuts are collected on a conveyor belt and transferred to a plastic bin directly into a gondola trailer. The second method may also involve shaking the trees, but in this case, the nuts fall to the ground. After this operation, pecans are swept into windrows, where they are picked up by a mechanical harvester and subsequently transferred to a hulling facility. Almonds are also swept up from the orchard floor but they may first be left on the ground for a short period to partially dry by solar radiation. The orchard floors have the potential to be contaminated with an array of both spoilage and pathogenic microbes that are deposited there by domestic-or wild-animal wastes or by runoff from adjacent contaminated land. Hence, nuts can be contaminated when they land on these contaminated sites, or contaminated soils picked up during collection of the nuts may contribute to contamination in the hulling facility.

Once at the hulling facility, both the pecans and almonds are separated from debris using forced air and sifting operations, but aerosols generated during these cleaning operations may contaminate other equipment in the facility. Pecans that are to be stored and sold in the shell are mechanically dried, whereas wetting of the pecans to soften the shell before cracking is used to minimize breakage and facilitate removal of the intact kernel halves. A wet process is also used to assist in removing the hulls of pistachios with an abrasive peeler. Good-quality pistachio nuts sink to the bottom of a float tank, where they are then moved to a heated drier. However, concerns with water-assisted hull removal are the same as those for washing of fruits and vegetables. Specifically, the lack of sufficient sanitizer in the water may

facilitate cross-contamination between contaminated and noncontaminated materials. In addition, salmonellae can subsequently infiltrate in-shell nuts and reach the kernel, where they survive after drying (88). However, mechanical separation of hulls from the kernel under dry conditions, as is done for almonds, is not without its own safety issues. In particular, large amounts of fine particulate matter are produced, and this matter is extremely difficult to eliminate from the processing environment and thus could potentially contribute to the microbial contamination of kernels.

Grains

Many different types of grains are grown throughout the world (e.g., barley, maize, millet, oats, quinoa, rice, rye, sorghum, soybeans, teff, and wheat), and each may be subjected to processing operations that vary from being very labor-intensive to highly mechanized. Hence, there are many permutations in the processing of grains that could be discussed. However, the focus of this section is on the processing of wheat and how those operations affect the fate of microbes and their toxins.

Three general operations are applied to wheat: cleaning, tempering, and milling. In the cleaning process, a sequence of operations, involving aspiration and screening, is applied to wheat to remove unwanted materials (e.g., sticks, stems, stones, unsound wheat kernels, attached soil, and insects) based on their shape, density, size, and magnetism. Depending on the level of contamination of these impurities with spoilage microbes, mycotoxins, and pathogens, the degree of their reduction is variable (89). For example, no differences in *Aspergillus* contamination were observed between unclean and cleaned Algerian wheat, with fungal counts ranging from 2.7 to 2.9 log CFU/g (90). In contrast, there was a 53 to 62% reduction in two mycotoxins after cleaning of durum wheat in a study by Pascale et al. (91).

The second stage of processing of wheat, tempering, involves spraying the grain with precise amounts of water to reach a desired moisture level and then holding the wetted material in tempering bins for specific holding periods (6 to 18 h) to soften the surface of the grain, toughen the bran, and soften the endosperm. Tempering is a critical conditioning step in processing of grain, because improper implementation of this step can encourage microbial growth either in the wheat or on the milling equipment.

Once the grain is conditioned, milling occurs, which involves a series of reduction, grinding, and sifting operations designed to separate the germ and the bran fractions from the endosperm. Overall, milling does not reduce microbial or mycotoxin contaminants in the grain; rather, it redistributes contaminants, with the milled fractions more commonly used for human

consumption (flour and semolina) having lower levels than the germ and bran, which have higher levels (92). In general, the extent of the redistribution depends on the variety of wheat, the degree of penetration of mycotoxin-producing molds, transfer of mycotoxins to the inner parts of the kernel, and flour extraction rate (93). Historically, higher microbial and mycotoxin levels in the germ and bran fractions were not of concern, as these fractions were less likely than flour to be used in food production. This practice has been changing as the consumer desire for more whole-grain products increases, owing to their known health benefits (94).

Once flour is produced, the products made from this ingredient generally involve the application of heat, including baking, frying, cooking, steaming, or extrusion. Such thermal treatments are sufficient to kill vegetative microbial cells and thus reduce the number of spoilage and pathogenic microorganisms that may be present. However, both mycotoxins and bacterial spores of the genus *Bacillus* are highly resistant to thermal treatment (95–97). Survival of *Bacillus* spores in baked goods is of concern from both a spoilage and safety perspective. In the former case, germination of heat-resistant *B. subtilis* spores may occur under conditions of prolonged cooling or improper storage that ultimately results in a ropy baked good. In the latter case, *Bacillus cereus* spores may germinate under similar improper storage conditions, with the vegetative cells producing toxins (96).

Over the past decade, a new problem that has come to the forefront of the food industry is the potential for enteric bacterial pathogens to contaminate flours. Although incapable of multiplying in this low-water-activity product, they are capable of surviving in a dormant state for long periods of time (30). Furthermore, they retain the ability to multiply when flour is incorporated into higher-moisture environments, such as batters and mixes. Pathogen contamination of flour would not be an issue if consumers were to abide by educational and label statements that indicate there is a health risk if flour-based products are eaten in the raw rather than cooked state (89).

Environmental Conditions

Environmental conditions (temperature, atmosphere, and relative humidity) have been used for decades to control the physiological activity of fruits and vegetables, with the intent of delaying ripening and senescence and prolonging shelf life (3). These conditions are now well established and have been extensively reviewed (1). For example, holding temperatures near 0°C are recommended for most commodities. The exceptions to this guideline are products of tropical origin and produce items that suffer from physiological disorders (chilling injuries) when stored at refrigeration temperatures. For

those products, the optimal storage temperature is close to 10°C. Fortunately, storage at these low temperatures is beneficial for reducing the growth of many spoilage and pathogenic microorganisms. However, maintaining a consistently low temperature throughout the distribution chain is challenging. For example, a study of the holding temperatures of fresh-cut leafy greens transported from California and Arizona to distribution centers across the United States revealed that all16 shipments would have been rejected based on the Food and Drug Administration (FDA) standard of maintaining leafy greens at 5°C during shipment (98). Furthermore, applying time-temperature profiles obtained during commercial transport, retail storage, and display to commercial bags of romaine lettuce mix contaminated with *E. coli* O157:H7 and *L. monocytogenes* led to an increase in pathogen population of nearly 2 log CFU/g (99).

Environmental storage temperatures have also been influential in affecting microbial survival and growth in the low-moisture food groups of nuts and grains, respectively (100, 101). For example, increased survival of *Salmonella* was observed as temperatures of pecans, hazelnuts, and pine nuts decreased from 25°C to 4°C (101). However, fungal growth exhibited varying minimum and optimal temperatures dependent on the species but ranged from 0 to 12°C and 20 to 35°C, respectively (100). Minimal temperatures for mycotoxin production by each of the mycotoxin-producing fungal species were in almost all cases higher than their minimal growth temperatures. In contrast, the optimal temperatures for mycotoxin production were either similar to or lower than those documented for optimal growth.

Another environmental condition that affects the quality of produce is relative humidity. For most produce, garlic and onions being examples of exceptions, the relative humidity should be 90 to 100% (3). Otherwise, water loss will occur and many produce items will lose their turgor and be limp. The disadvantage is that high humidity also favors the survival of microbes and potentially leads to growth if favorable temperature conditions and nutrients are also present. Hence, when nuts and grains are stored, the relative humidity should be maintained such that the water activity is below the minimum needed for growth of microorganisms. In line with this recommendation, the minimal and optimal aw for fungal growth/mycotoxin production range from 0.70 to 0.94 and 0.90 to 1.00, respectively (100). However, decreasing the a_w to extremely low values is not necessarily beneficial, as *Salmonella* survives longer in tree nuts at an a_w of 0.4 than at 0.6 (101).

Modified atmospheres are also used in combination with chilled temperatures to reduce the physiological activity of fresh produce. To achieve this state, CO_2 concentrations recommended for produce storage rarely exceed 10%, and 1 to 5% oxygen is tolerated (1). Higher CO_2 or lower O_2 concentrations often lead to

physiological disorders that, in turn, lead to loss in quality, but the level at which this occurs varies with the horticultural crop, as different products have different tolerances to these gases (1). Similarly, the response of microorganisms to these gases is not consistent (3). As a result, adjustments made to the atmosphere during storage of produce must consider their effects on both the plant tissue physiology and the microorganisms' metabolic systems.

Variation in Resident Microbes

Microbiological spoilage of foods involves the breakdown of the food through the action of hydrolytic enzymes released by microorganisms into the food. Although many microorganisms may be present in the food and contribute to the hydrolytic activity, each microbial strain could potentially express a different profile of hydrolytic enzymes. For example, in a study conducted by Al-Najada and Gherbawy (102), 33 fungal isolates were obtained from 40 fruit and vegetable samples and were subsequently assigned to 21 fungal genera through sequencing of their internal transcribed spacer regions. These isolates were then screened, and 85%, 79%, and 73% of the isolates produced xylanase, amylase, and cellulase, respectively. Although only a qualitative assessment for these enzymes was performed, it is likely that the enzymes produced by these isolates also had varying degrees of activity. Hence, it would be difficult to assess the potential damage that a population of fungi would exert if present in a food. Similarly, discovering that bread is contaminated with *Bacillus* does not provide sufficient proof that ropy spoilage could occur, as five of 15 strains of *B. subtilis* and two of six strains of *B. licheniformis* were unable to cause ropy spoilage of laboratory-based bread (103).

Even when only one microbial species may be responsible for a spoilage defect, wide ranges between isolates' metabolic capacities to create a spoiled state can occur. Notably, *Lactobacillus buchneri* is detrimental to preserving cucumbers by fermentation, as this bacterium metabolizes the lactic acid produced during fermentation into acetic acid and 1,2-propanediol (104). However, when eight *L. buchneri* isolates were compared, all were capable of degrading lactic acid, but they differed in their rate and extent of lactate degradation (105). Biodiversity in bacterial growth rates was also observed when three *Bacillus amyloliquefaciens* strains were inoculated into various pan bread recipes (106). Therefore, on the premise that biological diversity is an inherent characteristic of microorganisms in general, one should be cautious in comparing inactivation or spoilage rates from separate challenge studies when different isolates of a microbial species are used in those studies.

INTERVENTIONS

Since horticultural crops are usually grown in environments where there is constant exposure to external sources of microbial contamination, it is inevitable that the microorganisms will be transferred to those crops. Considerable research has been conducted on how to minimize crop losses due to plant pathogen activity, to delay loss of food quality due to spoilage microbe activity, and to minimize the distribution of unsafe product due to human enteric pathogen contamination. These intervention efforts are addressed below in two categories: prevention of contamination and reduction of postharvest contamination.

Prevention of Contamination

To address contamination challenges posed at the preharvest and postharvest stages during the production and processing of fruits, vegetables, nuts, and grains, the principles of GAPs, good manufacturing practices (GMPs), and hazard analysis and critical control points (HACCP) remain the prerequisite cornerstones of food safety management strategies to establish safe practices. Each of these programs has played a unique role toward achieving a safe food supply.

GAPs are considered guidance documents (i.e., not required by the government) and are written either by the industry or with input from the industry on recommended practices that growers should apply in the production of fresh produce. These documents focus on areas of production that potentially contribute to contamination of the product. In some cases, specific guidance (metrics) may be provided, but in general the guidelines have not been scientifically validated. Despite these limitations, growers have been encouraged to adopt GAPs to help assure buyers that the produce was safely produced and could offer some protection against liability claims.

In contrast to GAPs, which are strictly voluntary, GMPs are regulated by the U.S. FDA and apply to processed fruits and vegetables but not to fresh fruits and vegetables. GMPs include many operational conditions and procedures that are common among all food manufacturing facilities, including (i) the construction and layout of the site, (ii) condition of the external environment, (iii) adequate maintenance of equipment and utensils used with the facility; (iv) avoidance of foreign matter within the finished product; (v) waste disposal sites that are clearly identified; and (vi) a routine schedule for pest control and hygiene practices to ensure the cleanliness of the facility. GMPs have their greatest impact when the procedures are clearly described in written documents and records of their implementation are kept routinely. For

additional information on this topic, an extensive description of GMP-related control measures that may be used in low-aw-food production environments has been provided in a review by Beuchat et al. (30).

Although GMPs constitute the operational requirements necessary to enable a food business to produce food safely, they also are a requisite component of HACCP programs. However, for the commodities being considered in this chapter, HACCP is required by law only for producers of fruit juices. HACCP is considered a tool to identify within a food manufacturing facility specific hazards and the critical control points (CCPs) for controlling those hazards. Subsequent steps in applying HACCP are to monitor the CCPs, take corrective action on deficiencies in the CCPs, if needed, verify that the processing procedures are working appropriately, and maintain accurate records. However, HACCP programs are not feasible to apply to primary produce production because of the lack of applicable CCPs.

In lieu of HACCP, the U.S. FDA developed the produce safety rule, which is part of the Food Safety Modernization Act (FMSA) and has enforceable standards for growing, harvesting, packing, and holding produce for human consumption. This rule was enacted in 2011, but not all fresh-produce-growing operations will be subjected to the rule, as farms having less than $25,000 in sales are excluded.

Additional information on the produce safety rule is found in chapter 42 of this volume. Additional approaches that have been used to prevent microbial contamination of crops in the field are to alter the plant's susceptibility by applying treatments to the plants or by breeding plants to be resistant to the microorganisms causing the damage. An example of a treatment to protect plants from plant pathogen invasion is the application of calcium or a calcium solution directly to a fruit or vegetable such that absorption of the calcium by the plant's tissues reinforces their pectin bonds, thereby making their cell walls more resistant to the pectic enzymes of plant pathogens (107). Hence, soft rot caused by *Erwinia carotovora* subsp. *atroseptica* on potatoes and growth of *P. expansum* on apples are controlled. An example of applying plant breeding to prevent damage by plant pathogens is the breeding of peanuts to have increased tannin content, amino composition, waxy surfaces, and cell structure arrangement, thereby enabling the peanuts to resist fungal invasion (108). More recently, several acquisitions of germplasm resistant to the pathogens that cause blue mold and bitter rot of stored apples have been obtained and are envisioned to serve as a foundation for a conventional breeding program to develop apples resistant to blue mold rot (109).

Reduction of Contamination on Produce

One of the most prolific areas of research over the past decade has been the development and evaluation of interventions that would either reduce the extent of microbial contamination or prevent the growth of existing microbial contaminants in fruits, vegetables, nuts, and grains. These interventions can be grouped into three categories: physical, chemical, and biological. A fourth group of interventions consists of applications, in combination or in sequence, of two or more interventions from the three intervention categories, which provides a synergistic response by employing different mechanisms of inactivation of the microorganisms.

A brief overview of the mechanisms of action of each of the types of interventions and their advantages and limitations is provided below. Considering the large number of studies that have been conducted on interventions over the past decade, only selected studies are discussed to provide examples for each of the commodity groups. Additional information on these interventions is provided in a number of recent review articles that are listed in Table 7.5.

The efficacy of postcontamination interventions is assessed primarily by observing the initial microbiological reduction achieved as well as the continuation of this reduction during storage. However, the success of an intervention should not be judged solely by its efficacy. The occurrence of undesirable quality effects following application of the intervention would eliminate it as a useful treatment. Similarly, if the cost of the intervention increases the cost of the product to adversely affect sales, the treatment would not likely be applied for routine use. Hence, the interplay of these three criteria (i.e., microbiological efficacy, quality losses, and treatment costs) should be considered when the likely success of an intervention is being evaluated. Very few research papers address the costs of new interventions, ignoring the expense of equipment needed to perform an intervention treatment or the costs that recur per treatment. Under these circumstances, a risk assessment (described in more detail in “Risk Assessment” below) should be conducted to determine whether the public health benefits would warrant the implementation of an expensive intervention. If not, then continued research with that intervention would best address opportunities to reduce its cost.

Physical Treatments

Thermal treatments

Refrigeration has long been used as the most convenient and effective means to reduce physiological activities of plant tissues, to reduce the growth of spoilage and pathogenic microorganisms, and thus to extend shelf life without

compromising the safety of the product (24, 86). Alternatively, these benefits may be accomplished by heat treatments, but their applicability will depend on their effect on the targeted product. For example, one of the qualifying descriptors of fresh-cut fruits and vegetables is that heat not be applied, as it would alter their quality. Canned, blanched, and frozen vegetables and juices, on the other hand, are commonly subjected to heating operations that will reduce if not eliminate resident microbial populations. However, short-term exposure to heat (e.g., a few seconds at 60 to 62°C; 120 min at 45°C) has also been used with some success to reduce postharvest spoilage of a wide range of fresh produce, including green pepper, apple, and citrus (110, 111). Application of superheated steam (85°C for 240 s) to cantaloupe rind also decreased surface-inoculated *Listeria innocua* by nearly 4 log CFU/cm^2 (112). However, a limitation of that type of treatment is that heating also decreases the competitive indigenous microflora (e.g., yeasts, molds, and coliforms) to levels that would allow the proliferation of harmful microbes such as listeriae that may be introduced to the rinds postheating.

In crops such as nuts and grains, the application of heat has been routinely used as a treatment in their processing. There is renewed interest in applying these thermal treatments to low-a_w foods such as nuts and grains in which *Salmonella* and mycotoxins are known to have a high degree of heat resistance. For example, Beuchat and Mann (113) determined that hot-air treatments could not be relied upon to reduce *Salmonella* by 5 log CFU/g on raw pecan nutmeats without adversely affecting their sensory qualities, whereas temperatures and times typically used to oil-roast nutmeats were sufficient to reduce the pathogen by 5 log CFU/g. Similarly, hot-air drying of pistachios has been viewed as being insufficient to ensure their microbial safety, whereas sequential infrared drying, tempering, and hot-air drying could shorten the drying time while reducing the levels of a surrogate of *Salmonella* by ca. 6 log CFU/g kernel (114).

For grains, the application of dry heat postharvest generally can decrease the microbial load of spoilage organisms without compromising the grain's quality (115). However, such treatments require considerable amounts of energy and may last for several days. Furthermore, the conditions must be adjusted carefully to achieve sufficient microbial decontamination while maintaining grain quality. Other disadvantages to the use of dry heat for grain processing are its inefficiency in killing spores of heat-resistant bacteria and its inability to destroy mycotoxins produced in the field (115). In contrast, superheated steam is a more efficient treatment for microbial decontamination and mycotoxin degradation. For example, when naked oats were subjected to superheated steam (160°C for 2 min), the numbers of surface bacteria and mold were

reduced from 4.26 to 2.44 log CFU/g and from 4.02 to 2.34 log CFU/g, respectively, which were both below the acceptable safe storage numbers for this grain (116). Similarly, exposure of wheat grain for 30 s to superheated steam at 110°C or higher reduced mold populations to undetectable levels in the product, and higher temperatures (170 or 200°C) killed *Bacillus* spp. (117). For mycotoxin degradation, deoxynivalenol was reduced by up to 52% by treatment at 185°C for 6 min (118). However, optimal parameters for superheated steam treatments in different matrices still need to be determined. Additionally, the safety of the degradation products of mycotoxins thermally degraded by superheated steam is unclear, as the potential remains that they are as toxic as the original mycotoxin (115).

Table 7.5 Selected review articles (2017 to 2018) that address the efficacy of postharvest interventions applied to fruits, vegetables, nuts, and grains

Type of interventions addressed	Title (reference)
General	Application and kinetics of ozone in food preservation (161)
	Essential oils: sources of antimicrobials and food preservatives (163)
	The hurdle approach-a holistic concept for controlling food safety risks associated with pathogenic bacterial contamination of leafy green vegetables. A review (180)
Fruits and vegetables	Challenges in the microbiological food safety of fresh produce: limitations of post-harvest washing and the need for alternative interventions (124)
	Ready-to-eat vegetables: current problems and potential solutions to reduce microbial risk in the production chain (54)
	Recent developments in novel shelf life extension technologies of fresh-cut fruits and vegetables (4)
	Nonthermal physical technologies to decontaminate and extend the shelf-life of fruits and vegetables: trends aiming at quality and safety (131)
	A review on microbiological decontamination of fresh produce with nonthermal plasma (136)
	Postharvest ozone application for the preservation of fruits and vegetables (181)
	Plant-based edible coatings for managing postharvest quality of fresh horticultural produce: a review (165)
	Bacteriocins from lactic acid bacteria and their potential in the preservation of fruit products (182)
Grains	Current and future technologies for microbiological decontamination of cereal grains (132)
	Critical factors responsible for fungi growth in stored food grains and non-chemical approaches for their control (129)
	Recent advances in physical post-harvest treatments for shelf-life extension of cereal crops (115)

Type of interventions addressed	Title (reference)
	Mold spoilage of bread and its biopreservation: a review of current strategies for bread shelf life extension (183)
	Biocontrol of mycotoxins: dynamics and mechanisms of action (184)
	Mycotoxin occurrence in grains and the role of postharvest management as a mitigation strategies. A review (24)
	Deoxynivalenol and its masked forms: characteristics, incidence, control and fate during wheat and wheat based products processing-a review (185)

Modified atmosphere packaging

Modified atmosphere packaging (MAP) is packaging with modification of the gas composition surrounding the product inside, with the intent of extending the shelf life of the product. Used for over 90 years (4), the atmospheres that have been typically applied are discussed in "Environmental Conditions" above. Both active and passive MAP can be used to create the desired atmosphere, which is a function of the product type, the packaging materials, and the storage temperature. In active MAP, the target atmosphere is created by either displacing or replacing the gases in the package with those that are desired, or scavengers or absorbers are added within the package to remove a specific gas. In passive MAP, the desired atmosphere develops naturally as a result of the product's respiration and the diffusion of gases through the permeable packaging film (119).

Oxygen, CO_2, and nitrogen are the gases most commonly used in MAP. Nitrogen is considered an inert gas, with its primary function being to prevent the package from collapsing. Other gases that have been used as gas mixtures for MAP include carbon monoxide, argon, helium, and nitrous oxide (4). Due to the higher density of noble gases, increased diffusivity of O_2 and CO_2 from plant tissues is likely to occur and, through this mechanism, would affect plant tissue metabolism. For example, packaging fresh-cut watercress in noble gas-enriched MAP modified its respiration rates, whereas there was no effect on the growth of psychrotrophic microbes and *Enterobacteriaceae* (120). Another variation on the traditional gas atmospheres of MAP is the application of high-O_2 MAP in lieu of low O_2 concentrations, which can stimulate the growth of anaerobic psychrotrophic microorganisms. However, the success of this approach is dependent on the spoilage microbe of interest. For example, growth

of the yeast *Candida sake* on fresh-cut honeydew melon cubes was greatly suppressed by the presence of 50% or 70% O_2 in the atmosphere, whereas growth of the lactic acid bacteria *Leuconostoc mesenteroides* and *Leuconostoc gelidum* was affected to only a minor extent (121).

Irradiation

Gamma rays, X rays, and electron beams are all considered different forms of ionizing radiation because they are electronically charged atoms or molecules. They differ from each other in the manner in which they are produced. In the case of gamma irradiation, the rays are produced from the degradation of the radioisotopes of cobalt-60 or cesium-137. In contrast, X rays are produced by accelerating electrons through a potential difference onto a target material (e.g., tungsten) where, upon slowing, X rays are released. Electron beams are generated from machines capable of accelerating electrons very close to the speed of light at high energy levels (0.15 to 10 MeV) in a vacuum environment. Once formed, the mechanisms by which they interact with foods and microorganisms are similar. More specifically, the principal target of the ionizing radiation is water that breaks down to produce free radicals that, in turn, react with organic matter. Inactivation of microorganisms occurs when the free radicals disrupt the organisms' DNA structure and denature their enzymes and membrane proteins, resulting in the loss of their reproductive capabilities and other functions of the cell.

The main advantages of electron beam irradiation are faster operation, lower irradiation dosages, and the use of electricity rather than radioactive materials to generate the ionizing radiation. Hence, electron beam irradiation is considered more flexible and easier to use. Its chief disadvantage is that its penetration power is less than that of gamma irradiation, rendering the former a tool restricted primarily to surface disinfection. Despite this limitation, electron beam irradiation is allowed for the treatment of vegetables, fruits, and grains in many parts of the world, with the exception of Europe. However, there are restrictions on the levels of irradiation that may be applied. For example, the FDA caps the irradiation dose on most fresh fruits and vegetables at 1.0 kGy but allows fresh lettuce and spinach to be irradiated at 4.0 kGy. Doses of 0.1 to 0.5 kGy are permitted by the FDA for the treatment of insect infestations of wheat and wheat flour.

Many studies have during the past 2 decades addressed the irradiation of fruits and vegetables; examples are provided in the review articles listed in Table 7.5. The irradiation dose is positively correlated to the degree of killing of microorganisms; however, different microorganisms have different tolerances to this treatment. For example, among vegetative bacteria, Gram-positive

bacteria are more resistant to irradiation than Gram-negative bacteria (122). As another example, populations of *Salmonella enterica* serovar Poona were significantly reduced in fresh-cut cantaloupe by electron beam irradiation; however, at the same dosage, yeasts were not reduced significantly (123). Viruses, endospores, and enteric protozoa are also very resistant to irradiation (124). For example, an irradiation dose of 1 kGy reduced *E. coli* O157:H7 populations by 5 log CFU in bagged spinach (125), whereas spinach treated with a dose of 2.5 kGy resulted in <1 log reduction of rotavirus (126). The sensitivities of fungal species in grains, nuts, and fruits to irradiation also differ substantially (115, 127), although the form of fungal contamination (mycelium or spores) as well as the moisture content of the commodity may contribute to the reported differences (115). Uneven dose distribution may also be a contributing factor in disparities among irradiated nuts. Karagöz et al. (128) determined that the D_{10} values (dose required to achieve 1-log reduction in population) of *Salmonella* and *E. coli* inoculated on the flat surfaces of pecans were 13 to 44% greater than bacteria inoculated within the dorsal grooves of the pecan, which absorbed higher doses of irradiation. However, irradiation is generally regarded as being effective in controlling fungi (129). In contrast, many mycotoxins are not affected substantially by irradiation (130), with aflatoxin and ochratoxin being more insensitive to irradiation than *Fusarium* toxins (115).

Despite irradiation being recognized as an effective intervention step for microbial decontamination, its use commercially has been minimal. A major concern has been lack of consumer acceptance. Most irradiated foods must be labeled as having been irradiated, which deters consumers from consuming them. Hence, food processors are reluctant to irradiate foods, justifying their decision on the basis that consumers would not be willing to pay the additional costs for irradiating the food.

Cold plasma

Cold plasma is a new intervention for decontaminating foods under nonthermal conditions. Cold plasma is generated by applying an energy source (heat, electricity, laser light, radiation, etc.) to gases (air, O_2, N_2, or a mixture containing some proportion of noble gases). The resulting quasineutral ionized gases consist of UV photons, neutral or excited atoms and molecules, negative and positive ions, free radicals, and free electrons, that in combination have the capacity to inactivate microorganisms on the surfaces of fruits, vegetables, nuts, and grains (131–133). Additional potential applications include applying gas plasmas within MAP containing fresh produce (134) and detoxification of aflatoxins in hazelnuts (135).

Currently, cold plasma is still considered a new technology that is in an early stage of development (4). Attributes that are attractive for this technology include a relatively low operating temperature, low water consumption, low cost, timely production of the reacting agents, and a lack of residues during production compared to thermal and chemical treatments (136). Some of the limitations that need to be addressed are the long treatment times (300 s to 20 min) that are required (136) and the need for the food to be in close proximity to the plasma source (124). Additionally, more research is needed to address the physicochemical and functional changes that can occur in the treated food products.

UV light

Nonionizing radiation with wavelengths ranging from 100 nm to 400 nm is classified as UV light. UV light, in turn, is further subdivided into three categories: near-UV radiation, or UV-A (315 to 400 nm); midrange UV, or UV-B (280 to 315 nm); and far UV, or UV-C (100 to 280 nm). UV-C that encompasses the maximum germicidal wavelength of 254 nm is the most common type of UV light applied to fresh fruits and vegetables and serves as an antimicrobial agent, either by causing direct DNA damage to microorganisms or by inducing resistance mechanisms in the plant tissues against pathogens (137). However, microbial inactivation is limited to the food's surface due to UV-C's extremely low penetration in solid foods. Hence, with comparable UV doses, surfaces that are uneven (e.g., cantaloupes and berries) have pathogen reductions of only ca.1 log CFU, compared to reductions on the smooth surfaces of apples of 2 to 3 log CFU (138, 139). The application of UV-C is also limited by its adverse effects on the sensory and nutritional characteristics (e.g., browning and decreases in vitamin C content in fresh-cut pineapples [140]) of produce. UV light can completely degrade aflatoxin B_1; however, the degradation products remain cytotoxic (115).

Pulsed light

Pulsed-light interventions are an emerging technology for inactivating microorganisms which uses pulses of an intense broad spectrum (200 to 1,000 nm) rich in UV-C light. By storing the energy in a high-power capacitor over fractions of a second, the power is released within millionths or thousandths of a second, producing several high-energy pulses per second. The energy intensity is heightened through this process and contributes to the inactivation of both spoilage and pathogenic microorganisms (141). The major advantages of this intervention approach are substantial microbial reductions in very short treatment times, lower energy costs, flexibility, and the lack of residual chemicals (4). However, there are two main drawbacks to applying pulsed light

to grains and nuts: (i) their uneven surfaces, which protect microorganisms in shaded areas; and (ii) their abundance of proteins and fats, which absorb the effective wavelengths (142). Two approaches have been taken to address the former limitation. In one approach, strawberries and raspberries were exposed to the pulsed light while being immersed in agitated water (143). In the second approach, it was envisioned that light-emitting diodes (LEDs) that deliver UV at multiple angles could be constructed (144). In addition, LEDs could apply a range of different UV wavelengths, thereby providing a synergistic antimicrobial action (145).

High-pressure processing

High-pressure processing (HPP) is a process in which elevated pressures, typically in the range of 100 to 700 MPa, are applied to either an aqueous food product or a product that is suspended in a liquid medium, normally water. The pressure is transmitted isocratically throughout the food sample, thereby shortening the time necessary for the treatment independent of food shape or size (124). In general, yeasts and fungi are more sensitive to pressure than vegetative bacteria, and Gram-negative bacteria are more sensitive to pressure than Gram-positive bacteria (146). Pressure resistance also varies greatly between and within taxonomic groups and strains of viruses (146).

Investigations into potential HPP applications for plant-based foods have primarily focused on purees and juices (131). This process does not lend itself to treating cereals and nuts, as it would require an additional drying step after suspension of the product in a liquid medium for treatment (115). In contrast, HPP treatment of fresh produce is theoretically possible; however, there are barriers to commercial application that relate to cost, limited capacity, batch system, and incompatibility with produce packed using MAP (124). In addition, issues of texture loss through plant cell disruption and browning caused by chlorophyll degradation have been observed for leafy greens treated with HPP (124).

Chemical Treatments

Oxidizing treatments

A variety of antimicrobial chemical treatments have been used as postharvest interventions for the purpose of either product decontamination or the avoidance of cross-contamination. Chlorine is the most frequently used disinfectant, which is easily applied to water in controlled amounts, either as chlorine gas, which readily dissolves in water at room temperature to generate hypochlorous acid, or as a salt of hypochlorite. Concentrations used in produce

operations vary from 50 to 200 ppm, with contact times of less than 5 minutes. Under those conditions, typical reductions of vegetative bacteria on produce are 1 to 2 log CFU/g, whereas reductions on pecan nutmeats are even less. Hence, decontamination of these products with chlorine is only marginally better than washing the items in water (147). The ineffectiveness of chlorine for decontamination of tissue surfaces has been attributed to microbial cells being strongly attached to the tissue or embedded in biofilms or in cracks and crevices that are inaccessible to the chemical agent (86, 147). In contrast, chlorine is considered to be very effective against suspended vegetative bacteria, making it an excellent sanitizing agent for preventing cross-contamination during produce wash operations (54). However, it is imperative that chlorine levels in the water be monitored constantly, as chlorine not only oxidizes microorganisms but also is capable of reacting with other inorganic and organic molecules in the system to form disinfection by-products (DBPs) such as trihalomethanes. Many of these DBPs have been associated with adverse health effects (148) and have led several countries (e.g., Germany, Netherlands, Denmark, Belgium, and Switzerland) to prohibit the use of chlorine-based disinfectants in washing of fresh-cut produce (54). However, one conciliatory outcome of washing fresh-cut lettuce with chlorine has been that despite high levels of total trihalomethanes being observed in the wash water, no measurable amounts of the DBPs were detected on the fresh-cut lettuce (149).

Due to the limited effectiveness of chlorine in decontaminating tissue surfaces, alternative oxidizing agents have been evaluated. Chlorine dioxide is an example and is considered to have greater biocidal efficacy than free chlorine. Chlorine dioxide is an undissociated gas when dissolved in water and is relatively stable, as it is less likely than chlorine to react with oxidant demand substances. However, its aqueous concentration decreases if left in an open tank due to its volatility (150). Furthermore, chlorine dioxide is photoreactive in sunlight, producing the by-products of chlorate, chlorite, and chloride (151). Since adverse effects in humans have been associated with chronic exposure to chlorates, the European Food Safety Authority (152) has imposed maximum limits for this chemical of 0.7 mg/kg in foodstuffs and 0.7 mg/liter in drinking water. However, a recent pilot-scale study using chlorine dioxide at 5 and 3 mg/liter in the wash water revealed that the upper limits for both chlorate and chlorite were exceeded in the process water but not in the lettuce (153). These results, along with the requirement in the United States that chlorine dioxide treatment of fruits and vegetables be followed by either a potable rinse or another preservative method (154), may preclude extensive use of this disinfectant in washing fresh produce. A review of studies utilizing chlorine dioxide as a decontamination agent on a range of produce items revealed that

applying the agent as a gas is a more effective antimicrobial measure than applying it as a liquid because it has greater penetration ability as a gas (155).

Ozone is another oxidizing disinfectant that has been of increased interest to the produce industry in the past 2 decades. In 1997, ozone received GRAS (generally recognized as safe) status and was later approved in 2001 by the FDA as a secondary direct food additive and antimicrobial agent for all food types (156). Comparatively, aqueous chlorine and ozone have similar levels of effectiveness as a decontaminant of produce, as log reductions with ozone have been reported to range between 0.8 and 1.8 log CFU (155). However, the advantage to using ozone is that it decomposes into molecular oxygen and hence does not leave hazardous residues on the food product (157). Similar to chlorine dioxide, however, gaseous ozone is more efficacious than the antimicrobial applied in the liquid state. To support this assertion, Shynkaryk et al. (158) determined that the depth of penetration of aqueous ozone into lettuce was limited to several millimeters due to slow diffusion of the antimicrobial and self-decomposition, whereas gaseous ozone reached a depth of 100 mm.

An interesting application of ozone is its proposed use as a disinfectant/fumigant for produce and grains. In these cases, the product is often stored in large contained areas for lengthy periods of time. A study with spinach revealed that low concentrations of ozone diffused into 300-liter containers reduced *E. coli* O157:H7 populations by 1.4 log CFU/g (159). Replenishment of ozone into storage structures, on the other hand, has been advocated as the solution to ozone decay in the treatment of grains (129). Benefits to treatment of grains with ozone include inactivation of molds (160, 161) as well as degradation of mycotoxins (162).

Novel natural sources and applications

The consumers' desire for natural food ingredients has prompted the food industry to find alternatives to artificial additives. In response, natural antimicrobials found in edible plants have been identified as possible alternatives. Many plant-based compounds (e.g., albumins, lectins, protein hydrolysates, flavonoids, isoflavonoids, terpenoids, etc.) have potent antibacterial and antifungal activities. However, cost constraints as well as government regulations pose limits to their commercial application and restrict their application to foods as crude plant extracts. Other additional limitations associated with their use are discussed in chapter 42. However, these limitations have not hampered research in this area, as exemplified by the publication of several recent reviews addressing this topic (163–165). Future applications of this approach will likely be limited to very specific uses where

the costs associated with the incorporation of natural antimicrobials into products would be offset by increased sales or added shelf life or safety to the product. An example is the use of an extract of a legume (pea, lentil, and fava bean flours) hydrolysate as an ingredient in bread making. Not only did the bread have a longer shelf life than the control (based on the absence of mold growth), but the chemical, structural, and sensory characteristics of the bread were not affected by the addition of the extract (166).

Another approach to applying chemical interventions to produce is to improve the efficacy of the antimicrobials by modifying the method for delivery of the chemical to the agricultural crop. Examples include application of the antimicrobial in a nanoemulsion to lettuce (167), application of nisin-incorporated films onto minimally processed mangoes (168), application of antimicrobial sachets in the packaging of lettuce (169), and utilization of antimicrobial films to packaged apple slices (170).

Biological Treatments

Biological interventions are based on the application of either naturally occurring microorganisms that are inhibitory to spoilage organisms or pathogens or the antimicrobials that are produced by these microbial antagonists (e.g., bacteriocins) directly to agricultural crops. Recent examples include the application of antimicrobial peptides derived from *Lactobacillus plantarum* LR/14 for the prevention of grain spoilage (171), the application of *L. plantarum* and *Lactobacillus fermentum* to freshcut cantaloupe to reduce *L. monocytogenes* contamination (172), and the application of the bacteriophage Listex P100 to reduce *L. monocytogenes* contamination on fresh-cut melon slices (173). Many more examples are provided in the review articles listed in Table 7.5. However, it is important to recognize that although inhibiting microbial degradation is the purpose of the treatment, the application of these biological agents may cause adverse changes to the food quality. Therefore, it is important that the treated foods' quality characteristics be evaluated to determine if the treatment will yield a product that is acceptable to consumers. In addition, the safety of the product in which any new microbes such as lactic acid bacteria are to be used should be considered, as there has been an association of some lactobacilli with sepsis, endocarditis, and bacteremia (174).

Hurdle Treatments

Hurdle technology consists of using a sequence or combination of mild interventions to inhibit or inactivate food spoilage microorganisms or pathogens. A basis for using this approach is that it would control the microbes of interest but would have a lesser effect on the organoleptic quality of the

product than a single intervention. One of the more common recent applications of the hurdle approach involves using a physical intervention (e.g., ultrasound or UV light) in combination with chemical interventions (e.g., chlorine or organic acids). Another novel hurdle approach is to generate chlorine dioxide within the tissue of fresh produce instead of relying on it to migrate to those sites (175). This is accomplished by applying a sequential treatment that includes first submerging the produce item (cantaloupe rinds, cucumber peels, tomato stem scars, or spinach leaves) in a sodium chlorite solution for 10 or 30 min, drying for 20 min, and then soaking in a hydrogen chloride solution for 10 or 30 min. When the hydrogen chloride interacts with the sodium chlorite, chlorine dioxide is generated. Greater reductions of *Salmonella* and *L. monocytogenes* (4.8 log and 2.7 log CFU/g, respectively) occurred with the sequential treatment than with a control treatment in which the produce item was treated only with either sodium chlorite or hydrogen chloride.

RISK ASSESSMENT

Due to the complexity of the systems used to grow and process agricultural crops as well as the many factors that affect the microbiological profiles of the crops and subsequent products at any one point, there is a need to understand the impact of each individual factor within the system on the overall response (e.g., the risk of becoming ill from eating a contaminated product or the risk of the product's becoming spoiled and not suitable for consumption). To estimate these risks, quantitative microbial risk assessment (QMRA) is applied using mathematical models that are based on data sets for the statistical distributions of microbial concentrations as foods are subjected to certain conditions and interventions. Recent QMRA studies have addressed leafy greens (176, 177), raw produce (178), pistachios (179), and pecans (77). QMRA outcomes are not absolute values, as there is often variability or uncertainty associated with data sets used within the models. Hence, risk assessors may have to use surrogate data or make assumptions in order to generate an outcome, and therefore, the results should be used only as an indication of the level or degree of risk.

CONCLUDING REMARKS

Microbiological contamination of agricultural crops such as fruits, vegetables, nuts, and grains will continue to contribute to preharvest crop losses, postharvest spoilage losses, and contamination of food with enteric pathogens. Understanding the sources and the factors that contribute to survival of resident microorganisms provides the basis for implementation of

management programs that will keep plants healthy and in a state that will provide near-optimal quality during storage and distribution. The analytical tools for detection of microorganisms continue to improve. With these advancements, our knowledge of plants' microbial ecosystems will increase and will help to provide a road map on how to cost-effectively utilize postharvest interventions for controlling or eliminating the microbes that cause spoilage or illness.

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III
Foodborne Pathogenic Bacteria

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8
Epidemiology of Foodborne Illnesses

Foodborne illnesses result from the dynamic interaction of agents (i.e., bacteria, viruses, and parasites), hosts (humans and animal reservoirs), and the environments in which these interactions occur (Fig. 8.1). The depiction of these relationships is known as the epidemiological triad. It serves to illustrate the need to integrate information from multiple disciplines to understand the occurrence of foodborne illnesses. It also suggests why the epidemiology of foodborne illnesses is constantly changing. As new foods and new sources of foods become available, new opportunities for foodborne illness transmission often follow. As the population ages, more people are at risk for serious illness from common foodborne pathogens. The use of antimicrobial agents in production environments or clinical settings may select for the development or promotion of antimicrobial resistance. These are some examples of changes in one area that will change the pattern of illnesses which are observed. The dynamic nature of our food systems results in a constantly changing epidemiology of foodborne illnesses.

The ability to monitor changes in the patterns of foodborne illnesses in the population is the subject of epidemiology, and such monitoring is one of the primary functions of public health agencies. Public health surveillance for foodborne illnesses has been rapidly evolving with the development of new laboratory methods to identify agents in clinical specimens, food, and the environment. In particular, whole-genome sequencing (WGS) is increasing the sensitivity of outbreak detection and simultaneously increasing the specificity of outbreak investigation, much as pulsed-field gel electrophoresis (PFGE) did 20 years ago. However, the ability of WGS to link isolates from human, food, and

environmental sources presents new opportunities and challenges to foodborne illness surveillance. Laboratory evidence alone is not sufficient to establish a causal relationship. Detailed exposure assessments and epidemiologic evaluation of the exposures are critical. Developing and maintaining a framework to effectively incorporate WGS into public health surveillance and food regulatory activities is a priority that requires the ongoing engagement of public health and food regulatory officials and food safety specialists in industry and academia.

This chapter provides an introduction to epidemiology and epidemiologic methods as they are applied to problems of the surveillance and control of foodborne illnesses. It also discusses major advances in the world of foodborne illness outbreak detection and the role of surveillance in source attribution of foodborne illnesses. An understanding of epidemiology is important, because despite our best efforts to prevent foodborne illnesses, humans remain the ultimate bioassay for low-level or sporadic contamination of agents of foodborne illness in our food supply. Epidemiologic methods of foodborne illness surveillance are needed to detect outbreaks, identify their causes, and assess the effectiveness of control measures. Epidemiologic data are also important in establishing food safety priorities, allocating food safety resources, stimulating public interest in food safety issues, establishing risk reduction strategies and public education campaigns, and evaluating the effectiveness of food safety programs. The examples provided in this chapter are drawn from recent literature, outbreak reports, and multi-stakeholder collaborations. They were chosen to illustrate key concepts and emerging approaches. Epidemiologic data relevant to individual foodborne illnesses are presented in their respective chapters.

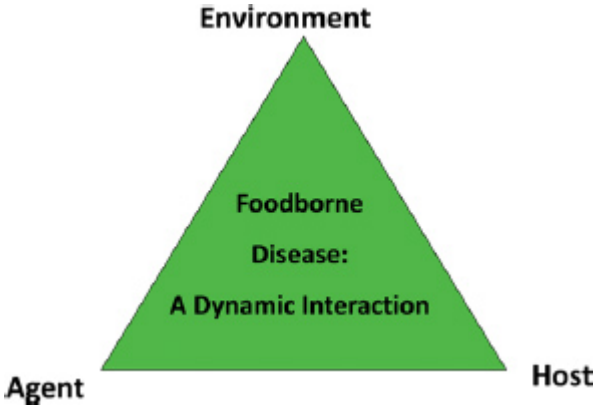


Figure 8.1 Illustration of the epidemiological triad, depicting the dynamic interaction of agents (i.e., bacteria, viruses, parasites), hosts (humans and animal reservoirs), and the environments leading to the occurrence of foodborne illnesses.

EPIDEMIOLOGY

Epidemiology is the study of events in populations. Events are usually thought of in terms of illnesses. For example, the CDC established an Active Surveillance Network for Foodborne Diseases (FoodNet) to determine how many laboratory-confirmed infections caused by *Campylobacter*, *Escherichia coli* O157:H7, *Salmonella*, and selected other pathogens occur in the populations under surveillance (https://www.cdc.gov/foodnet). By actively ascertaining all diagnosed infections in the FoodNet catchment area, FoodNet is able to establish reliable trends in the occurrence of these illnesses by age, gender, geographic area, and time. A second major FoodNet activity has been to conduct periodic surveys to provide an epidemiologic context that relates the specific foodborne pathogens under surveillance to the overall burden of diarrheal illnesses in the population. Population surveys have assessed how many diarrheal illnesses occur in the population and how often ill people seek medical care. Physician surveys have assessed how often and why physicians order diagnostic tests. Clinical laboratory surveys have assessed the availability and use of various laboratory test methods. By comparing the incidence and

proportion of specific diseases attributable to foodborne transmission with the frequency of medical evaluation and laboratory testing, it becomes possible to determine what proportion of diarrheal illnesses may be foodborne. These data provided the epidemiologic framework for the approach taken by Scallan and colleagues to determine that 48 million foodborne illnesses occur each year in the United States (1). Technical appendices to Scallan's landmark publication provide a rich compilation of information on disease characteristics and surveillance data for known foodborne pathogens.

Epidemiology also includes the study of factors associated with the occurrence of illnesses. The same population survey that FoodNet conducted to assess the frequency of diarrheal illness also surveyed the population to find out how often people have potential exposures, such as eating undercooked hamburger, eating sprouts, failing to wash their hands after handling raw chicken, or making a salad on the same cutting board they used to cut up raw chicken. Many of these factors are assessed in the context of outbreak investigations to enable identification of the factors actually contributing to the occurrence of an outbreak. FoodNet's compilation of these in their Atlas of Exposures (https://www.cdc.gov/foodnet/surveys/foodnetexposureatlas0607_508.pdf) has been an important resource for hypothesis generation in outbreak investigations, as described below.

Descriptive Epidemiology

The careful description of events in populations is generally called descriptive epidemiology. This process of determining characteristics of person, place, and time associated with illness occurrence forms the basis of all surveillance systems and outbreak investigations. The defining example of this process was the work of John Snow, during the London cholera epidemic of 1860. He located cases on a map of London, saw a cluster around the Broad Street pump, and pulled the pump handle. The contaminated water source was shut down, and the local outbreak ended. Such dramatic

examples of consequential epidemiology are not common. However, surveillance of foodborne disease, based on the principles of descriptive epidemiology, remains a cornerstone on which other food safety activities are built.

Surveillance of foodborne illnesses is a basic function of public health agencies. It involves the systematic collection and analysis of data on the cases and characteristics of the illnesses to guide public health actions, as described at length below. At the time of Snow's investigations, cholera was diagnosed by its typical clinical symptoms and disease progression. Thus, it represented a clinical syndrome rather than a microbiologically defined disease. As laboratory methods have developed, laboratory characteristics of the isolates have become important elements of descriptive epidemiology. In mid-September 1994, a nationwide outbreak of *Salmonella* illness associated with commercially distributed ice cream was detected when the Minnesota Department of Health's Public Health Laboratory noted an increase in the number of *Salmonella enterica* serovar Enteritidis isolates being identified. Initially, cases appeared to be clustered in southeastern Minnesota, and the full scope of the outbreak became apparent only after the investigation allowed cases to be linked to the outbreak based on their exposure to the nationally distributed food item.

In the mid-1990s, PFGE became a standard part of surveillance for *Salmonella*, *E. coli* O157:H7, and *Listeria monocytogenes*, and a national network of public health laboratories, PulseNet, was established. Because PFGE is more discriminant than genus, species, and serotype, PulseNet led to a marked increase in the number of outbreaks detected. This is turn led to a reduction in the number of sporadic cases of these illnesses that were reported. An evaluation of PulseNet's economic impact is a landmark demonstration of the value of increasing the sensitivity of public health surveillance (2). The current replacement of PFGE by WGS is leading to a similar gain of function for foodborne-disease surveillance and detection of clusters that likely represent outbreaks of foodborne illness.

As surveillance has evolved from detecting clusters of cases defined by clinical syndrome to microbiologic characteristics of genus, species, and serotype to molecular subtype defined by PFGE pattern and WGS, the size of detectable clusters has decreased, and the likelihood that cases within the cluster may share a common source of exposure has increased. Descriptive features of the cases in the cluster may provide important clues as to the source of the outbreak. Reviewing that descriptive epidemiology is the first step in hypothesis generation during outbreak investigations.

Analytical Epidemiology

In contrast to the mere description of events, the comparison of different rates at which they occur between groups is known as analytical epidemiology. This provides the means to test hypotheses about the source of an outbreak. For etiologic studies to determine the cause of an outbreak, the critical measure is the difference between the risk of illness among those exposed to the "contaminated" food and the risk of illness among those who were not exposed. This is customarily presented as an odds ratio or risk ratio, depending on the epidemiologic study design. As the development of molecular laboratory methods has transformed laboratory-based surveillance of foodborne illnesses, there has been a similar evolution in the development of epidemiologic methods to analyze outbreaks.

Community-Based Case-Control Studies

The classic tool of analytical epidemiology for foodborne diseases is the use of a case-control study. Comparing food exposures among cases in an outbreak to those among healthy control subjects has great power to identify foods that are associated with the illnesses. For example, in the nationwide outbreak of salmonellosis associated with ice cream, epidemiologists constructed a questionnaire to ascertain what foods individual patients had eaten in the 5 days before they became ill. The same questionnaire was used to interview healthy individuals of about the same age from

the same communities. By comparing the responses of the cases to the responses of the healthy individuals, who served as controls, it was rapidly determined that the outbreak was due to consumption of commercially manufactured ice cream. Ten (67%) of 15 cases, but only 2 (13%) of 15 controls, had eaten brand A ice cream (matched odds ratio = 10.0; 95% confidence interval [CI] = 1.4 to 434). While results of the case-control study implicated an exposure source within 3 days, regulatory testing to confirm the source of contamination required an additional 10 days.

The magnitude of the odds ratio is a measure of the strength of the association between illness and exposure (Table 8.1). The strength of this association left little doubt that the ice cream was the source of the outbreak. A public health intervention was initiated days before *Salmonella* was actually isolated from the suspected product. In addition, results of the case-control study suggested that the problem likely involved multiple ice cream flavors and days of production. In response, a nationwide product recall was initiated. Further investigation led to the determination that the ice cream premix had been contaminated by raw egg residues in tanker trailers used to haul the premix from two other suppliers. Ultimately, what initially appeared to be a regional outbreak in southeastern Minnesota involved an estimated 224,000 illnesses, with cases reported from 41 states. The published report of this investigation provides a useful model for how an outbreak investigation can identify the root cause of an outbreak and identify effective measures to prevent its recurrence (3).

Table 8.1 Evaluating the strength of association between illness and exposure in epidemiologic studies

Odds ratio or relative risk	Strength of association[a]
1	No association
2-5	Relatively weak
5-10	Relatively strong
>10	Very strong

[a]Other key factors to consider when evaluating the association include the following. Was time between exposure and illness onset consistent with a reasonable incubation period? Was a dose-response behavior observed? Do results of product tracing suggest a common source?

Although they may provide very useful information, large community-based case-control studies such as the one described above are no longer routinely conducted in outbreak investigations. Because of growing difficulties in identifying and interviewing suitable controls, they have become too expensive to conduct and are too slow to produce actionable results in a timely manner. However, smaller, focused case-control studies should be conducted when a subcluster of cases in a multistate outbreak share exposure to a common retail food establishment. As described below, an ingredient-specific case-control study among establishment patrons may be critical to identifying the cause of the larger outbreak. Because these case-control studies are limited in scope to foods served by the establishment, interviews are quicker and well meal companions can serve as controls.

Case-Case Comparisons

The development of molecular subtype-specific surveillance based on PFGE, and more recently WGS, has made it possible to compare cases caused by an outbreak-associated strain with cases caused by unrelated strains. Because the occurrence of an outbreak implies a common source, the cases caused by unrelated strains with many different likely sources of exposure make a very efficient control

group. This is particularly true when patients representing sporadic cases are routinely interviewed with detailed food exposure questionnaires. Case-case comparison studies have been critical for investigating *Listeria* outbreaks, where the risk for illness is dependent on underlying health conditions. For example, during the summer of 2011, an outbreak of listeriosis was identified by the Colorado Department of Public Health and Environment. Food exposures from reported cases were compared to aggregated exposures of nationally reported cases collected by the CDC's *Listeria* Initiative. Outbreak-associated case patients had much higher rates of consuming cantaloupes. Cantaloupe source tracing identified a common grower, and microbiological testing confirmed the contamination of the whole cantaloupes within a week of the outbreak's recognition. Similar methods are also being applied to outbreaks of *E. coli* O157:H7 and *Salmonella* infections.

Case-case comparisons produce the same measures of association as do case-control studies. Although there are theoretical reasons to believe that the unrelated cases used for comparison may have different exposure distributions than would a group of community controls, neither comparison group would likely share the common exposure that caused the outbreak. Thus, from a practical standpoint, these studies are interpreted in the same way. The increased stringency of WGS to discriminate outbreak-associated from unrelated cases makes case-case comparisons a desirable alternative to case-control studies when aggregate case exposure data are available.

Binomial Comparison Studies

A second derivation of case-comparison studies has become the *de facto* standard of practice for investigating clusters and outbreaks identified by pathogen-specific surveillance. The use of this method was pioneered by the late William Keene, an epidemiologist with the Oregon State Health Department and founder of the International Outbreak Museum (www.outbreakmuseum.com/). This approach requires interviewing cases with a detailed exposure

questionnaire, aggregating case exposure data, and comparing them to exposure data compiled from a suitable reference population. The proportion of cases with a particular food exposure can be compared to the population exposure proportion. However, the method does not produce an odds ratio or other measure of the strength of association. Keene developed a simple binomial calculator to test the significance of differences between the case and population exposure proportions. His use of this method in 2006 helped identify bagged spinach as the source of a large multistate outbreak of *E. coli* O157:H7 infections.

FoodNet's Atlas of Exposures has been the most commonly used source of population exposure data. However, there are several limitations to its use. The most recently published FoodNet exposure data are from 2006. Because dietary patterns change over time, novel food exposures may require validation form other sources. The FoodNet population survey was reinitiated in 2017. Similarly, FoodNet exposure data were aggregated across participating sites, and geographic differences in dietary patterns are also apparent. Thus, identifying local population exposure data is preferred.

As with other analytical epidemiology methods, the binomial comparison identifies associations that must be confirmed by product source tracing and corroborated by other investigation findings. In this regard, binomial comparisons should be considered more as advanced hypothesis generation than as hypothesis testing. Because binomial comparisons emulate very large case-control studies, there is an increased chance of producing spuriously significant results. Thus, the results must be cautiously interpreted to avoid errors in identifying the source of an outbreak.

Cohort Studies

A closely related tool of analytical epidemiology is the cohort study. In outbreak investigations, retrospective cohort studies are conducted when an entire group (or population) with a common

exposure can be identified. Individuals can be interviewed without prior knowledge of whether they were ill. Identifying groups based on their exposure status rather than their illness status allows direct calculation of a risk ratio for specific exposures. The risk ratio is the percentage of exposed persons who were ill divided by the percentage of unexposed persons who were ill.

Typical settings for cohort studies involve banquets, wedding receptions, and other events where a list of all persons attending can be obtained. For specific exposures, such as a particular food item, the percentage of persons who became ill after eating the food can be determined. Hence, in addition to implicating the food item, it is possible to determine the attack rate among persons who ate it. Information on attack rates may also help in evaluating the factors contributing to the occurrence of the outbreak. For example, for many bacterial foodborne agents, the attack rate may be related to the exposure dose. A high attack rate may imply high levels of contamination, such as may occur after prolonged temperature abuse.

For example, the CDC's *Morbidity and Mortality Weekly Report* recently reported on an outbreak of diarrheal illness and abdominal pain among employees who attended a catered Thanksgiving lunch that was reported to a local health department. An online survey was prepared and sent to all employees who attended the lunch. The use of the online survey allowed rapid collection of illness and exposure data, from which investigators simultaneously established a clinical case definition, estimated food exposure illness rates, and associated illness with consumption of turkey and stuffing that had been served together. Subsequent laboratory studies and environmental assessments confirmed that the outbreak was caused by *Clostridium perfringens* due to improper temperature control of the cooked turkey.

P Values and CIs

In both case-control and cohort studies, much attention is paid to the precision of the calculated estimate, as measured by the *P* value or 95% CI. Most conventions treat a *P* value of <0.05 as being significant. Similarly, 95% CIs are expected to exclude 1.0 when a significant exposure occurs. While these criteria have served as useful guides to data analysis over the years, it must always be noted that the main contributor to the *P* value and CI is the size of the sample. The larger the sample size, the more likely it is that a statistically significant result may be obtained. Investigators should not discard potentially important associations just because they fail to achieve a *P* value of <0.05. Conversely, "highly significant" results may be totally spurious. The concern is that bias in sampling may have introduced reasons other than contamination of food for the observed difference in risk of illness.

In particular, if food items are usually consumed together, one food item may be identified while the other is overlooked. Mistakes in the identification of the source of two large foodborne outbreaks appear to have resulted from this type of error. In 1996, outbreaks of illness caused by *Cyclospora* were initially associated with strawberries from California, while in 2008, a nationwide outbreak of *S. enterica* serovar Saintpaul infections was associated with tomatoes. In both outbreaks, subsequent epidemiologic analyses identified the actual vehicles: raspberries and jalapeno peppers, respectively. The harm done by this type of error to the affected producers and to confidence in the public health system may be substantial. However, when potential sources of bias are properly controlled and accounted for, epidemiologic methods have demonstrated an impressive ability to accurately identify the sources and contributing causes of foodborne outbreaks.

Limitations in the Use of Epidemiology

As powerful as epidemiologic methods are, they do have limitations. In an outbreak setting where everyone ate a contaminated food item, there may be no opportunity to demonstrate a difference in risk of illness between those who were exposed and those who were

not. Alternative approaches, such as looking at the amount eaten or when the food item was eaten, may allow some epidemiologic discrimination, but results are not likely to be "significant." For example, in two outbreaks of shigellosis in Minnesota that were associated with parsley from a common source, parsley was associated with illness at one restaurant, but not at the other. Chopped parsley was used on so many dishes at the second restaurant that most of the cases and the controls ate it. Thus, the absence of an association does not necessarily mean that the food item or exposure in question is safe.

AGENTS AND TRANSMISSION

Information about specific agents and their transmission is contained in their respective chapters. However, in discussing the epidemiology of foodborne illnesses, it is important to keep in mind the chain of infection. This includes the agent, the reservoir that contains the agent, a means of escape from the reservoir, a mode of transmission to a susceptible host, and a means of entry to the host. These elements contribute to the epidemiologic triad of agent, host, and environment interactions that results in the occurrence of illness.

Specific agents vary with respect to the types of illness they cause, from self-limited gastroenteritis associated with bacterial enterotoxins to invasive and life-threatening diseases associated with *L. monocytogenes*. Some agents, such as norovirus, are cleared from the gastrointestinal tract in a matter of days, whereas others, such as *Giardia*, may cause prolonged carriage, over several weeks. Infectious doses may range from ingestion of a few *E. coli* O157:H7 cells to hundreds of thousands of *C. perfringens* cells. Incubation periods range from a few hours for staphylococcal intoxication to weeks for viral hepatitis type A. Some agents, such as *Shigella*, infect only humans, whereas *Salmonella*, *Campylobacter*, and *E. coli* O157:H7 primarily originate from animal sources. Many agents are associated with typical food vehicles associated with their

animal reservoirs, such as *S.* Enteritidis with eggs and *E. coli* O157:H7 with ground beef, or with food vehicles susceptible to environmental contamination from the animal reservoir, such as leafy green vegetables and *E. coli* O157:H7.

The characteristics of specific agents affect the potential sources of contamination during food production and preparation and form the basis for hazard analysis for specific foods. Knowledge of these patterns contributes to hypothesis generation. It helps investigators focus on likely sources of transmission. However, most foodborne pathogens may be transmitted by multiple routes of exposure, and outbreak investigations are critical for identifying new food hazards and exposure pathways. As described at length below, the results of investigations into foodborne illness outbreaks are also increasingly being incorporated into food attribution models.

FOODBORNE ILLNESS SURVEILLANCE

Public health surveillance for foodborne illnesses is a governmental function that is specifically authorized by law. In the United States, foodborne illness surveillance is established within the framework of state and local communicable disease reporting rules. There is considerable variation in these rules with respect to what illnesses are reportable, how quickly they need to be reported, and whether isolates need to be submitted to the public health laboratory. While the Food and Drug Administration (FDA) and USDA's Food Safety and Inspection Service (FSIS) have regulatory authority over foods in interstate commerce, there is no corresponding authority over the multistate outbreaks that may result from the interstate shipment of contaminated foods. On a national level, the CDC coordinates surveillance activities and multistate outbreak investigations and compiles data from states. However, they have no authority to intervene in any state's investigation. The Council to Improve Foodborne Outbreak Response (CIFOR), which includes representation from local, state, and federal agencies' epidemiologists, environmental health specialists, and public health

laboratorians, has developed guidelines for foodborne disease outbreak response to establish effective outbreak investigation coordination and communication (http://cifor.us/products/guidelines). The CDC has played a key role in developing, improving, and promoting new laboratory methods and epidemiologic practices, as described below.

Purposes of Foodborne Illness Surveillance

Surveillance involves the systematic collection of data with analysis and dissemination of results. Surveillance systems may be passive or active and may be national or regional in scope or based on a sentinel system of individual sites designed to provide estimates that can be generalized. Types of systems, their use, and their limitations are discussed below.

There are four primary purposes for conducting foodborne disease surveillance:

- Identify, control, and prevent outbreaks of foodborne illness
- Determine the causes of foodborne illness to identify preventable hazards
- Monitor trends in occurrence and attribution of foodborne illness
- Quantify and reduce the burden of foodborne illness

“Pulling the pump handle” is the goal of all public health epidemiologists conducting foodborne disease surveillance. Hence, there is a sense of urgency in every investigation to rapidly identify the source of the outbreak and implement control measures to stop transmission and prevent additional illnesses. For products with a long shelf life, such as cured meat products, frozen foods, or cereals, even a relatively long investigation can identify a contaminated product quickly enough to remove it from the marketplace in time to prevent many illnesses. For highly perishable products, such as fresh fruits and vegetables, often the outbreak will have run its course before the food item can be implicated.

Even if it is impossible to directly intervene to control the outbreak, there is still value in determining the causes of foodborne illness. Identifying contributing factors and root causes can help prevent future outbreaks of foodborne illness. Under the Food Safety Modernization Act (FSMA), food manufacturers must establish preventive controls to abate food safety hazards in their operations. Many of these hazards, in terms of either specific agents, specific food ingredients, or various agent-food interactions, were originally determined as a result of foodborne illness surveillance. Because food sources and foodborne disease agents are constantly changing, hazard analysis is an ongoing process that requires continuous support from public health surveillance of foodborne diseases. Additionally, Scallan and colleagues' estimates of the burden of foodborne illness suggest that most foodborne illnesses are caused by unspecified agents. As the experience with *Cyclospora* illness outbreaks from Guatemalan raspberries reveals, foodborne disease surveillance may be the only way to identify both new causes of foodborne disease and potential foodborne hazards.

A third purpose for conducting foodborne disease surveillance is to monitor trends in occurrence and attribution of foodborne illnesses to specific commodities or production practices. This is important to identify priorities for new food safety activities and to monitor the effectiveness of existing programs. For example, Painter and colleagues at the CDC developed a detailed classification scheme for food commodities and used it to attribute sources of foodborne outbreaks from 1998 to 2008 (4). Their approach was the first to quantify the importance of fresh produce as a source of foodborne illnesses. They attributed 46% of outbreak-associated illnesses to produce. They also attributed more deaths to poultry than to any other commodity. Beyond indicating the need for efforts to prevent contamination of produce and poultry, they established a methodological framework that has been used by the CDC, FDA, and FSIS in their Interagency Food Safety Analytics Collaboration in their development of foodborne illness source attribution. They are

developing models to incorporate data from outbreak investigations, sporadic illnesses, and pathogen prevalence in animals and foods.

FoodNet surveys of the population, physicians, and clinical laboratories linked to active surveillance of laboratory-confirmed infections provide the most comprehensive data available from which to estimate the burden of foodborne diseases. These burden-of-illness estimates have been used as a basis for setting the public health priorities reflected in the Healthy People 2020 goals. FoodNet trend analysis for specific agents has been used for evaluating the effectiveness of prevention measures. These analyses have demonstrated the success of interventions to reduce *E. coli* O157:H7 in ground beef, *L. monocytogenes* associated with ready-to-eat meats, and *S. enterica* serovar Typhimurium overall. However, not every trend has demonstrated success. Several *Salmonella* serovars associated with produce and poultry have become more abundant. In addition, significant gaps remain in our knowledge base. Scallan and colleagues concluded that approximately 80% of estimated foodborne illnesses cannot be attributed to known agents. Moreover, timely estimates for the burden of illness associated with all major foodborne pathogens, not just those under FoodNet surveillance, are needed to improve our evaluation of prevention measure effectiveness.

Methods of Foodborne Illness Surveillance

Foodborne disease surveillance requires close collaboration between acute-disease epidemiologists, public health laboratories, and environmental health specialists to determine the likely sources of exposure and routes of food contamination. Methods of surveillance of foodborne illness have been changing in response to the development of new molecular diagnostic methods, including WGS, and rapid developments in information technology and social media. These advances have had important implications for each of the four primary components of foodborne disease surveillance (Table 8.2).

Investigation of Outbreaks Associated with Events and Establishments

Approximately three-quarters of foodborne outbreaks are reported to local or state health departments by ill persons who were part of the outbreak. These include the vast majority of outbreaks caused by norovirus, *C. perfringens, Staphylococcus aureus*, and other agents for which there has not been routine clinical laboratory testing. There is a bias towards detecting outbreaks at events such as a wedding, where a large group with a common exposure experiences illnesses and has some reason to discuss it later. People eating at a restaurant may never know that their illness was also experienced by other restaurant patrons, who are unknown to them. However, multiple independent complaints may identify the same establishment.

Foodborne illness complaints are usually collected by local environmental health programs or health departments. Most individual complaints identify the meal most recently eaten out of the home, usually within a few hours, as the suspected source of the illness. Because many foodborne illnesses have incubation periods in the 1-to 3-day range, collecting a 3-day food history increases the likelihood of identifying a relevant exposure. Entering this exposure information into an electronic database also increases the likelihood that a common exposure can be identified in relation to multiple complaints.

Table 8.2 Primary components of foodborne disease surveillance and surveillance systems and impact of recent developments in laboratory and epidemiologic methods

Component	Surveillance systems	Impact of recent developments
Investigation of outbreaks associated with events and establishments	NORS, NEARS	Database management of complaint systems and social media-based surveillance increase outbreak detection. CIDT of clinical, food, and environmental samples increases confirmation of etiology and contributing factors.
Pathogen-specific surveillance to identify clusters of cases	PulseNet, NORS	WGS increases sensitivity of outbreak detection, specificity of outbreak investigation, ability to link human, food, and environmental isolates. CIDT increases diagnostic testing but decreases sensitivity of outbreak detection and specificity of outbreak investigation. CIFOR, COE, FoodCORE, and FDA's RRT demonstrate and promote effective outbreak investigation measures.
Determining risk factors for sporadic cases of infection with common foodborne pathogens	FoodNet	Large community-based sporadic case-control studies limited by available resources. Case exposure aggregations through the *Listeria* Initiative and the COE Project Mercury leverage routine data collection.

Component	Surveillance systems	Impact of recent developments
Population surveillance to determine frequency of gastrointestinal illnesses, health care-seeking behavior, food consumption, and personal prevention measures	FoodNet	FoodNet population surveys are being reinstituted to provide context for interpreting results of other surveillance activities.

Several investigators have explored the potential use of social media to identify foodborne outbreaks. Restaurant reviews posted to social media sites such as Yelp!, Facebook postings, and Twitter feeds identifying symptoms of foodborne illnesses have all demonstrated potential to identify unrecognized foodborne outbreaks. For the most part, attempts to use these sources have required considerable effort for a small increase in outbreaks detected. Several cities have developed systems to identify suspicious Tweets and encourage the sender to report the illness to their conventional complaint system. Further development and refinement of machine learning algorithms could reduce the need for health department staff to review suspect messages and increase the usefulness of the approach. One organization has developed a system to promote crowd sourcing to identify foodborne outbreaks. Iwaspoisoned.com has developed a simple online complaint reporting interface and forwards complaints to local health departments. However, most complaints do not include detailed food exposures or complete contact information. Local health departments have raised concerns that this system could compete with their own complaint systems, leading to a net loss of information and missed opportunities for

public health interventions. Because independently operated complaint systems require the same individual-level awareness and motivation as do complaint systems run by local public health agencies, they are unlikely to fundamentally increase outbreak detection.

Outbreaks associated with events and establishments require prompt and thorough investigation to identify both the agent and the source. These outbreaks are usually recognized because of the occurrence of common symptoms, such as diarrhea and vomiting, and are reported to public health officials before a specific diagnosis has been established. With the development and use of norovirus testing by many state public health laboratories, the proportion of outbreaks with an unknown etiology has declined from 67% from 1998 to 2002 to 23% in 2015. However, the shift in attribution to norovirus was not solely due to improved access to laboratory testing. The proportion of outbreaks that were not confirmed but were suspected based on clinical and epidemiologic features increased from 0% from 1998 to 2002 to 27% in 2015. Such epidemiologic profiling of outbreaks is useful to guide laboratory testing as well, and has been incorporated into CIFOR Outbreaks of Undetermined Etiology Guidelines (http://cifor.us/products/oue-guidelines).

Although clinical laboratories and many public health laboratories have not historically tested for the presence of enterotoxigenic *E. coli* or other uncommonly reported foodborne pathogens, the development of new culture-independent diagnostic tests (CIDT) and multiagent test panels is transforming our ability to confirm the etiology of outbreaks. The use of CIDT in outbreak investigations may increase the proportion of outbreaks with a confirmed etiology and provide an important framework to evaluate the usefulness of the tests. Confirming CIDT results by culture in public health laboratories can identify potential concerns with sensitivity and specificity of the tests. It can also provide an opportunity to assess the importance of codetection of multiple pathogens within the same sample.

The investigation of outbreaks associated with events is the oldest form of foodborne disease surveillance and accounts for approximately three-quarters of reported foodborne outbreaks. These outbreak investigations are responsible for most of the contributing factor data reported to the National Outbreak Reporting System (NORS). Contributing factors and the environmental antecedents or root causes that precede them are necessary for foodborne attribution studies based on outbreak data. This is a new application for these data and warrants efforts to improve standardization of environmental assessment and reporting. The CDC's Environmental Health Branch has established the National Environmental Assessment Reporting System (NEARS) to complement outbreak data reported to NORS (www.cdc.gov/nceh/ehs/nears/). Moreover, these outbreaks remain the primary way to identify "new" foodborne pathogens that may not be identified by clinical laboratories.

Across much of the developing world, recognition of outbreaks associated with events or establishments, usually involving large groups and short incubation periods, is the extent of foodborne disease surveillance available. This basic level of foodborne disease surveillance requires very little public health infrastructure. However, the development of multipanel diagnostic kits and point-of-use testing will transform the utility of even this basic level of foodborne disease surveillance.

Pathogen-Specific Surveillance To Identify Clusters of Cases

Although most outbreaks are detected through complaints, most multistate and high-profile outbreaks caused by *Salmonella*, Shiga toxin-producing *E. coli* (STEC), and *L. monocytogenes* are detected through identification of clusters by pathogen-specific surveillance. Thus, improving pathogen-specific surveillance has been a major focus of public health agencies.

Laboratory advances

Twenty years ago, the standardization and widespread use of PFGE revolutionized surveillance for *Salmonella*, *E. coli* O157:H7, and *L. monocytogenes*. The introduction of PulseNet created a national framework for surveillance that enabled the detection of widespread outbreaks, even though few cases were reported from any individual state. PFGE greatly improved the investigation of outbreaks by distinguishing unrelated sporadic cases from the main outbreak-associated strain. PulseNet took advantage of the combined revolutions in molecular biology and information technology. Highly reproducible PFGE patterns were generated under standardized conditions, and the PFGE patterns were transmitted electronically between participating laboratories. PulseNet became the backbone of a public health surveillance system, providing truly national surveillance for a variety of foodborne pathogens in a manner timely enough to be an early warning system for outbreaks of foodborne illness. As a result of PulseNet activities from 1994 to 2009, it was estimated that 270,000 foodborne infections caused by *Salmonella, E. coli* O157:H7, and *Listeria* were prevented each year, with a net savings of $500,000,000 (2).

PFGE subtyping improved the specificity of the case definition and made it more likely that the source of the outbreak could be identified. However, PFGE was a relatively crude technique that provided no real information about the organism beyond the subtype pattern. The rapid development of genetic sequencing technology (known as next-generation sequencing) and informatics has made WGS a new standard method for public health surveillance of foodborne pathogens (5). Beginning in 2013, the CDC began using WGS for surveillance of *L. monocytogenes*. *Listeria* was the first pathogen chosen because it causes severe illnesses in a high proportion of cases, a *Listeria* initiative had been established to ensure that all clinical isolates would be forwarded to public health laboratories, and there were fewer than 1,000 cases annually reported in the United States. Almost immediately, the

benefits of routine WGS became apparent, with more clusters being detected, outbreaks being solved with fewer cases, and novel food vehicles being identified and linked to cases. WGS has entirely replaced PFGE for surveillance of *Listeria* in the United States. The transition of routine *Salmonella* surveillance from PFGE to WGS is now under way. While surveillance moves from PFGE to WGS, PulseNet will remain the public health network through which this surveillance is organized.

A key factor in the success of PulseNet was the ability to standardize laboratory procedures so that PFGE patterns in one laboratory could be reliably compared to those in another laboratory. To maintain this same functionality with WGS for *Listeria*, the CDC is developing core genome multilocus sequence typing (cgMLST) to provide a standard reference of gene loci for the core genome of the pathogen. With cgMLST surveillance, the number of allele, or gene, differences can be compared to the reference standard, allowing a strain-specific allele code to be generated. Isolates with the same or closely related allele code can be identified and compared to historic frequencies, similar to surveillance by PFGE pattern. This approach can be extended to the whole genome by including accessory genes in the analysis. In addition, sequence information codes for other important factors, such as genes associated with serotype, virulence factors, or antimicrobial resistance. Thus, the size and potential severity of the cluster can be simultaneously identified, to help establish priorities for investigation.

A complementary approach to WGS involves the analysis of high-quality single nucleotide polymorphisms (hqSNP). With hqSNP analysis, closely related isolates are compared to determine the number of SNP, or base pair differences, between isolates. This is a highly discriminatory practice that is useful to sort out relationships among isolates within an outbreak investigation. However, because results generated using different references cannot be directly compared, it may not be as generally applicable as cgMLST for routine surveillance. As with PFGE, there are common cgMLST

sequence types for which hqSNP analysis could provide further discrimination.

There are limitations to the use of WGS. Sequencing is not currently as rapid as PFGE. Thus, there may be delays in detecting clusters during the transition from PFGE to WGS. In addition, some *Salmonella* serotypes appear to diversify their genomes faster than others. Thus, inherent variability could affect cluster definitions. For outbreaks that extend over prolonged periods, outbreak-associated cases may have more differences than would cases in a point source outbreak. If an outbreak involves polyclonal contamination, outbreak-associated clusters may not be recognized. These are problems that also limited the usefulness of PFGE. However, the greater discriminatory power of WGS will lower the threshold of investigating clusters. This should largely compensate for these other shortcomings.

The other major laboratory development that impacts pathogen-specific surveillance is the increase in the use of CIDT. These are tests that detect the presence of the agent by identifying specific toxins, antigens, or gene sequences in clinical material without isolating the organism itself. They provide rapid results that are not very specific. A variety of CIDT have begun to be used in clinical laboratories for the primary diagnosis of diarrheal diseases. CIDT targeting STEC or specific parasites have been in use for several years. Positive CIDT results indicating the presence of STEC have required the secondary culture and isolation of the organism for confirmation and subtyping. This secondary culture of the original clinical sample that provided the positive CIDT result is known as a reflex culture. More recently, multipathogen panels have been developed and are becoming widely used in clinical laboratories. CIDTs are popular because they provide results in hours rather than days, which provides information to clinicians that they can use to manage patient care. Multipathogen panels provide results for a wider range of agents than conventional culture methods and require fewer trained microbiologists to use and interpret. Thus,

they may be cheaper to use, in addition to being faster and more informative.

However, the lack of a pathogen isolate to subtype represents a considerable challenge to surveillance. Much of the increased utility of WGS will be lost if CIDT results cannot be confirmed with culture. The CDC and state public health officials are making concerted efforts to have clinical laboratories perform reflex cultures themselves or to forward clinical samples to the public health laboratory for culture. In 2017, the CDC reported a 176% increase in CIDT-positive reports of *Salmonella*, compared to the numbers in 2014 to 2016. However, because of the high frequency and success of reflex cultures, only 9% could not be confirmed (6). For other agents, the loss of isolates was more pronounced, as 36% of *Campylobacter* and 27% of STEC isolates were either culture negative or not cultured. In addition, the process of reflex culture considerably delays the availability of useful subtype data and represents a large cost to both clinical and public health laboratories.

The changing patterns of CIDT use have considerably complicated the CDC's efforts to track trends in the occurrence of foodborne pathogens under surveillance. The dynamic nature of change in the frequency of use and characteristics of individual CIDT performance will need to be assessed to provide a framework to adjust incidence rates over time. On the positive side, if the benefits of CIDT result in more patients being tested and diagnosed, public health officials will see a net gain in surveillance data. While the approaches needed to understand the results from an epidemiologic perspective may change, more data should result in better surveillance and enhanced detection of outbreaks.

Epidemiologic advances

The public health utility of incorporating molecular subtyping into routine surveillance of *E. coli* O157:H7, *Salmonella*, and *Listeria* has been repeatedly demonstrated. However, many subtype cluster

investigations initiated on a national basis fail to identify a common source of exposure. This may reflect the difficulties in conducting timely multistate outbreak investigations. For example, in December 2017, an outbreak of *E. coli* O157:H7 illness was identified. Twenty-five cases across 15 states were identified, and the outbreak-associated strain was linked to a contemporaneous outbreak in Canada, which Canadian public health officials had linked to romaine lettuce. By the end of the outbreak, only 15 of 25 case patients in the United States had been interviewed to ascertain detailed food exposure histories. While leafy greens were suspected, no source could be identified.

In order to improve the level of performance for foodborne illness surveillance and outbreak investigations, the CDC and its state and local partners have initiated several coordinated activities. In 2006, the CDC, the Council of State and Territorial Epidemiologists, and the National Association of City and County Health Officials convened a working group representing local, state, and federal public health and food regulatory agencies. CIFOR began to develop a set of guidelines for foodborne disease outbreak response to standardize and improve outbreak investigation practices. The guidelines were originally published in 2009, and the third edition was published in 2018.

In 2009, the CDC developed a pilot program to explore how to improve disease surveillance and outbreak investigation practices in three states. In 2011, it was named the Foodborne Diseases Centers for Outbreak Response Enhancement (FoodCORE), which expanded to 10 centers by 2014. FoodCORE has promoted timely and comprehensive submission of clinical isolates to public health laboratories for molecular subtyping, rapid interviewing of reported case patients by teams of student workers using detailed food exposure questionnaires, and enhanced coordination and communication with environmental health specialists. The usefulness of many of these practices has been revealed over the past 20 years by the Minnesota Department of Health. Federal

support through the CDC is enabling other states to employ the same methods.

With the passage of the FSMA in 2011, Congress mandated that federal public health and food regulatory agencies work with state and academic partners to improve surveillance for foodborne illnesses. The FSMA established a network of Integrated Food Safety Centers of Excellence (COE) in five states to empower health departments and academic partners to assist other states improve their outbreak investigations. The COEs coordinate with foodborne illness surveillance programs at the CDC to evaluate the effectiveness of surveillance measures and provide training to build the public health workforce.

Following the passage of the FSMA, the FDA established the Coordinated Outbreak Response and Evaluation Network (CORE), a multidisciplinary team of FDA specialists charged with trying to assist the CDC and states to investigate multistate outbreaks. CORE coordinates with the FDA's rapid response teams (RRTs). These are multiagency, multidisciplinary teams that use Incident Command System principles to respond to human and animal food emergencies.

CORE and the RRTs have been particularly involved in product tracing activities. Although product tracebacks are frequently started only after a food item has been implicated, detailed product information is actually critical to epidemiologic analysis, and tracebacks intended to provide source information that can be used in epidemiologic analyses are critical to the identification of the contaminated food item. The importance of this point is further emphasized by the increasing occurrence of outbreaks associated with foods, such as salads and salsas, that routinely contain multiple ingredients. Had source tracing of tomatoes been pursued before tomatoes were implicated as the source of a large *S.* Saintpaul outbreak in 1998, the lack of convergence for tomato sources might have led to consideration of other produce items frequently combined with tomatoes. However, it is likely that

investigators were anticipating tomatoes to be identified as the source, given the frequency of tomato-associated outbreaks and the apparent strength of the association based on the results of an initial case-control study.

The importance of product tracing early in the course of outbreak investigations is emphasized by the major changes that are occurring in outbreak investigation methods. Increasingly, routine interviews of all patients with *Salmonella*, STEC, and *Listeria* illness are being conducted by teams of student workers using detailed hypothesis-generating questionnaires. As case clusters are being identified, frequencies of case food exposures are being compiled and compared to expected values from population surveys or sporadic case aggregations. The differences in these proportions are being tested by simple statistics, and likely exposure sources are being identified. Because most of these aggregated data sources do not contain fine product details, product tracing is conducted to ascertain a common source for the suspected exposure. This approach is proving efficient and reliable and will likely be the standard of practice for the foreseeable future.

Integration of surveillance on a global level

The Global Foodborne Infections Network has been established by the World Health Organization (WHO) to promote pathogen-specific surveillance for *Salmonella*, *Campylobacter*, and *E. coli* O157:H7 and provide training to international partners. Declining public health resources in developed and developing countries may severely limit the usefulness of this system and the international distribution of PulseNet. Building the capacity to conduct molecular subtype surveillance is necessary to detect widely dispersed outbreaks caused by common serotypes and to track the emergence of global food safety threats. Failure to invest in these advanced surveillance systems will consign developing countries to a basic level of surveillance.

Determining Risk Factors for Sporadic Cases of Infection with Common Foodborne Pathogens

Determining risk factors for sporadic cases of infection with common foodborne pathogens is a third major component of foodborne disease surveillance. Population-based case-control studies of sporadic infections are needed to supplement outbreak data for attribution studies. They can help identify targets for intervention and provide a basis to evaluate its effectiveness. Case-control studies of sporadic *S.* Enteritidis infections in the United States and Europe helped establish the role of grade A shell eggs in the epidemiology of these infections.

Case-control studies of *Campylobacter* infections helped establish chicken as a primary source of *Campylobacter*. Similarly, case-control studies of sporadic *E. coli* O157:H7 infections confirmed the importance of ground beef, particularly undercooked ground beef, as a source of the pathogen. However, case-control studies of this type are expensive to conduct and may require several years to complete. Thus, they may not be sensitive to rapid changes in patterns of food sourcing or consumption, both of which can shift over short time periods. In addition, because common foodborne pathogens may have many potential sources of exposure, these methods are inherently limited with regard to evaluation of exposure to commonly eaten food items.

FoodNet has conducted population-based, case-control studies for *E. coli* O157:H7, *Salmonella* serogroups B and D, *Campylobacter*, *Cryptosporidium*, and *L. monocytogenes*. These have provided updated and original estimates of the proportion of these infections that are attributable to specific food items. Results of sequential *E. coli* O157:H7 case-control studies revealed a decrease in the risk of *E. coli* O157:H7 infections associated with eating hamburgers at a fast-food restaurant. This validated the fact that fast-food restaurants were thoroughly cooking the hamburgers they serve. At home, or at table service restaurants, where consumers have a choice to order an undercooked hamburger, eating undercooked

hamburgers remained associated with illness. Case-control studies of *Campylobacter*, *S.* Enteritidis, and *S. enterica* serovar Heidelberg infections also revealed associations with eating out, not at home. Other interesting findings included associations between *S.* Enteritidis infection and chicken consumption, a finding that was subsequently documented through a series of outbreak investigations. Similarly, a FoodNet case-control study of sporadic *L. monocytogenes* infections identified eating melons at a commercial establishment as a risk factor for illness before the 2011 outbreak was associated with cantaloupe. Cantaloupe was included on food exposure questionnaires because of the results of the case-control study.

Population Surveillance

The final important component of foodborne disease surveillance is population surveillance to determine the frequency of gastrointestinal illnesses, health care-seeking behavior, food consumption, and personal prevention measures. This type of syndrome-specific surveillance is not usually conducted by state or local health departments. However, these measures formed the basis of the CDC's estimates that 48 million foodborne diseases occur each year in the United States. These data are also useful for understanding the results of active surveillance. By describing when and why people seek medical care when they are ill, these data can help to estimate how many illnesses occur for each one that gets a specific diagnosis. With the development and use of CIDT, this type of information is critical to understanding foodborne illness trends. Finally, the population-based food consumption data are being used to estimate the proportion of specific illnesses attributable to specific exposures in the FoodNet case-control studies, which are used to help develop foodborne illness attribution models.

Following models developed to build on FoodNet findings, international collaborations have been coordinated through the WHO to strengthen disease surveillance and to determine the global burden of acute gastroenteritis (7). The Foodborne Disease Burden

Epidemiology Reference Group (FERG) found that the most frequent causes of foodborne illness were norovirus and *Campylobacter* spp., with non-typhoidal *Salmonella enterica* being a leading cause of deaths. Forty percent of the burden of foodborne illnesses was borne by children under 5 years of age, with the greatest burden in Africa.

Results of the FERG study support integrating food safety policies into poverty reduction programs targeting food security and nutrition. Encouraging international investments to promote access to safe drinking water and sanitation in developing countries is a global public health issue. Reducing the burden of foodborne illness in these countries will provide global benefits through the reduced risk of contamination of foods imported into developed countries.

SURVEILLANCE FOR FOOD HAZARDS

Risk assessments for specific pathogens, such as *E. coli* O157:H7 in ground beef or romaine lettuce, *S.* Enteritidis in shell eggs or chicken meat, or *L. monocytogenes* in soft cheeses or frozen vegetables, require making measurements or assumptions about many parameters. At every step from farm to table, potential sources of contamination can be identified, measured, and modeled to determine the relative contribution to the overall risk of foodborne illness.

Environmental Monitoring

For many foodborne pathogens of public health importance, contamination of finished food products is a sporadic and rare occurrence. While end-product pathogen testing is difficult to justify as a primary prevention measure, it provides important information as part of a comprehensive quality assurance testing program. Given the scale of modern agriculture and food production systems, contamination levels below the statistical and microbiological threshold can still be large public health problems.

For example, internal contamination of eggs with *S.* Enteritidis is rare, occurring in an estimated 1 in 20,000 eggs on a national basis in the United States. Despite this sporadic occurrence, consumption of contaminated shell eggs is still an important cause of foodborne illness. For this reason, surrogate measures of contamination, such as identifying infected hens or environmental contamination in egg laying houses, have been widely accepted as being more efficient and effective than testing individual eggs.

Similarly, environmental sampling of generic *Listeria* spp. in food processing environments has been critical to manage and reduce the risk for contamination by *L. monocytogenes*. Identification of potential harborage areas for generic *Listeria* spp. has allowed manufacturers to target sanitation measures to eliminate potential source of contamination. Successful application of environmental monitoring in plants that process ready-to-eat meats, along with product formulations that inhibit growth of *Listeria*, has helped reduce the number of outbreaks associated with deli meat products. This has contributed to incidence rates of *Listeria* infections in the United States that are at all time low levels.

WGS is providing new opportunities to monitor patterns of environmental contamination in food manufacturing facilities. Environmental isolates can be subtyped and monitored over time to identify strains that may have taken up residence in the facility. Identifying these resident organisms may indicate the need for special corrective actions or sanitation measures to eradicate these persisting sources of contamination.

Finished-Product Testing

Although food manufacturers may avoid finished product testing, routine food testing is conducted as part of larger food safety verification programs operated by the FDA, FSIS, and state agriculture departments. WGS is routinely performed on these food isolates, with gene sequences uploaded to the National Center for Biotechnology Information (NCBI) at the National Institutes of

Health. These data are compared to human cases in the PulseNet database to identify potential links. This type of cross-referencing between food, environmental, and human isolates has become an important tool to identify previously unrecognized outbreaks and to generate hypotheses during cluster investigations. For example, *L. monocytogenes* isolated from an ice cream sample in South Carolina was traced to a manufacturing facility in Texas that was subsequently linked to a cluster of cases that occurred in a Kansas hospital in 2015. These isolates were all determined by WGS to be closely related. During the investigation of ice cream products at the Kansas hospital, an additional ice cream product from a separate manufacturing facility in Oklahoma was found to be contaminated with *L. monocytogenes*. The *Listeria* isolate from this second product was different from the original outbreak strain. However, a search of PulseNet identified five other cases from three states with onset of illness from 2010 to 2014 that were caused by closely related strains of *Listeria*. At least one of these case patients had a history of consuming ice cream from the source plant.

The example of the ice cream-associated listeriosis outbreak highlights several important considerations for food and environmental testing in the WGS era. Because results of WGS data can be stored electronically in searchable databases, links can be made across space and time with unprecedented ease. Once a record is established, it may be retrieved years later. In this outbreak, WGS linked cases that had occurred 5 years previously to a manufacturing source. However, the outbreak investigation provided an epidemiologic context to the link between cases and source. Just identifying an isolate from a case that is closely related to a food isolate by WGS does not imply that the food was the source of the illness. For every potential WGS link that is made between food or environmental isolates and human cases, an epidemiologic investigation must be conducted to establish the relationship between source and case.

Behavioral Risk Factor Surveillance

Behavioral risk factors for foodborne disease range from choosing to eat alfalfa sprouts or undercooked ground beef to failing to wash hands between using a toilet and making a salad. These factors are evaluated and can be identified during the course of outbreak investigations. The frequency of these behaviors in the general population can be assessed through surveys, such as those being conducted as part of FoodNet. Information of this type can provide important perspectives for understanding the occurrence of foodborne disease outbreaks and sporadic infections. It also serves as the basis for developing and modifying public education campaigns to improve food safety, such as the Partnership for Food Safety Education's national public education campaign, Fight Bac!.

Environmental Hazard Surveillance

Factors contributing to the occurrence of foodborne disease outbreaks are compiled by the CDC from national surveillance data and by individual states. National data are published in annual foodborne outbreak surveillance summaries. Leading contributing factors include bare-hand contact by a food worker who is suspected to be infectious and contamination of raw foods intended to be eaten raw. These factors are associated with norovirus transmission routes. Cross-contamination and food preparation practices that support proliferation were more frequently cited as contributing to *Salmonella* outbreaks. Unfortunately, contributing factors have been reported from only 50 to 60% of all outbreaks reported to the CDC. Bias in what is reported is the largest systematic problem for interpreting national foodborne disease outbreak surveillance data.

To address the lack of contributing factor information reported to NORS, NEARS, which collects outbreak data on restaurants and other retail food service establishments, collects data on environmental factors related to the occurrence of the outbreak and on the reasons that these factors occurred. Relating contributing factors to their environmental antecedents may provide a better understanding of corrective actions that can be applied to help food service operators prevent foodborne illness in their establishments.

NEARS is intended to be a companion to NORS, so that information on the same outbreaks can be reported to both and linked. The ability to link outbreaks between NORS and NEARS creates a unique analytical resource that can be especially useful to explore the role of restaurants in the epidemiology of foodborne illnesses.

The improvements that NEARS will bring to contributing factor reporting highlight a critical need for addressing likely food hazards: first, to determine the proportion of illnesses and outbreaks attributable to the hazard, and then to estimate the predictive value of the hazard for causing disease. In a restaurant setting, we may know how many times bare-hand contact with foods or failure to wash hands is identified as a contributing factor in an outbreak of foodborne illness, but what is the frequency of this failure in restaurants? The FDA began to address these data needs in 1997 with the creation of the FDA Retail Food Program Database of Foodborne Illness Risk Factors. This was followed by surveys in 2003 and 2008 to assess trends (8). Results of these surveys revealed better compliance with hand washing in fast-food restaurants than in full-service restaurants. Both fast-food and full-service restaurants increased compliance with provisions regarding bare-hand contact with ready-to-eat foods. While both fast-food and full-service restaurants improved on holding times and temperatures, these were out of compliance in approximately half of full-service restaurants. Approximately one-third of full-service restaurants had potential contamination issues. These studies provide data to address important environmental questions using epidemiologic methods applied to surveillance for the hazards. Current attribution models focus on commodity-level attributions. However, since more than 50% of outbreaks occur in restaurant settings, a better understanding of how restaurants modify commodity-level risks is imperative.

FOODBORNE DISEASE SURVEILLANCE AND ATTRIBUTION

Public health surveillance of foodborne disease is critical to the performance of food safety systems that are based on HACCP (hazard analysis and critical control point) plans. Surveillance is required to identify new hazards. It also provides the ultimate feedback on the efficacy of HACCP plans. In the 1994 outbreak of salmonellosis due to commercial ice cream, cross-contamination during transportation was identified as a hazard that had not been addressed in the manufacturer's HACCP plan.

As noted earlier, case-control studies of sporadic outbreaks of *E. coli* O157:H7 infection revealed that public health recommendations regarding thorough cooking of hamburgers appeared to reduce the risk of eating hamburgers in fast-food restaurants. Following the 2002 FSIS notice to manufacturers of raw ground beef products that they must consider *E. coli* O157:H7 a potential hazard in their HACCP plans, the percentage of positive samples at retail decreased markedly, and the incidence of *E. coli* O157:H7 infections decreased as well, achieving Healthy People 2010 goals.

The FoodNet surveillance network was established, in part, to provide a tool to evaluate the public health impact of the USDA's implementation of meat processing plant HACCP systems. FoodNet sites provide population-based measures of the incidence of illnesses caused by several important foodborne pathogens. The reductions in the occurrence of *E. coli* O157:H7 infections demonstrated the success of coordinated activities by ground beef producers, FSIS, and public health officials to reduce the risk of infections associated with ground beef. Currently, while overall rates of salmonellosis have remained stable for the past 20 years, there have been considerable declines in *S.* Typhimurium and *S.* Heidelberg infections over the past 10 years. This is important because these were among the serotypes targeted by the poultry industry and FSIS measures. Furthermore, the declines in numbers of human cases correlate with declines in numbers of positive chicken meat samples. Thus, these results suggest that these coordinated activities have reduced the burden of disease associated with these food-pathogen pairs.

As noted above, food attribution models being developed by the Interagency Food Safety Analytics Collaboration incorporate outbreak data using a standardized food categorization scheme. This model is being updated to incorporate statistical modeling approaches to evaluate temporal changes in attribution estimates. Given the examples of changes observed by FoodNet, having attribution models that can be updated to reflect current conditions in the food system is critical to maintaining effective food control. In addition, because attribution measured at the point of processing may have different results than attribution at the point of service, efforts are being made to estimate food source attribution along the farm-to-table continuum. In this regard, restaurant operations will be a particularly important focus area. The final area of great effort is to incorporate WGS and case exposure data for food source attribution. The high degree of granularity with WGS may allow more stable associations between specific clonal groups of pathogens and food animal or environmental reservoirs. Initial efforts to characterize source associations with *S.* Enteritidis illnesses may serve as a model that can be expanded into a larger range of serotypes.

SUMMARY OF MAJOR ADVANCES IN THE EPIDEMIOLOGY OF FOODBORNE ILLNESS

Humans remain the ultimate bioassay for low-level or sporadic contamination of our food supply. Epidemiologic methods of foodborne disease surveillance are powerful tools because they take advantage of events that are occurring throughout the population. This population-based lens, focused by advances in molecular subtyping and information technology available to public health laboratories, is particularly well suited to dealing with foodborne diseases associated with mass-produced and widely distributed food products.

WGS is becoming the standard for public health surveillance. It offers greater sensitivity for outbreak detection and greater specificity for outbreak investigation than previously used methods. Public health officials will be challenged to provide the epidemiologic resources to investigate and solve the anticipated growth in small outbreaks. WGS links between cases and foods or environmental samples will require exposure assessment. Ascertainment of detailed information on food source and production is needed to confirm the cause of the outbreak and identify contributing factors.

In contrast, CIDT threaten public health surveillance with a loss of case specificity, requiring that the local cluster of illnesses, such as that caused by *Salmonella*, be recognized based on a common exposure to a retail food establishment. The increased use of CIDT by clinicians may offset the loss of specificity in the test result by increasing the numbers of cases reported.

Streamlined approaches to outbreak investigations focused on detailed exposure assessment and comparison to expected values, followed by product tracing to confirm the source, will increase efficiency, but the results need careful evaluation to avoid spurious associations.

Better outbreak investigation methods, including environmental assessments, will provide more useful data to evaluate the effectiveness of food safety policies and improve food attribution models. Improving the science base for policy development should lead to more effective food control activities.

The same methods of observation and analysis that form the basis of foodborne disease surveillance in the United States are also being practiced across the world in developed countries and are being adapted for use in many developing countries. Since national food supplies are rapidly becoming global in origin, the need for an international system for foodborne disease surveillance exists as well. Models such as PulseNet provide opportunities to conduct multinational surveillance for at least the major bacterial foodborne

disease agents. Because foodborne disease problems imported into one country may represent disease problems endemic to the food-producing country, growing awareness of these problems could stimulate investment in interventions that improve public health in both countries. In particular, the global trade in fresh produce, spices, aquacultured fish and seafood, and seed stock requires safe water distribution and sanitary sewage disposal systems wherever these products are handled.

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9
Salmonella

CHARACTERISTICS OF THE ORGANISM

Salmonella, a rod-shaped facultative anaerobic member of the family *Enterobacteriaceae*, is a ubiquitous microorganism able to infect a multitude of hosts, including animals, plants, and protozoa, and colonize diverse environments. The versatility of this pathogen resides in its ability to swiftly adapt to broad shifts in temperature, pH, water activity, and oxygen concentrations (1). These bacteria are generally motile and are peritrichous, except for the nonflagellated serovars *Salmonella* Pullorum and *Salmonella* Gallinarum (2). Some metabolic traits of the species of this genus that distinguish it from other Gramnegative rod-shaped enterobacteria are production of catalase, the absence of the cytochrome *c* oxidase, and the inability to ferment lactose and produce urease and indole.

The unique *Salmonella* environmental pervasiveness, its ability to cause disease in humans and animals, and the multiple routes of infection make this pathogen a public health threat that cannot be eradicated, only identified and contained. In developed and industrialized countries, *Salmonella* spp. contaminate mainly animal products and produce, whereas, in developing countries, waterborne transmission and person-to-person transmission play a more important role. There are as many as 130 million cases of nontyphoidal salmonellosis worldwide each year, and of those, about 80 million are foodborne. Considering that there is an estimate of 2.8 billion diarrheal diseases worldwide, this makes *Salmonella* responsible for 3% of them (3). In the United States, *Salmonella* is responsible for approximately 1.02 million illnesses and more than 378 deaths each year. These estimates make nontyphoidal *Salmonella* (NTS) serovars the leading cause of bacterial foodborne illness (4).

CLASSIFICATION AND TAXONOMY

Nomenclature

The first microorganism belonging to the genus now called *Salmonella* was first isolated in 1884 by Theobald Smith, a trained physician working at the time as inspector with the U.S. Bureau of Animal Industry, and described by Daniel E. Salmon, who was the veterinary surgeon chief of the Bureau and the first recipient of a degree in veterinary medicine in the United States. The bacillus-shaped microorganism was isolated from porcine intestines during an outbreak of what was thought to be hog cholera and, as a result, named *Bacillus choleraesuis*. In 1900, the director of the National Institute of Bacteriology in Buenos Aires, Argentina, Jose Leon Marcel Lignières, first proposed placing all the bacteria causing hemorrhagic septicemias into a single group, called *Pasteurelloses* (5). However, the following year, recognizing that such a general classification was too vague, he proposed separating the group into two taxa, *Pasteurella* and, to honor the lead discoverer, *Salmonella* (6).

In 1923, Bergey first physiologically (7) defined the members of this genus as follows: “Motile forms occurring in the intestinal canal of animals in various types of acute inflammatory conditions. Attacks numerous carbohydrates with the formation of both acid and gas. In general, does not form acetyl-methyl-carbinol.” This definition was modified in 1930 to exclude lactose, sucrose, and salicin from the fermented carbohydrates and again in 1934 to further restrict the genus definition to organisms that form acid only in carbohydrate media (8). In 1934, *Salmonella* was recognized as a genus by the *Salmonella* subcommittee of the Nomenclature Committee of the International Society for Microbiology. The committee also decided that the most efficient way to classify the members of this

new genus was through the discriminating criteria based on serotyping first proposed by Bruce White in 1929 and expanded by Fritz Kauffmann in 1931.

Based on this system, each new serological type identified by O (somatic) and H (flagellar) antigen characterization received a name according to the disease caused, the animal affected, or the name of the place of its first occurrence. These serological varieties (serovars) were to be considered different species if they showed distinctive cultural characteristics. In 1966, Kauffmann proposed the one serovar-one species concept by which every serovar should be considered a separate species (9). Kauffmann also divided the genus *Salmonella* into four subgenera (I to IV) based on biochemical characteristics without making a formal nomenclature proposal (10). This proposal was only one of several classification systems used for epidemiological purposes, but, if adopted, it would have resulted in more than 2,500 different species. At the same time, phenotypic studies and numerical taxonomy showed that *Salmonella* serovars within each subgenus were biochemically indistinguishable. As a result, Kauffmann's subgenera were considered species: "*S. kauflmannii*" (subgenus I), "*S. salamae*" (subgenus II), *S. arizonae* (subgenus III), and "*S. houtenae*" (subgenus IV) (11). In the early 1970s, the development of new DNA-based techniques allowed new approaches to understanding the taxonomy of the *Salmonella* genus and its nomenclature.

In 1973, Crosa et al. determined by DNA-DNA hybridization that virtually all the serovars of *Salmonella* were highly related from the genetic standpoint and supported the classification of all the *Salmonella* serovars and species and all serotypes of the genus *Arizona* thus far identified as one species (12). This genetic similarity, however, conflicted with the broad biochemical and serological variability, host range, and pathogenicity characteristics of the genus. As a result, Crosa et al. supported the three-species system proposed by Ewing in 1972, consisting of *Salmonella choleraesuis*, *S. typhi*, and *S. enteritidis* (13). Because "choleraesuis" as a designation denoted a species as well as a serovar, thus possibly leading to classification confusion, "*Salmonella enterica*" was suggested in 1986 as the designation of the *Salmonella* type species by the Subcommittee of Enterobacteriaceae of the International Committee on Systematic Bacteriology at the XIV International Congress of Microbiology (14). In 1987, Le Minor and Popoff proposed dividing *Salmonella* into seven subspecies: *S. enterica* subsp. *enterica* (I), *S. enterica* subsp. *salamae* (II), *S. enterica* subsp. *arizonae* (III), *S. enterica* subsp. *houtenae* (IV), *S. enterica* subsp. *bongori* (V), and *S. enterica* subsp. *indica* (VI) (15). Subsequently, biochemical and genetic differences led to the split of subspecies III into *S. enterica* subsp. *arizonae* (IIIa) and *S. enterica* subsp. *diarizonae* (IIIb) and gave *S. enterica* subsp. *bongori* (subspecies V) separate species status. Specifically, the latter decision was based on DNA relatedness studies and multilocus enzyme electrophoresis techniques (16).

This system was accepted by several organizations across different countries, including the American Society for Microbiology, the World Health Organization (WHO), and the Centers for Disease Control and Prevention (CDC), but it was rejected by the Judicial Commission of the International Committee of Systematic Bacteriology. The rejection was based on the possibility of overlooking the clinical importance of the serovar *Salmonella* Typhi by making it a serovar of *S. enterica* subsp. *enterica*. More importantly, the changes proposed by Le Minor and Popoff implied a restructuring of the taxonomy of the genus, and the commission did not and does not have the authority to rule on such changes.

In 2002, the commission reconsidered the nomenclature proposal by Le Minor and Popoff and, after considering recent taxonomic analyses and subsequent requests for changes submitted by several scientists, issued an opinion (Opinion 80) that was made official in 2005 and clarified by Tindall et al. (17). Based on this opinion, the type species of the genus *Salmonella* Lignieres 1900 is *S. enterica*, and the epithet *enterica* should be conserved over all earlier epithets that may be applied to this species (e.g., *choleraesuis*). The ruling recognized that *S. enterica* should be divided into six subspecies: *S. enterica* subsp. *enterica*, *S. enterica* subsp. *S. enterica* subsp. *salamae*, *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diarizonae*, *S. enterica* subsp. *houtenae*, and *S. enterica* subsp. *indica*. It also implicitly accepted the taxonomic proposal by Reeves et al. (16), thus establishing two species, *S. enterica* and *Salmonella bongori*.

Serovars

In most bacterial species, a serovar is defined by a combination of surface antigens that produce serological reactions, leading to an antigenic formula (e.g., *Escherichia coli* O157:H7). In *Salmonella* species and subspecies except *S. enterica* subsp. *enterica*, antigenic formulae are assigned using the simplified Kauffmann-White-Le Minor scheme based on the O and H antigens. In *Salmonella*, the H antigens of flagellin can undergo rapid mutations and switch immunologic reactivity, in a phenomenon called phase variation. This is a method deployed by this and other pathogens to evade the immune response. For this reason, the antigenic formula includes antigen classification for H in phase 1 and 2. According to this scheme, the subspecies name is written in roman numerals (e.g., II, IIIa, IIIb, IV, and VI), including the species *S. bongori* (V) (mainly for consistency with the formula system) followed by a space, the O antigens (separated by a comma if needed) followed by a colon, H antigens from phase 1 followed by a colon, and H antigens in phase 2, whenever present (for example, *Salmonella* serotype II 6,7:Z_6:1,7 refers to *S. enterica* subsp. *salamae*, O antigens 6 and 7, H antigen Z_6 in phase 1, and H antigens 1 and 7 in phase 2). The official serological classification system is maintained by the WHO and is published in *Antigenic Formulae of The* Salmonella *Serovars* (18).

S. enterica subsp. *enterica* has the largest number of serovars (more than 2,500), and most them are of human relevance. For this reason, using the antigen formula for their identification could cause confusion. Therefore, the modern nomenclature rules assign names to serovars of this subspecies based on the name of the geographic area where a serovar was first isolated. There are a few exceptions where the historical names based on the illness caused or the habitat or host of the serovar are still maintained (i.e., Typhimurium, Typhi, Paratyphi, and Gallinarum).

Concerning proper naming in publications and official documents, the first mention of a serovar should contain the full species name (*Salmonella enterica*) followed by the subspecies (*enterica*), which must be italicized (*Salmonella enterica* subsp. *enterica*). This is then followed by the word "serovar" along with the name of the serovar (e.g., *Salmonella enterica* subsp. *enterica* serovar Agona). Note that the name of the serovar is given in roman letters with the first letter capitalized. Subsequent mentions of the name can be condensed to "*Salmonella*" followed by just the serovar name, for example, *Salmonella* Agona. In scientific writing, abbreviation of a genus name in front of anything but a species epithet is strongly discouraged, as it could lead to confusion of that name for a species. Therefore, a serovar name should not follow an abbreviated genus (e.g., *S.* Agona) (18, 19).

Isolation

Isolation of *Salmonella* from foods and surfaces can often be challenging because of low cell numbers and the competing microbiota present. The variety of foods that can be contaminated by this foodborne pathogen, including eggs, candies, spices, and pig ears, makes the development of a standard isolation method particularly challenging. Currently, the U.S. Food and Drug Administration (FDA) publishes a laboratory methods manual that contains the procedure accepted for food and environmental testing (20). Sampling and homogenization of the food is always dependent on the type of food considered and can vary substantially between foods. However, for most of them, there are different specific broths (i.e., lactose broth, Trypticase soy broth enriched with ferrous or potassium sulfate, tetrathionate broth, and universal pre-enrichment broth) used to resuspend, homogenize, and pre-enrich the sample. This first preenrichment is generally followed by enrichment in a slightly acidic and hyperosmotic medium, Rappaport-Vassiliadis (RV) broth, and tetrathionate broth. If the presence of *Salmonella* Typhi is suspected, selenite cysteine broth is used instead of RV broth, as this serovar is inhibited in RV. Both enrichments are streaked for single colonies on three selective and differential media: bismuth sulfite (BS), xylose lysine deoxycholate (XLD), and Hektoen enteric (HE) agar. Following incubation for 24 h, typical *Salmonella* colonies look brown, gray, or black with sometimes a metallic sheen and a brown or black halo on BS, pink with or without black centers on XLD, and blue-green to blue with or without black centers on HE. If present, two typical *Salmonella* colonies are picked from each plate for subsequent analysis. Occasionally there can be atypical colonies that should also be recognized and characterized. Some *Salmonella* strains can produce green colonies with little or no halo on BS agar or yellow colonies with or without black centers on HE and XLD agars. These colonies typically grow more slowly than normal and should also be collected for identification.

At this point, colonies from the plates are usually characterized for their reaction on triple sugar iron (TSI) slants and lysine iron agar (LIA). In TSI, *Salmonella* typically produces an alkaline (red) slant and acid (yellow) butt, with or without production of H_2S and blackening of agar. In LIA, *Salmonella* usually produces an alkaline (purple) reaction at the bottom of the tube. Only a clearly yellow butt of the tube should be considered acidic and, therefore, negative, as other shades or colors can still be indicative of *Salmonella*. The LIA reaction is the most important in this phase for the identification of *Salmonella*. As a result, all the isolates that are positive for the LIA test should be kept for subsequent analyses, regardless of the TSI results. The presumptive *Salmonella* isolates should be tested for urease activity (negative), dulcitol fermentation in red phenol tubes (positive with gas formation), inability to grow in KCN broth, negative malonate broth reaction (green or unchanged color), and negative indole test (no deep red/pink color at the top of the tube). These cultures should be also serologically tested for flagellar type. Any culture either positive in the indole test and negative in the serological flagellar (H) test, or positive in the KCN test and negative in the lysine decarboxylase test, is not *Salmonella* and is discarded. Table 9.1 provides a list of biochemical reactions distinctive for *Salmonella* species.

Table 9.1 Biochemical and serological reactions of *Salmonella* (reproduced from reference 20)

	Result		
Test or substrate	**Positive**	**Negative**	***Salmonella* reaction[a]**
Glucose (TSI)	Yellow butt	Red butt	+
Lysine decarboxylase (LIA)	Purple butt	Yellow butt	+
H_2S (TSI and LIA)	Blackening	No blackening	+
Urease	Purple-red color	No color change	–
Lysine decarboxylase broth	Purple color	Yellow color	+
Phenol red dulcitol broth	Yellow color and/or gas	No gas; no color change	+[b]
KCN broth	Growth	No growth	–
Malonate broth	Blue color	No color change	–[c]
Indole test	Red color at surface	Yellow color at surface	–
Polyvalent flagellar test	Agglutination	No agglutination	+
Polyvalent somatic test	Agglutination	No agglutination	+
Phenol red lactose broth	Yellow color and/or gas	No gas; no color change	–[c]
Phenol red sucrose broth	Yellow color and/or gas	No gas; no color change	–
Voges-Proskauer test	Pink-to-red color	No color change	–
Methyl red test	Diffuse red color	Diffuse yellow color	+
Simmons citrate	Growth; blue color	No growth; no color change	v

[a] +, 90% or more positive in 1 or 2 days; —, 90% or more negative in 1 or 2 days; v, variable.

[b] The majority of *Salmonella* Arizonae cultures are negative.

[c] The majority of *Salmonella* Arizonae cultures are positive.

Core Genome versus Pangenome

Biochemical and serological characterizations are useful and effective when determining the identity of isolates that have already been characterized. However, only after the development of genome-based phylogenetic reconstruction was it possible to clearly define the members of a genus. This shows that in bacteria, these identification techniques based on phenotypic characteristics lack taxonomic accuracy and, consequently, are not appropriate for identifying the relationship of a new, unknown strain to others. This inadequacy is mainly due to the genetic nature of the traits used by serological and biochemical tests. In fact, these phenotypic characters are linked to genes that have high levels of mutations (e.g., flagellar genes) or horizontal transfer between congeneric species (21).

DNA-based methods such as DNA-DNA hybridization, 16S rRNA gene sequencing, pulsed-field gel electrophoresis, multilocus sequence typing, and other molecular-marker-based techniques have been developed to make identification more reliable and better linked to the taxonomy of this genus (21, 22). Over the past 2 decades, the development of cost-effective high-throughput sequencing techniques and the exponential increase of computational power have produced an ongoing paradigm shift that challenges our definition of species. This is particularly true for prokaryotes, as these microorganisms have generally smaller genomes than eukaryotes and a high frequency of horizontal transfer that calls into question our concept of cohesive species (23). The comparison of the genomes of different strains of *E. coli* and other well-known bacteria has shown that certain chromosomal regions and the genes contained therein are highly conserved at different taxonomic levels, whereas other sections appear to be highly variable and sometimes uniquely linked to a single species or population (23). The genes that are common to all the species members of a genus are defined as part of the core genome of that genus (e.g., the *Escherichia* core genome accounts for about two-thirds of any species genome belonging to it). Similarly, the genes shared by all the subspecies or populations of a species constitute the core genome and define that species genetically (e.g., *S. enterica* has an estimated core genome comprising 3,200 gene families over an average genome size of 5,000 genes [23]). Conversely, the genes that are present in only some of the taxa of a certain group are considered "accessory genes." The sum of all the accessory genes across all the members of a taxonomic group and the core genome is the pangenome of that group. Usually the taxonomic ranks considered for the identification of core and pangenomes in bacteria span genus to serovar (24).

The addition of new complete genomes has shown that certain species have a "closed" pangenome. This means that the species is confined to a homogeneous group of strains with a relatively large core genome and a restricted number of accessory genes. Variation among these strains is usually limited to genetic mobile elements and single nucleotide polymorphisms (25). *S. enterica* and *S. bongori*, with large stretches of shared virulence factor-carrying genomic islands (*Salmonella* pathogenicity islands [SPI]), belong to this group of organisms, with an estimated core genome of about 2,800 genes and a pangenome of 10,000 genes (26). Other bacterial species have what are called "open" pangenomes; these groups have very variable genome sizes, generally small core genomes, and an ever-expanding number of unrelated accessories. Although closely related to *Salmonella* spp., *E. coli* is a good example of a species with an open pangenome. The difference between open and closed pangenomes does not depend on the number of sequenced genomes available, as the phenomenon is still significant even when the relative number of genomes analyzed per species is taken into account.

The fact that two closely related genera, such as *Salmonella* and *Escherichia*, represent a closed and an open pangenome indicates that horizontal gene transfer mechanisms are not directly responsible for this difference, as both genera rely on the same mechanisms. In all bacterial genomes, there is a certain level of redundancy and repeated sequences that inflate the genome size. However, the number of functional genes and pathways that are present correlates well with the diversity of growth conditions an organism can endure. Therefore, an intriguing possibility is that *Salmonella*'s success as an animal pathogen is based on a fine balance between trading sources of genomic variability and niche expansion for specialization and adaptation to specific hosts.

PHYSIOLOGY

Salmonella normally grows at temperatures between 5.5 and 45°C, depending on the specific serovar and the environmental conditions. It needs a pH ranging between 6.5 and 7.5 for optimal growth; however, it is able to grow at a much broader range of pH values (3.99 to 9.5) by using pH-dependent acid resistance (AR) mechanisms that are specific to the stationary phase or a low-pH acid tolerance response (ATR) that can be induced in stationary or exponential phase (27–30). This response is characterized by the specific expression of several proteins, mostly membrane related, regulating the growth rate of the cells and the pH balance within the cell. These changes have been shown to induce protective mechanisms against other stresses, such as low water activity (a_w) and heat stresses. Exposure to sublethal temperatures of less than 50°C for 15 to 30 minutes can provide increased tolerance to higher temperatures in *Salmonella*, completely independent of ATR (28, 29). Similarly to the ATR, heat pretreatment also induces the expression of heat shock proteins that can increase the heat resistance of the cells. This stress response system involves several general stress transcriptional regulators and might have a crossover protection effect against other environmental conditions as well.

Desiccation Resistance

One of the most important challenges that *Salmonella* encounters in environmental habitats, such as soil and lowmoisture foods, is water loss through evaporative drying or through the matric potential generated by the adhesion of water to small particles present in soil. These processes decrease the water available to participate in chemical reactions and, ultimately, lower the water activity in the environmental system. *Salmonella* achieves protection against low a_w by slowing the evaporation of water and preventing complete desiccation by specific mechanisms that protect membranes and proteins from the denaturation caused by the loss of water. Although the genetic and physiological responses to desiccation stress in *Salmonella* have not been thoroughly studied, there are strong indications that they overlap with other stress response networks triggered by similar chemophysical signals (e.g., osmotic, thermal, and oxidative stress) (31).

On the surface of the bacterial cell, there is usually a layer of exopolysaccharides and associated proteins, forming an extracellular matrix, referred to as the glycocalyx. This surface structure is known to have a variety of protective functions, including preventing complete desiccation by holding water and limiting the amount lost through evaporation. Also, extracellular cellulose associated with curli amyloid fiber has been shown to play a major role in desiccation resistance and survival in *Salmonella* Typhimurium (32). This extracellular cellulose has been shown to be modified by a phosphoethanolamine. The presence of this modification makes the polymer a zwitterion, or inner salt, which is an overall neutral molecule bearing positive and negative ionic groups. Ionic charges increase water retention of this modified cellulose. The enzymes responsible for the formation of this modified cellulose are encoded by the *bcsEFG* operon. Mutants with mutations in any of these genes are unable to form a fully structured biofilm and lose their community behaviors (33).

Loss of water is harmful to bacteria because it causes denaturation of proteins by allowing the exposure of hydrophilic amino acids and disrupting the bilayer structure of the membranes. Disaccharides like trehalose and betaine not only act as solutes, thus counterbalancing the osmotic pressure, but also maintain the structure and function of proteins and membrane lipids under conditions of extreme desiccation by replacing water and acting like glass in a process called vitrification (34). This process lowers the diffusion rates within the cell, thus preventing the diffusion of harmful reactive oxygen species generated by the stress (35). Trehalose has also been shown to protect *Salmonella* against other forms of stress associated with desiccation (e.g., thermal stress and oxidative stress). Trehalose biosynthesis involves the enzymes trehalose-6-phosphate phosphatase and trehalose-6-phosphate synthase, encoded by the genes *otsB* and *otsA*, respectively. The *otsBA* operon is controlled by the general stress response sigma factor σ^S, which also controls a wide range of physiological functions necessary for resistance to osmotic stress, temperature stress, and oxidative stress (31, 34).

Osmotic stress can be seen as a consequence of dehydration, where the water loss begets an increase in solutes concentration. Bacterial cells must maintain the appropriate turgor pressure during osmotic

upshifts or downshifts, and they achieve this goal through increases or decreases in the concentration of solutes in the cytosol and/or periplasm. First, immediate countermeasures are deployed to quickly balance the osmotic pressure, and then they are replaced by long-term readjustment if the osmotic conditions are sustained in the surrounding environment. Within only a few minutes, a loss of turgor pressure or increases in intracellular ionic strength induce the transcription of the *kdpABC* operon, encoding a high-affinity K^+transport system. Potassium transport is followed by an increase in glutamate, as a counterion. This first temporary mechanism is followed and replaced by an accumulation of organic compounds known as compatible solutes (e.g., proline, glycine betaine, ectoine, or trehalose) that can be obtained from the extracellular environment or synthesized *de novo* (34).

It has been observed that desiccation and high osmolality elevate the upper limit of growth temperature of bacteria and increase their survival at otherwise lethal temperatures and that mutations in *otsAB* increase the sensitivity of *Salmonella* to higher temperatures (21). Recently, a group of virulence genes was found to be involved in desiccation tolerance by using RNA sequencing, and mutants in *sopD*, a virulence effector, and *sseD*, a component of the injectisome, were unable to form a proper biofilm, thus highlighting the importance of this extracellular structure (36).

Thermal Resistance

Although treatment with heat has deep historical roots—for instance, ancient Romans used to preserve quince fruits by soaking them in honey and cooking them to a hard paste—the systematic use of thermal treatments in food processing is relatively recent. The use of heat to preserve food was first published in 1810 by Nicolas Appert. Appert was a French confectioner and distiller who started experimenting with ways to preserve foods using reinforced jars sealed with waxed corks in 1795. This specific way to thermally treat foods preceded pasteurization, the process invented by Louis Pasteur, by several decades. It became a strategic asset for the Napoleonic army, as it allowed the transport of food over longer routes and times. For this reason, Nicolas Appert could not make his invention public until 1810 (37). Thermal treatment is one of the main technologies that is currently used to inactivate spoilage and pathogenic bacteria in food and favored by international regulatory agencies. There is a clear distinction between treatment based on the intensity of the heat used and its goal (e.g., sterilization or reduction of the load of spoilage organisms). Food sterilization differs from medical sterilization, as it does not imply the complete removal of microorganisms present in the food. For instance, thermophilic bacteria are usually not removed because they do not have any impact on public health. This process generally employs temperatures higher than 100°C and direct or indirect exposure to the heat source, and it is usually performed in the final container. The heat can either be dry or wet, and the product can be held at high temperatures for a short time or at low temperatures for a long time. Sterilization usually allows a prolonged shelf life (3 months to a year). Treatment at mild temperatures for prolonged times is typical for pasteurization and provides a stable food product that will last for a few weeks under refrigeration. Thermal processing of foods is a field unto itself and beyond the scope of this chapter (38). For this reason, we focus on *Salmonella*'s response to thermal stress.

In *Salmonella*, thermal stress elicits a cytoplasmic heat shock response under the control of the heat shock sigma factor σ^H and an extracytoplasmic response regulated by the sigma factor σ^E. The σ^H regulon includes more than 30 heat shock proteins, which function as molecular chaperones and proteases to decrease the heat-induced denaturation of proteins. The intracellular concentration of σ^H is regulated at the translational level. Elevated temperatures (42°C) denature the secondary structure of *rpoH* mRNA, allowing ribosomal binding and translation. Increased levels of σ^H inhibit the cytoplasmic heat shock response in a negative feedback loop that leads to the overexpression of heat shock proteins. Expression of *rpoH* is also under the control of σ^E, allowing a coordinated response to thermal and, possibly, envelope stress (39, 40).

The alternative sigma factor σ^E (encoded by *rpoE*) controls multiple cell envelope, or extracytoplasmic, functions that mitigate the effects (e.g., accumulation of misfolded proteins) of high temperatures on

the extracytoplasmic compartment as well as other factors causing envelope stress. Members of the σ^E regulon include periplasmic chaperones (e.g., SkpA, SurA, and DegP), peptidyl-prolyl isomerases (e.g., SurA and FkpA), (serine) proteases (e.g., DegP), and other factors involved in outer membrane biosynthesis and function (41).

Continuous osmotic and/or heat stresses induce several genes belonging to the oxidative stress response network. These genes include *sodC* (superoxide dismutase), *katE* (catalase), *osmC* (peroxidase), *puu* genes (putrescine catabolism), and *cusCFBA* (copper efflux system). At least nine genes, mainly iron related, are induced by the two stresses combined: *acnA* (aconitase A), *bfr* (bacterioferritin), *ibpA* (heat shock chaperone), and *degP* (heat shock serine protease) genes. Also, proteomic studies confirmed that the Dps, KatE, AcnA, and OsmC proteins are induced during the initial phase of osmotic adaptation. A number of genes, including the *suf-ABCDSE* operon (which encodes components of a secondary pathway of iron-sulfur cluster assembly), are induced when both stresses are applied but not by one stress only (42).

Acid Resistance

Salmonella is an enteric bacterium and, like many bacteria that transit through the human gastrointestinal tract, is a neutralophile with optimal growth within a pH range of 7.00 to 7.50. However, depending on the characteristics of other environmental factors (e.g., temperature, salinity, and presence of organic or inorganic acids), the pH range allowing the growth of this pathogen can be much broader, from 3.99 to 9.5. This wide range reflects the variety of environmental pH conditions this pathogen encounters. For example, in the gastrointestinal tract, proton concentrations can range from mildly acidic in the saliva (pH 6.3 to 7.3) to extremely acidic in the stomach (pH 1.5 to 3.5). The difference in proton concentration between those two body sites ranges between a thousand-and a million-fold (27–29).

In food, the ability to avoid the damage caused to proteins and DNA by low pH is also extremely important. The consumption of fermented foods is an ancient, common, and widespread practice among humans. Acidic fermentation of food products (e.g., milk, meat, fish, grains, fruits, and vegetables) containing different end products, such as lactic acid, propionic acid, carbon dioxide, and ethanol, improves organoleptic characteristics, digestibility, and bioavailability of several nutrients. Furthermore, it has been particularly important for food preservation from spoilage and contamination because it limits the growth of the local microbial communities or possible pathogenic contaminants.

Similar to *E. coli*, *Salmonella* can deploy for its protection from low pH AR and ATR mechanisms (28). However, differently from *E. coli*, the AR mechanisms do not include a glutamate-dependent response (AR2) but rely instead on arginine, ornithine, and lysine decarboxylase systems. Comparative studies of these decarboxylase systems have shown that the arginine decarboxylase is mainly involved in the response to extreme pH, but its activity is negligible under moderately acidic conditions. The ornithine decarboxylase system is not important for survival and is active under anaerobic and mildly acidic conditions, whereas lysine decarboxylase has a broad impact on survival, being essential at pH of 2.3 and supporting growth at 4.5. Lysine and ornithine decarboxylase systems are also involved in controlling the intracellular pH (43, 44).

The ATR is a network of mechanisms that are induced by growth in mildly acidic conditions (habituation) or by exposure to moderately low pH (adaptation). Physiological conditions have a large impact on the ways these mechanisms are activated and mainly can be divided into log-phase and stationary-phase ATRs. Regardless of the physiological phase of the bacterium, several types of acid shock proteins are induced to prevent or repair the macromolecular damage caused by acid stress. Most of these proteins are involved in cellular regulation, molecular chaperoning, energy metabolism, transcription, translation, synthesis of fimbriae, regulation of the cellular envelopes, colonization, and virulence (45, 46).

During the logarithmic phase, ATR is mainly regulated by σ^S, Fur, and the two-component system PhoPPhoQ. The σ^S sigma factor is encoded by *rpoS*, regulates several oxidative stress genes, and controls the expression of at least 10 different acid shock proteins in the log-phase ATR; most of these

are of unknown function except SodCII, which is a periplasmic Cu^{2+}/Zn^{2+}-superoxide dismutase (1, 47). Fur is a negative regulator that mainly controls iron homeostasis, and therefore, its role during oxidative stress has been well characterized and is dedicated to preventing the production of hydroxy radicals through the Fenton reaction. Recently, a clear link between different Fur-regulated antioxidant enzymes, such as Mn superoxide dismutases and catalases, and resistance to the deleterious effects of organic acids was shown (48). Indeed, Fur has also been shown to directly regulate a subset of acid shock proteins in an iron-independent manner, contributing to ATR under organic acid stress (44). Acid stress caused by weak organic acids is particularly damaging for the cell, as their undissociated form can easily diffuse through the membrane, through either passive or active transport, and dissociate within the cell, thus lowering the intracellular pH. This characteristic is extremely important in the food industry, as it is one of the reasons that organic acids such as lactate and acetate are widely used in preventing spoilage of several food products.

PhoPQ is a two-component system where PhoP is the response regulator and PhoQ is the sensor kinase. At low extracellular Mg^{2+} concentrations, PhoQ can be activated by autophosphorylation and transphosphorylation of PhoP, which regulates genes for Mg^{2+} transport and virulence. At high Mg^{2+} concentrations, PhoPQ can be induced by moderately acidic pHs, possibly sensing the acidic pH by conformational changes induced by H^{+}at the Mg^{2+} ion-binding site. PhoQP activation is essential for *Salmonella* Typhimurium resistance to inorganic acid stress and survival within macrophages (44, 47).

PATHOGENICITY AND VIRULENCE FACTORS

Immune Response to *Salmonella* Infection

Upon ingestion, *Salmonella* can survive the low pH of the stomach and travel to the distal ileum of the small intestine. Once in the small intestine, the bacteria must penetrate the mucus layer to make contact with the intestinal epithelium. This mucus layer, composed of mucin glycoproteins, is secreted by epithelial goblet cells and, in addition to being a barrier to the epithelium, also contains antimicrobial peptides secreted by epithelial and Paneth cells (49). Figure 9.1 presents a schematic representation of *Salmonella* Typhi infection in humans.

Increased production of some antimicrobial peptides and mucin by goblet cells is induced by the host inflammatory response to the detection of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors. Typical microbial PAMPs expressed by *Salmonella* include lipopolysaccharide, type III secretion systems (TTSS), fimbriae, and flagellin. However, *Salmonella* is capable of combating antimicrobials such as lipocalin-2, which sequesters bacterial siderophores, thus starving microbial invaders of needed iron (50). To evade iron starvation, *Salmonella* expresses *iroBCDE* and *iroN*, which encodes an enzyme to alter its siderophore enterobactin so that it can no longer be sequestered by lipocalin-2 (51). In addition to the *iro* locus, *Salmonella* also contains the two-component regulator PhoPQ, which can sense cationic antimicrobial peptides and upregulate mechanisms to modify lipid A by either acylation or attaching an amino-arabinose moiety to confer resistance to these antimicrobials (52–54).

Once *Salmonella* makes contact with the epithelium, the bacteria invade intestinal epithelial enterocytes and M cells. *Salmonella* can then colonize the lymphatic tissue of the intestines called Peyer's patches through the invasion of epithelial M cells. Once in the Peyer's patches, *Salmonella* can travel to the mesenteric lymph nodes, where many phagocytic cells reside (55–57). Gastroenteritis is caused by a localized *Salmonella* infection of the intestinal mucosa and mesenteric lymph nodes and is associated with an intestinal inflammatory response and an increase in intestinal fluid secretion (58).

The detection of PAMPs by pattern recognition receptors also induces the expression of proinflammatory cytokines and chemokines that activate and recruit neutrophils and macrophages to the infected tissue as a part of the innate immune response. Host complement proteins and antibodies are opsonins that can coat the bacterium to promote phagocytosis of the bacterium by phagocytes such as neutrophils, macrophages, and dendritic cells (59). Once the bacterium is engulfed, it resides inside

a phagosome that is designed to destroy the microbial invader. However, *Salmonella* is an intracellular pathogen that can survive after being phagocytized within modified phagosomes known as *Salmonella*-containing vacuoles (SCV) by altering its environment to more favorable conditions and inducing a dormant state. *Salmonella* can survive intracellularly within host macrophages by increasing the expression of important virulence genes in response to the acidification of the intracellular environment within the membrane-bound compartment of the phagosome (60). For further reading, Broz et al. provide a detailed review of the innate immunity against *Salmonella* infection (61).

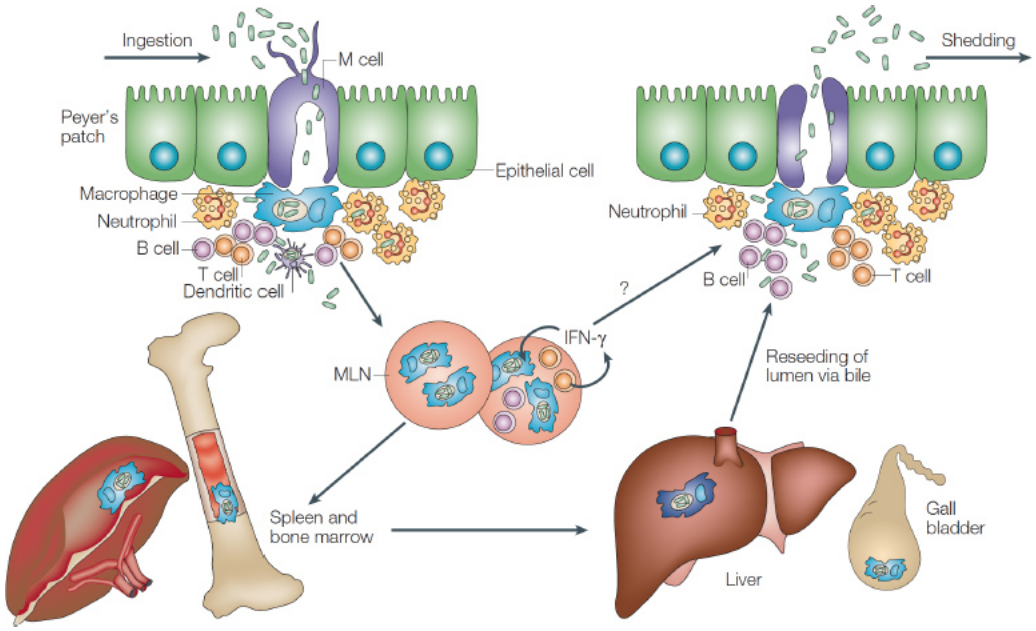


Figure 9.1 Schematic representation of persistent infection with *Salmonella* Typhi in humans. Bacteria invade the M cells of the Peyer's patches of the intestinal tract mucosal surface. This induces the inflammatory response, including recruitment of neutrophils and macrophages, phagocytosis of bacteria, and deployment of T and B cells. In systemic *Salmonella* infections, such as typhoid fever, the pathogen targets host cells, such as dendritic cells and/or macrophages, which allow systemic spread through the lymphatic and blood circulatory systems to the lymph nodes present in the mesenteries. This then leads to transport to the spleen, bone marrow, liver, and gallbladder. Bacteria can colonize all these tissues and organs and periodically can restart shedding from the mucosal surface. $IFN_{-\gamma}$, which can be secreted by T cells, has a role in maintaining persistence by controlling intracellular *Salmonella* replication. Interleukin 12, which can increase $IFN_{-\gamma}$ production, and the proinflammatory cytokine tumor necrosis factor alpha also contribute to the control of persistent *Salmonella* (not shown).

Production of the cytokine gamma interferon ($IFN_{-\gamma}$) by macrophages, neutrophils, and lymphoid cells is also an early form of cell-mediated innate immune defense against *Salmonella* infection (62). $IFN_{-\gamma}$ is detected by most host immune cells with specific $IFN_{-\gamma}$ receptors and causes an increase in the major histocompatibility complex (MHC) class I and class II molecules. MHC class I molecules present antigens from intracellular microbes on the surfaces of antigen-presenting cells to $CD8^+$ cytotoxic T cells. $CD8^+$ cytotoxic T cells can then produce cytokines to this $IFN_{-\gamma}$ and tumor necrosis factor alpha. In addition to cytokines, $CD8^+$ cytotoxic T cells produce cytotoxic granules and cause the activation of the caspase cascade, both of which lead to apoptosis of the infected cell (63). However, it has long been known that *Salmonella* is adept at preventing the fusion of the lysosome to the phagosome (64), which leads to the destruction of the bacterium and the release of the microbial antigens needed for MHC class I presentation. In addition to induction of MHC molecule expression, $IFN_{-\gamma}$ also upregulates the expression of inducible nitric oxide synthase and promotes antigen presentation by macrophages (63).

B cells can recognize extracellular antigens with their B-cell receptors, take up the antigen through endocytosis, and degrade it. Once degraded, the antigen is presented on the surface of the B cell on MHC class II molecules to $CD4^+$ helper T cells, which can then activate the B cell and initiate an adaptive immune response. Through this mechanism, Th1 helper T cells and B cells have been shown to play an important role in clearing *Salmonella* infection (65). Th1 helper T cells also produce $IFN_{-\gamma}$, which will stimulate undifferentiated $CD4^+$ T cells to differentiate into Th1 helper T cells, creating a more robust immune response.

If the infection is not contained within the lymphatic tissues in the intestines, *Salmonella* can then disseminate throughout the body by using the lymph system and reach the blood (causing bacteremia) and systemic tissues. These types of systemic infections are common in immunocompromised individuals with nontyphoid serovars of *Salmonella* and in otherwise healthy individuals with *Salmonella* Typhi and *Salmonella* Paratyphi A to C. The well-known disease typhoid fever is a systemic infection caused by *Salmonella* Typhi or *Salmonella* Paratyphi A to C. During systemic infection, the salmonellae spread to the spleen, liver, and bone marrow, where the pathogen can be engulfed by resident phagocytes. As *Salmonella* Typhi and *Salmonella* Paratyphi A to C have host specificity only for humans and other higher primates, *nramp1*-deficient mice infected with the related pathogen *Salmonella* Typhimurium are used as models of infection for typhoid fever. Nramp1 (natural resistance-associated macrophage protein 1) is a cation transporter in late phagolysosomes of macrophages, neutrophils, and dendritic cells. During *Salmonella* infection, Nramp1 reduces intracellular iron levels to limit the availability of free iron to internal pathogens (66). Other animal models of *Salmonella* infection are reviewed by Santos et al. (67).

SPI and TTSS

As described above, the abilities to invade the intestinal epithelium and to survive intracellularly within phagocytes are major factors for the pathogenicity of *Salmonella*, and these abilities are mediated by TTSS encoded on SPI. Pathogenicity islands are mobile clusters of genes encoding a collection of virulence factors that are typically located near tRNA regions and are present in pathogenic bacteria but absent in their nonpathogenic relatives (68–70). It is hypothesized that pathogenicity islands were acquired through horizontal gene transfer, because the G+C contents of the pathogenicity islands diverge from that of the surrounding DNA (71). Figure 9.2 shows the organization of SPI-1 through SPI-5.

Currently, 21 SPI have been discovered and include genes for important virulence factors, such as TTSS and effector proteins that aid in invasion and persistence of *Salmonella* in its host (72, 73). TTSS are needle-like bacterial membrane structures that create a translocon pore in host cells to inject effector molecules into host cells. SPI-1 and SPI-2 encode TTSS-1 and TTSS-2, respectively, and are found in all serovars of *S. enterica* (74). TTSS-1 is required for the invasion of nonphagocytic intestinal epithelial cells and causes intestinal inflammation. The SPI-1-encoded transcription factor HilA regulates the needle-like component of TTSS-1 as well as the SPI-1-encoded transcription factor InvF, which in turn regulates SPI-1-encoded effectors. The TTSS-1 translocases SipB, SipC, and SipD are effectors that aid in the attachment of bacterial TTSS-1 to the host cell. Once the bacteriumhost cell connection is established, effectors can then be injected into the host cell. Secretion of the effectors SipA, SipC, SopB, SopE, and SopE2 into the host cell by *Salmonella* induces membrane ruffles by host actin rearrangement and results in the internalization of the bacterium (75, 76). Once inside nonphagocytic cells, the TTSS-1 effector SptP returns the host cell's actin structure to its original state. In addition to the SPI-1-encoded effectors, several TTSS-1 effectors are encoded on other SPI, such as SopE, encoded on SPI-7, and SopB, encoded on SPI-5 (75, 77).

Besides aiding in *Salmonella*'s internalization by intestinal epithelial cells, several TTSS-1 effectors also induce an inflammatory response. SopE, SopB, and SopE2 induce the expression of proinflammatory cytokines, SipA and SopA induce the expression of chemokines to recruit phagocytic cells to the site of infection, and SipB activates the caspase cascade. Once inside the SCV, the bacterium secretes additional effectors, such as SspH1, AvrA, SpvC, and SseL, to inhibit NF-κB-regulated apoptosis of the

infected cell. Other effectors such as SopB, SopD, and SipC help form the SCV and prevent its fusion with lysosomes. Once the SCV is formed, effector SptP interacts with the SCV and induces bacterial proliferation (73, 76, 78).

SPI-2-encoded TTSS-2 is important in the invasion of phagocytic cells, such as macrophages and dendritic cells, and the subsequent intracellular survival of the bacterium. Experiments with green fluorescent protein and luciferase promoter fusions have shown several genes in SPI-2 that are upregulated in not only the intracellular environment of macrophages but also epithelial cells (79, 80). The SPI-2-encoded two-component system SsrA-SsrB responds to signals within the intracellular environment, such as the acidification of the SCV, and upregulates the TTSS-2 genes and the associated SPI-2-encoded effectors. Expression of the SPI-2-encoded translocases SseB, SseC, and SseD aids in the formation of the TTSS-2 translocon, which is needed to secrete effector proteins out of the SCV into the host cell's cytosol. The TTSS-2 secretes the effectors SseF, SseG, SifA, and SpiC, which aid in the repositioning of the SCV to the perinuclear region adjacent to the Golgi apparatus and the maintenance of that position. At this location, the effectors SifA, SseJ, and SopD2 assist in the formation of tubular structures known as *Salmonella*-induced filaments that fuse the SCV with Golgi-derived vesicles for the acquisition of nutrients. There are many more TTSS effectors that are important for the virulence and survival of *Salmonella*, and these effectors are reviewed by van der Heijden and Finlay (81).

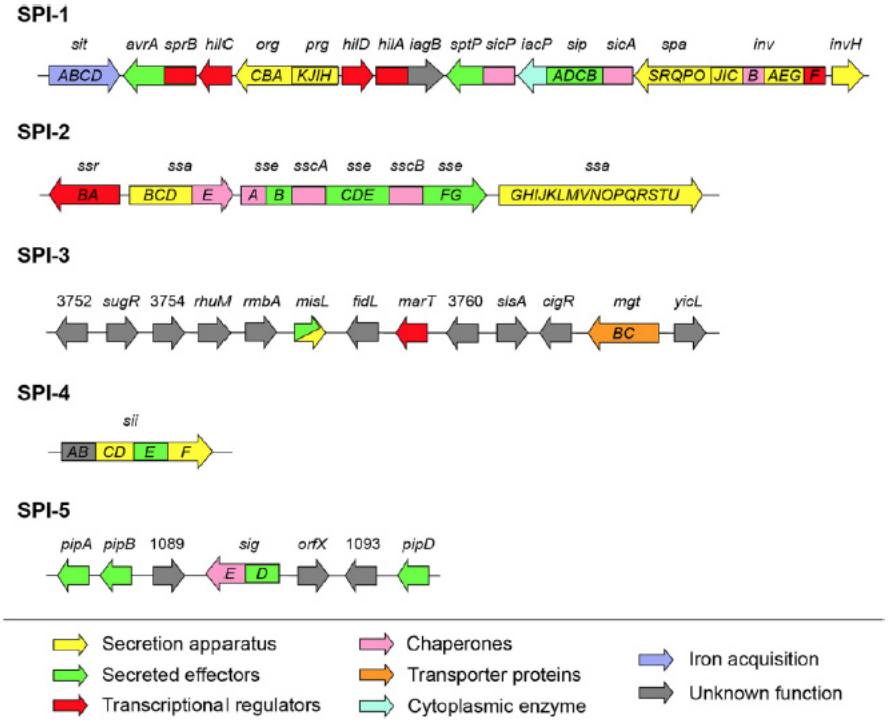


Figure 9.2 Schematic representation of the genes carried within the five SPIs and their putative virulence-related functions.

Although SPI-1 and SPI-2 are the most conserved and most extensively characterized SPI, other SPI play a role in the virulence of *Salmonella*. The *mgtC* gene encodes an inner membrane protein, MgtC, that is highly induced when *Salmonella* is inside a macrophage and is a required virulence factor for intracellular survival and growth in low-Mg^{2+} environments. The *mgtC* gene is within the *mgtCBR* operon on SPI-3 that also encodes an Mg^{2+} transporter (MgtB) and a peptide that promotes the degradation of MgtC (MgtR). It has been shown that inactivation of *mgtC* attenuates the virulence of *Salmonella* in a murine model (82), while the prevention of the degradation of the *mgtC* transcript causes hypervirulence in *Salmonella* (83). This indicates that the levels of MgtC must be carefully controlled during *Salmonella* infection.

SPI-7 is a large excisable pathogenicity island that is found in *Salmonella* Typhi and *Salmonella* Paratyphi C and some *Salmonella* Dublin strains. SPI-7 contains genes encoding type IVb pili, which have a role in macrophage infection, the TTSS-1-secreted effector protein SopE, and the Vi capsular antigen. The Vi capsule is a polysaccharide that inhibits the complement system and opsonization of the bacterium and is important for systemic infection by *Salmonella* Typhi (84–86).

In addition to encoding effectors that are secreted by TTSS-1 and TTSS-2, other pathogenicity islands contain genes for other important virulence factors, such as the SPI-4-encoded type I secretion system. This secretion system secretes SiiE, which aids the bacterium in adhering to epithelial cells (87). Besides the SPI-2 genes, SPI-11 carries *pagD* and *pagC*, whose products have a role in the survival of *Salmonella* within macrophages (88). As already mentioned, the SopB TTSS-1 and TTSS-2 effector is encoded on SPI-5, but also on this pathogenicity island is the SopB chaperone SigE (89). SPI-6 encodes the first discovered type VI secretion system in *Salmonella* (72) along with the fimbrial gene operons *saf* and *tcf* and the *pagN*-encoded invasin (90, 91). SPI-16 has been associated with murine intestinal persistence, though the genes within this island have yet to be characterized (92). Region of difference 21 (ROD21) is also an excisable pathogenicity island that is present in *Salmonella* Enteritidis, *Salmonella* Gallinarum, *Salmonella* Dublin, and *Salmonella* Typhi. This pathogenicity island contains genes encoding the TlpA protein, which is known to interfere with Toll-like receptor signaling, a potential type IV pilin, and genes involved in conjugation (93, 94).

Virulence Plasmids

Besides the TTSS and other virulence factors encoded on pathogenicity islands, some serovars of *S. enterica* contain a large (50-to 285-kb) virulence plasmid that has been shown to be important for bacterial multiplication in the reticuloendothelial system. Many clinically relevant *S. enterica* serovars contain a virulence plasmid, such as *Salmonella* Enteritidis, *Salmonella* Typhimurium, *Salmonella* Choleraesuis, and *Salmonella* Dublin. However, some notable serovars, such as *Salmonella* Typhi and *Salmonella* Paratyphi A and B, lack virulence plasmids altogether (95). Some larger (~140-kb) virulence plasmids also include antibiotic resistance genes. For plasmid-containing serovars, the curing of the plasmid reduces overall virulence, as was shown in strains of *Salmonella* Typhimurium (96). However, the virulence of serovars that do not natively include a virulence plasmid does not seem to benefit from their being transformed with a known *Salmonella* virulence plasmid (97, 98).

On the *Salmonella* virulence plasmid, the highly conserved *Salmonella* plasmid virulence (*spv*) region carries five virulence genes split into two operons, *spvR* and *spvABCD*. SpvR is a positive regulator of these genes, including its own gene, and is homologous to the LysR regulator family. SpvB, SpvC, and SpvD are effector proteins exported by the SPI-1-and SPI-2-encoded TTSS, and SpvA is an outer membrane protein with an undefined function. During intracellular infection, SpvB modulates the formation of the *Salmonella*-induced filament tubular structures on the surface of the SCV by inhibiting actin polymerization by ADP-ribosylation. SpvC is a phosphothreonine lyase that removes phosphate groups from mitogen-activated protein kinases to inhibit proinflammatory signaling. Likewise, SpvD also inhibits proinflammatory signals by inhibiting the upregulation of promoters by NF-κB (99). Though all the *spv* genes are regulated by SpvR, their expression levels are upregulated at different times of infection. The genes *spvR* and *spvA* are upregulated in the early stages of intracellular infection (100), while expression of the effector genes *spvB* and *spvC* is higher during the later stages of intracellular infection (101). On the other hand, the gene *spvD* was shown to be expressed constitutively during intracellular infection (101).

In addition to the *spv* genes, these virulence plasmids also contain the fimbrial *pef* and *fae* operons and *tra* conjugation genes. *Salmonella* fimbriae have been shown to induce the proinflammatory response, aid in cell adhesion to epithelial cells, and play a role in biofilm formation. Some *Salmonella* virulence plasmids contain an intact *tra* operon, such as pSTMV of *Salmonella* Typhimurium, which allows the plasmid to be horizontally transferred through conjugation. However, as a result of sequencing of several virulence plasmids from different serovars combined with the variation in plasmid size among different serovars, it has been concluded that most *Salmonella* virulence plasmids have been inherited through vertical transfer from parent to daughter cell rather than through horizontal means, such as

conjugation (95). This appears to be true partly because of the degradation of the *tra* operon within these nontransmissible plasmids. The large size variation between virulence plasmids appears to be due to the inclusion, or lack thereof, of these fimbriae and conjugation operons.

Toxins

In addition to the lipopolysaccharide lipid A endotoxin, which is common to Gram-negative pathogens, *Salmonella* species can produce several exotoxins during the course of an infection. There is only a limited understanding of *Salmonella*'s exotoxins, which are known to include several different cytotoxins and an enterotoxin.

Salmonella cytotoxins were first identified by their ability to lyse a specific line of mammalian cells (Vero cells derived from African green monkey kidney) and first thought to belong to the same group as the Shiga toxins. However, although they share some traits (e.g., they are heat labile and trypsin sensitive), they were not neutralized by antibodies against the *Shigella* Shiga toxin or *E. coli* Shiga-like toxins I and II (102). A *Shigella dysenteriae* 1-like toxin has been reported in *Salmonella* Enteritidis, *Salmonella* Kapemba, and *Salmonella* Thompson and was shown to be neutralized by antibodies against *S. dysenteriae* 1 toxin (103). It is now clear that several *Salmonella* serovars can produce toxins belonging to the same class of toxins as Shiga toxins. All the members of this class possess a catalytic A subunit linked to a pentameric B subunit (AB_5) and are classified into four groups according to the homology of the A subunit sequence, their catalytic activity, and the structural organization of the holotoxin. The B subunit pentamer attaches to specific glycan receptors on the host cell surface, allowing the internalization of the holotoxin. The most recently identified AB_5 toxin family is the subtilase cytotoxin B subunit-like proteins (104). The homologs for the B subunit of this toxin are present in different serovars of *S. enterica*, and the putative AB5 toxin has been named ArtAB (ADP-ribosylating toxin) (105). Although no cytotoxic phenotype has been detected for ArtAB, its homolog from *Salmonella* Typhi (also referred to as PltAB) has been reported to associate with a cytolethal distending toxin (CDT) (CdtB) and deliver it to target cells from an intracellular host compartment by autocrine and paracrine pathways (106).

The *Salmonella* CDTs are cytotoxins that cause DNA damage that results in cell cycle arrest, cellular elongation, and apoptosis in affected host cells (107). Typical CDTs need three subunits to be completely functional. The A subunit, CdtB, is the actual source of the toxic effects, while the B subunit, composed of CdtC and CdtA, aids the A subunit with gaining entry into the target cell. Interestingly, the typhoid toxin produced by *Salmonella* Typhi is a CDT that contains only CdtB with no associated subunit for toxin transport. In *Salmonella* Typhi, the expression of CdtB is induced after the bacterium in engulfed and internalized into the target phagocytic cell (108, 109).

Low-passage-number clinical *Salmonella* isolates have been reported to have hemolytic activity for the salmolysin encoded by *slyA*. The *slyA* gene was found to be homologous to genes in *Shigella* and enteroinvasive *E. coli*. The *slyA* gene encodes a positive regulator of *clyA*, which itself encodes a pore-forming cytotoxin that is present only in *Salmonella* Typhi and *Salmonella* Paratyphi A (110).

The *Salmonella* gene *stn* is thought to encode an enterotoxin that is found encoded in the genomes of all known *S. enterica* serovars and appears to be unique to *Salmonella*. However, the questions of whether this gene product is an enterotoxin and what its exact role in pathogenesis is are controversial. In a few studies, Stn was shown to induce fluid secretion in rabbit (111) and murine (112) ileal loop models, supporting the claim that Stn is an enterotoxin that causes diarrhea in the host. However, in another study, it was shown that a *stn* mutant was not deficient in its ability to invade or survive intracellularly in macrophages and retained its ability to induce fluid secretion in a murine ileal loop model (113). The same group did show that Stn can interact with the outer membrane protein A (OmpA) directly in order to aid in the localization of OmpA and stabilize membrane integrity (113). Overall, the specific role of Stn in *Salmonella* pathogenesis is unclear.

Other Factors that Contribute to Virulence

Examples of other important virulence factors of *Salmonella* are the abilities of this bacterium to form biofilms, convert to a persister state, and scavenge free iron from the environment. The formation of a biofilm is an important phase of growth for pathogenic bacteria and, through several different mechanisms, aids in the organism's resistance to antibiotics. Biofilm formation in *Salmonella* has been shown to improve the pathogen's survival in nutrient-limiting, acidic, and low-and high-temperature environments. Proteinaceous curli fimbriae and exopolysaccharide cellulose are the major components of *Salmonella*'s extracellular matrix and are important for the pathogen's ability to form biofilms (114). Carriers of *Salmonella* Typhi often have an infection of the gallbladder containing gallstones. It has been shown *in vitro* that *Salmonella* Typhi and *Salmonella* Typhimurium can form biofilms on human gallstones and that biofilm formation is bile dependent (115), showing how biofilm formation can possibly play a role in the persistence of the carrier state.

Persister cells are cells that can survive many different antibiotic treatments by virtue of the fact that these cells are not actively growing. Persisters play an important role in a biofilm's resistance to antibiotic treatment and can cause relapsing infections. It has been shown that *S. enterica* cells produce a subpopulation of persister cells outside biofilm formation when they are engulfed by macrophages. This persister state is regulated by a class II toxin-antitoxin mechanism (116) where a stable nonsecreted toxin arrests growth and, under certain conditions, an unstable antitoxin is produced to resume growth.

The procurement of iron is an important part of the survival and virulence of *Salmonella* within its host. In response to infection, the host sequesters free iron to starve microbial invaders by proteins such as Nramp1 (see above). However, *Salmonella* produces iron chelators called siderophores to scavenge iron and transport it to the bacterium. The importance of the *S. enterica* siderophores enterobactin and salmochelin has been shown in *Salmonella* Typhi. Leclerc et al. showed that enterobactin and salmochelin are upregulated in *Salmonella* Typhi within host macrophages and are important for virulence (117). This group also showed the importance of the Fur regulator of these iron siderophores and the regulation of siderophores during infection is discussed in the next section. In addition to the virulence factors discussed here, *Salmonella* also has other important virulence factors that include production of flagella within macrophages for the purpose of escape (118), the use of superoxide dismutase to combat host-produced reactive oxygen species (119, 120), and the uptake of other important metal ions, such as K^+ and Zn^{2+}, which have been shown to be important for intracellular survival (121, 122).

Virulence Gene Regulation

Salmonella has several important regulatory mechanisms to control the expression of its virulence factors. As mentioned above, PhoPQ is a two-component regulator that senses extracellular cationic antimicrobial peptides produced by the host immune system. However, PhoPQ can also sense low concentrations of divalent cations and low pH, which are both indicative of the SCV intracellular environment. In response to its regulatory signals, the sensor kinase PhoQ phosphorylates the DNA-binding response regulator PhoP. PhoP represses the expression of SPI-1 genes, including the SPI-1 regulator gene *hilA*, and upregulates genes involved in intracellular survival, such as the *mgtCBR* operon on SPI-3, SPI-2 genes, and the *spv* operon on the virulence plasmid (123, 124). PhoPQ upregulates the expression of the SPI-2 genes through the upregulation of the SPI-2-encoded two-component positive regulator SsrAB (123).

Expression of *srrAB* has also been shown to be regulated by another global two-component system, OmpREnvZ. EnvZ is the sensor kinase that detects the low osmolarity and pH of the SCV, and OmpR functions as its response regulator. Both OmpR-EnvZ and PhoPQ are required for optimal expression of *ssrAB* once the bacterium has reached the intracellular environment of the host cell (125, 126). OmpR-EnvZ is also known to upregulate *Salmonella* biofilm development through binding to the promoter region of the biofilm regulator *csgD* (127).

As mentioned in the previous section, biofilm formation is an important component of *Salmonella* virulence. The ability to produce biofilms is regulated by CsgD (curli subunit gene D), a transcriptional

regulator that upregulates both curli fimbria expression and cellulose biosynthesis. CsgD upregulates *adrA* transcription, which expresses AdrA, a diguanylate cyclase that generates the signaling molecule cyclic di-GMP. Cyclic di-GMP then activates cellulose biosynthesis. CsgD is also a positive regulator of *bapA* (biofilm-associated protein A) and of its own operon, *csgDEFG*, which includes genes for curli fimbria production (128).

In addition to its regulation by PhoPQ, the intracellular survival virulence factor gene *mgtC*, carried on SPI-3, is also upregulated by the presence of high levels of cytosolic ATP. This ATP is sensed by the leader region of the *mgtC* mRNA, and mutation of this leader region leads to reduced expression of *mgtC* and attenuates virulence in a mouse model (129).

Iron uptake homeostasis is also an important regulatory signal in *Salmonella*. This signal is sensed by the transcriptional repressor ferric uptake regulator (Fur). Fur binds DNA and represses the associated gene when Fe^{2+} is bound. In an iron-limiting environment, there is not enough free Fe^{2+} to bind Fur; Fur releases the DNA, and the associated gene is activated. In response to sensing low Fe^{2+} concentrations, Fur derepresses the production of siderophores such as enterobactin and salmochelin that bind free Fe^{2+} and genes involved in iron transport and storage (130, 131).

The alternative sigma factor RpoS is a stationaryphase regulator that plays an important role in the regulation of *Salmonella* genes during stress response to starvation, oxidative stress, osmotic pressure, heat, and low pH. Many of these environmental stimuli sensed by RpoS are present within the phagosomes of macrophages. First discovered in *E. coli* as a regulator of the catalase gene *katE* and named KatF (132), the RpoS sigma factor has also been shown to be important for virulence (133) and also responds to the expression of cationic antimicrobial peptides produced by the host immune system (134). Some of the many genes that are a part of the RpoS regulon include the virulence factor gene *csgD*, the virulence plasmid genes *spvRABCD*, and the superoxide dismutase gene *sodCII* (135). The expression of *rpoS* itself is regulated by the small RNAs RprA, DsrA, ArcZ, and OxyS posttranscriptionally (136–138). These small RNAs with the RNA chaperone Hfq target the 5′ untranslated region of the *rpoS* transcript in order to regulate its expression. The small RNAs RprA, DsrA, and ArcZ upregulate *rpoS* expression by stabilizing the transcript and aiding in the initiation of translation. On the other hand, the sRNA OxyS is a negative regulator of *rpoS*. There are many more regulators of *Salmonella* virulence, which are reviewed in detail by Fabrega and Vila (139).

CHARACTERISTICS OF DISEASE

Routes of Infection

Salmonella is typically spread through contaminated water or food via the fecal-oral route. While infection with *Salmonella* can occur via contact of open wounds or mucosal parts of the body with contaminated products, surfaces, or water, the primary cause of *Salmonella* outbreaks in developed countries remains food. Routes of infection tend to be associated with specific serotypes of *Salmonella* based on their ecology (140). For example, *Salmonella* Enteritidis is found among egg-laying poultry and has been the cause of illness outbreaks associated with shell eggs. While the ecology of many serovars of *S. enterica* remains unknown, a list of serovars, known ecologies, and associated outbreaks can be found in Table 9.2 (140). This table also highlights the flexibility of *S. enterica* serovars in particular to be associated with a primary host or ecology (i.e., chickens or pigs) and yet cause infection indirectly through food or other animal species unassociated with the primary host/ecology.

Environmentally, *Salmonella* serotypes have been detected in surface water, water storage tanks, piped water systems, irrigation water, and soil (141, 142). The level of prevalence depends on source of fecal contamination, distance from the contaminated site, frequency of contamination inputs into the site, efficiency of decontamination procedures, and flow between site and the source of contamination, which might be mediated by climatic factors such as rain, flooding, or human and animal interactions. Within the human-built environment, control of *Salmonella* has been particularly difficult in hospital settings. It has been detected on equipment, supplies, soiled laundry, hospital kitchens, and even the

hospital water supply (143). Ecology, human and animal movements, and the natural and human-built environments all serve to facilitate transmission and infection with *Salmonella* serovars globally.

Once *Salmonella* has entered the human body, the initial site of attachment is the intestinal epithelium of the small intestine, with invasion into nonphagocytic cells. Invasion can be mediated via two mechanisms, termed the trigger and zipper mechanisms. The trigger mechanism is carried out by a TTSS located on SPI-1 and involves cytoskeletal rearrangement and membrane ruffling. The zipper mechanism is TTSS independent, involves the Rck locus, and is host receptor mediated. The mechanisms differ in their mode of activation: the trigger mechanism is activated by bacterial effectors delivered by secretion systems from inside the cell, whereas the zipper mechanism is activated through host cell receptors on the outside of the cell (144, 145). Figures 9.3 and 9.4 illustrate the dissemination of infection of *Salmonella* in the human body and the trigger and zipper mechanisms of invasion of the cell by *Salmonella*.

Infectious Dose

The infectious dose is defined as the minimum number of bacteria required to cause illness. This can be dependent on current medications, the susceptibility of the host, the food matrix ingested, and virulence factors, which are often serovar dependent. Early studies using volunteers suggested that an infectious dose of 10^6 organisms was necessary to illicit infection; however, recent work has revised this estimate to an average of 10^3 to 10^4 CFU. It has been suggested through outbreak modeling that as few as 10 to 36 CFU can cause infection, depending on outbreak conditions and host susceptibility. Additionally, the probability of illness or attack rate has been shown to be linked to infectious dose. In 2002, the WHO estimated that the probabilities of illness given average doses of 1, 10, and 100 organisms were 0.2%, 2.2%, and 13%, respectively (146). While no study has established a gold standard of infectious dose for *Salmonella*, it is generally agreed that higher levels of *Salmonella* are more likely to cause infection. Research is ongoing to develop and evaluate methods to quickly enumerate *Salmonella* in environmental samples and food products to identify samples or products with a high bacterial load.

Symptoms and Treatment

The incubation time following successful invasion of the cellular tissue by *Salmonella* prior to manifestation of symptoms varies from a few hours to 2 days. Inflammatory gastroenteritis (salmonellosis) is the most common result of NTS infection, with symptoms including nausea, vomiting, abdominal cramps, diarrhea, fever, chills, headache, and blood in the stool. Symptoms can last 2 to 7 days, with diarrhea lasting up to 10 days. Though symptoms resolve, it may take months before the bowels return to normal. NTS infection can manifest without gastrointestinal symptoms in both adults and children, instead presenting as a febrile illness. Respiratory infection, anemia, and hepatosplenomegaly may be useful in diagnosing NTS; however, these clinical features are also seen in a variety of infections caused by other pathogens (147). This nonspecific clinical presentation makes diagnosis and treatment challenging, especially in settings where resources are limited. NTS infections can be diagnosed using blood or bone marrow cultures, PCR, or enzyme-linked immunosorbent assay (past infections). Treatment of NTS infections primarily consists of electrolyte replacement and rehydration (supportive care). Antimicrobial treatment is challenging and typically discouraged now that there is increasing prevalence of antibiotic-resistant serotypes and strains (see “Antibiotic Resistance” below). Currently, there are no vaccines licensed for human use for NTS; however, current vaccines for livestock and poultry are available and more are being tested (148).

Table 9.2 *S. enterica* serovars found in human Samples[a]

Serovar	Resistance type[b]	Ecology or host[c]	Vehicle of outbreak	Country and year(s)[d]
Salmonella Agona	GSCefCfxCafANTCSuSxt	Avian, swine	Infant formula Papayas	France, 2004–2005, 2017–2018
	Amc		Meat	USA, 2011, 2017 England, Scotland, Wales, Northern Ireland,200
			Cereal	USA, 1998, 2008
			Tea	Germany, 2002
			Chicken	USA, 2006
			Turkey	USA, 2004
			Goat	USA, 2002
			Seafood	USA, 2001
Salmonella Anatum	*AgBlSuTSxt*	Bovine	Hot peppers	USA, 2016
	AAmcNSuSxtTCCinDMPSpS		Papayas	USA, 2017
			Cantaloupe	USA, 2001
			Orange juice	USA, 1999
			Salsa	USA, 2002
			Potato salad	USA, 2001
			Roast beef	USA, 2004
			Pork	USA, 2006
			Duck	USA, 2004
			Tomatoes	USA, 2004
			Grilled meat	Japan, 2005
Salmonella Berta	AGSK	Chicken	Soft cheese	Canada, 199
			Dairy-based dessert, eggs	Italy, 2008
			Beef	USA, 2001, 2004
			Pork	USA, 2006
			Fresh fruit (grapes, melon)	USA, 2002
			Tomatoes	USA, 2006

Serovar	Resistance type[b]	Ecology or host[c]	Vehicle of outbreak	Country and year(s)[d]
Salmonella Baildon	ND	Unk	Raw tomatoes	USA, 1998–1999
			Mexican restaurant	USA, 2010
			Salsa	USA, 2007
			Salad	USA, 1998
Salmonella Bareilly	Ctx	Avian	Ground tuna	USA, 2012
	V		Bean sprouts	UK, 2010
	SN		Iced tea	USA, 2006
Salmonella Blockley	*AgBlSuTSxt*	Chicken	Smoked eel	Germany, 1998
			Meat, rice, potatoes	South Africa 2011
			Egg yolks in ice cream	USA, 1967
			Tomatoes	USA, 2008
Salmonella Braenderup	*AgBlSuTSxt*	Chicken	Mangoes	USA, 2012
	TSxtSuSAmcCfxFoxG		Live poultry	USA, 2016–2017
	Tob		Nut butter	USA, 2014
	KACTSNSxt		Meat pies	Switzerland, 1993
	ANitT		Roma tomatoes	USA, 2004–2005, 2008
			Eggs	USA, 1999, 2018
			Chicken, 2003, 2005	USA, 1999, 2001, 2007
			Bean sprouts	USA, 2005–2006
			Pork	USA, 2008
			Lettuce	England, 2011
			Lunchboxes	Japan, 2008
Salmonella Bredeney	ACCpKNGAprNitSTSuSxt	Chicken	Peanut butter	USA, 2012
			Meat	USA, 1998
			Chicken	Ireland, 199
			Mangoes	USA, 2012

Serovar	Resistance type[b]	Ecology or host[c]	Vehicle of outbreak	Country and year(s)[d]
Salmonella Chester	AKNetTGNSSuSxtC	Unk	Headcheese	Canada, 201
			Tap water	Australia, 2005
			Turtle meat	Australia, 1988
			Cantaloupe	USA, 1989
			Frozen chicken/rice meal	USA, 2010
			Sprouts	USA, 2003
Salmonella Derby	GSCefCfxCafANCpTC	Swine	Dried pork sausage	Spain, 2011
	SuSxt		Grilled meat	Japan, 2005
	C		Powdered milk	Trinidad, 1973
			Eggs	USA, 1963
Salmonella Dublin	ACFNeoOSuSxtT	Cattle	Unpasteurized milk/cheese	England and Wales, 1989
			Milk	Ireland, 197
			Cheese	USA, 2006
Salmonella Enteritidis	ATNSuSxtAmcCFSKGAkCpCfzCefCfxCaf	Chicken[e]	Truffle oil	USA, 2015
	AgBlSuTSxt		Eggs	USA, 1998–2008, 2011; England, 2011; Austri 2010; Belgium, Denmark, Luxembour Netherlands Norway, Sweden, United Kingdom, Croatia, 201
			Frozen/fresh chicken	USA, 1998–1999, 2001, 2003, 2005, 2007–2008, 2014–2015
			Cream cakes	Singapore, 2007

Serovar	Resistance type[b]	Ecology or host[c]	Vehicle of outbreak	Country and year(s)[d]
			Almonds	Canada and USA, 2000–2001, 2003-2004
			Pine nuts	USA, 2011
			Chicks/live poultry	USA, 2015–2018
			Pet guinea pigs	USA, 2018
			Sprouts	USA, 2001–2002, 2011, 2014
			Ground beef	USA, 1998–2000, 2003, 2008, 2012
			Unknown restaurant	USA, 2012
			Turkey	USA, 1998–1999, 2000-2001, 2005, 2008
			Gefilte fish, salmon	USA, 2000
			Crab	USA, 1998
			Melon	USA, 1999
			Lettuce	USA, 2003
			Pork	USA, 2004
			Tomatoes	USA, 2005
			Seafood	Singapore, 2011
Salmonella Goldcoast	SNeoSuT	Swine, bovine	Whelk	England, 2013
			Salami	Italy, 2009
			Sausages	Germany, 2001
			Pate	England and Wales, 1984
Salmonella Hadar	*AgBlSuTSxt*	Chicken, turkey	Meat salad	Italy, 1994
	NSNeoTSuSxt		Live poultry/chicks	USA, 2012, 2014–2017
			Turkey	USA, 2007–2008, 2011
			Roast rabbit	Italy, 1997

Serovar	Resistance type[b]	Ecology or host[c]	Vehicle of outbreak	Country and year(s)[d]
			Chicken	USA, 1999, 2004; Spain 2005
			Pork	USA, 1999
Salmonella Hartford	ND	Unk	Mexican restaurant	USA, 2010
			Refried beans	USA, 2002
			Ground beef	USA, 2005
			Salsa	USA, 2008
			Chia powder	USA, 2014
			Orange juice	USA, 1995
Salmonella Havana	SxtCTGAKCepCfxNCpFSSu	Chicken	Alfalfa sprouts	USA, 1998
			Hospital	Iran, 1982
Salmonella Heidelberg	CefAmcAFoxCtxSSuTSxtNCG	Chicken	Chicken products	USA, 1999, 2004–2006, 2008, 2011, 2013–2014
	Fs		Turkey	USA, 2004, 2011
			Dairy calves	USA, 2016
			Pudding	USA, 1999
			Beef	USA, 2002, 2004
			Egg	Australia, 2001; USA, 2002–2006, 2008
Salmonella 1,4, [5],12:i:–	GSCefCfxCafANAprCpTC	Bovine, chicken	Kratom[f]	USA, 2018
			Frozen rodents	USA, 2010
			Sprouts	USA, 2011
			Pot pies	USA, 2007
			Pork	USA, 2015; France, 201
			Frozen coconut	USA, 2018
			Live poultry	USA, 2017
			Small turtles	USA, 2013
Salmonella Infantis	GSCefANCpTCSuSxt AkTzpCfxCafAtm	Chicken, swine	Pork	USA, 2004, 2007–2008, 2015; Germany, 2013

Serovar	Resistance type[b]	Ecology or host[c]	Vehicle of outbreak	Country and year(s)[d]
			Beef	USA, 2000, 2002, 2016
			Fish	USA, 2000
			Potatoes	USA, 2005
			Chicken, vegetable protein	USA, 2016
			Porchetta, roasted meat	Italy, 2011
			Dry dog food/treats	USA, 2012, 2017; Canada, 1999, 2012
			Live poultry	USA, 2012–2013
			Papayas	USA, 2012–2014, 2016–2018
Salmonella Javiana	AT	Equine, turkey	Tomatoes	USA, 2002, 2004
	TK		Cantaloupe	USA, 2008
			Potato chips	Germany, 1993
			Amphibian	USA, 2001
			Lettuce	USA, 2006
Salmonella Kedougou	TSxtGSuS	Swine, avian	Salami	Norway, 2006
	AAmcAprCefCCpCoFGN		Mushrooms	Ireland, 200
	NeoSpSSuTSxt	Turkey	Unknown, 1992	
		Infant formula	Spain, 2008	
Salmonella Kiambu	ACSSuT[g]	Unk	Papayas	USA, 2017
			Beef jerky	USA, 2003
Salmonella Kottbus	SxtRCpNSu	Avian, equine	Sprouts	USA, 2001
	AkCpAmcATzpCfxCafNT		Bottled water	Spain, 2006
	Atm		Human milk	USA, 1977
Salmonella Manhattan	AmCfxFoxGTob	Unk	Meat products	France, 200
			Pork	Denmark, 1998; Italy, 2009

Serovar	Resistance type[b]	Ecology or host[c]	Vehicle of outbreak	Country and year(s)[d]
Salmonella Mbandaka	*AT*	Chicken	Peanut butter	Australia, 1996
	ANCpFGSSuTSxtTobFoxAk-Cfz		Tahini paste	USA, 2013, 2017
			Live poultry	USA, 2013, 2016
			Sprouts	USA, 1999, 2007
			Pork	USA, 2000
			Cereal	USA, 2018
Salmonella Montevideo	*K*	Bovine, chicken	Pistachios	USA, 2009
	SuAmcASxtSRNeo		Red/black pepper meat	USA, 2010, 2016
	AmcACepGKST		Tahini paste	USA, 2010, 2013, 2017; Australia an New Zealan 2002–2003
			Live poultry	USA, 2012, 2018
			Beef	USA, 2006
			Dietary food supplement	Germany, 2010
			Chicken	England, 1996
			Turkey	USA, 2004
			Pork	USA, 2007
			Sprouts	USA, 2007
			Cheese	USA, 2007
			Frozen coconut	USA, 2018
Salmonella Muenchen	ACSSuTAmcKCepCtxG ACSSuTAmcK	Bovine, chicken, swine	Raw pork	Germany, 2001, 2013–2014
			Sprouts	USA, 1999, 2016
			Orange juice	USA and Canada, 199
			Melon	USA, 2003
			Corn beef	Australia, 1997

Serovar	Resistance type[b]	Ecology or host[c]	Vehicle of outbreak	Country and year(s)[d]
			Hospital	USA, 2004; England, 1980
			Turkey	USA, 1999, 2002
			Pet geckos	USA, 2015
			Live poultry	USA, 2017
Chicken	USA, 1999			
Salmonella Muenster	TACpNGSxtCAmcSuCfxFox-TobR	Chicken	Goat cheese	France, 200
			Roast beef	Saudi Arabia 1980
			Live poultry	USA, 2016
			Pork	USA, 2006
Salmonella Newport	Bl	Bovine	Cucumbers	USA, 2014
	GNeoAFSxtSuTKAmcSAk-CfxTob		Pistachios	USA, 2009
	AmcACafCtxCepCST		Peanuts	UK, 2001
			Tomatoes	USA, 2002, 2005–2007
			Sprouts	USA, 2010
			Chia powder	USA, 2014
			Leafy greens	USA, 2004
			Potato	USA, 2000, 2006
			Milk	USA, 2001, 2004
			Beef	USA, 2002–2003, 2007-2008
			Turkey	USA, 2002, 2004
			Pork	USA, 2002–2003, 2007
			Frozen coconut	USA, 2018
			Papayas	USA, 2017
			Live poultry	USA, 2012–2014
			Melon	USA, 2003, 2006, 2012
			Small turtles	USA, 2012–2013

Serovar	Resistance type[b]	Ecology or host[c]	Vehicle of outbreak	Country and year(s)[d]
			Fruit (mango)	USA, 1999
			Cilantro	USA, 2002
			Chicken	USA, 2006
Salmonella Oranienburg	CefCpACKSSuTAmcCepSxtGN CefCpACKSSuTAmcCepSxt CepACarNitAk-CfxGNetCCtxPef	Chicken	Shell eggs	USA, 2016
			Chocolate	Germany, Sweden, Netherlands Finland, Denmark, Belgium, Austria, 200
			Chia powder	USA, 2014
			Fruit salad	USA and Canada, 200
			Sprouts	Australia, 2005
			Dry squid	Japan, 1999
			Cantaloupe	Canada, 199
			Gelato	Australia, 1998
			Shrimp broth or melon	Mexico, 201
Salmonella Panama	ACSSulTSpAKG	Unk	Cantaloupe	USA, 2011
	SuSpSTKAG		Beans	USA, 2001
	SuSpST		Lamb	USA, 2002
	AT		Fruit	USA, 2008
Salmonella Paratyphi B	APipCepFoxCafAkG	Humans	Sprouted nut butter	USA, 2015
	TobNit		Frozen raw tuna	USA, 2015
	AmcACfxFoxCtxCpGNitTTobSxtEF		Baby spinach	Sweden, Denmark, UK, Finland Norway, Netherlands USA, 2007
			Frozen eggs	England, 1955
			Soybean tempeh	USA, 2012
			Pet turtles	USA, 2011–2012

Serovar	Resistance type[b]	Ecology or host[c]	Vehicle of outbreak	Country and year(s)[d]
			Tuna	USA, 2007
Salmonella Poona	TCpNSxt	Turtles, chicken	Melon	USA, 2000–2002
			Cucumbers	USA, 2015
			Small turtles	USA, 2012–2013, 2015
Salmonella Rubislaw	TCpNGSxtAmc AFoxKSSxtTCep	Unk	Potato chips	Germany, 1993
			Pet lizards	Australia, 2010
			Meat	USA, 2008
Salmonella Saintpaul	ASuCTSNCfxGFr	Turkey, swine	Cucumbers	USA, 2013
			Potato chips	Germany, 1993
			Sprouts	USA, 1999, 2003, 2009; Australia, 2016
			Jalapenos	USA, 2008; Canada, 2008
			Raw produce	USA, 2008
			Milk	USA, 2016
			Tap water	Australia, 1999, 2005
			Cantaloupe	Australia, 2006
			Turkey	USA, 2008
			Fruit (mango)	USA, 2001
			Infant formula	USA, 2001
			Orange juice	USA, 2005
Salmonella Schwarzengrund	*CpAgBlSuTSxt*	Chicken	Dry pet food	USA, 2007
	AkCefFoxCpGNitTTob	Live poultry	USA, 2007	
	SxtE		Human	USA, 1996
Salmonella Senftenberg	TAmcASxtS	Turkey, chicken	Papayas	USA 2017

Serovar	Resistance type[b]	Ecology or host[c]	Vehicle of outbreak	Country and year(s)[d]
	ACepG		Pistachios	USA 2009, 2014, 2016; Canada, 2016; Mexico, 2016; Peru, 2016
			Salami	USA, 2010
			Basil	England and Wales, 2007
			Sprouts	USA, 1997–1998
			Baby cereal	England, 1995
			Leafy greens	USA, 2007
			Live chickens	USA, 2018
			Grapes	USA, 2001
			Beef	USA, 2007
Salmonella Stanley	GSCefCfxCafANCpTCSuSxt	Turkey, swine	Peanuts	UK, 2001
	AmcO		Cashew cheese	USA, 2014
			Turkey	USA, 1999; Hungary, Austria, Germany, England and Wales, Belgium, Czech Republic, Sweden, Italy, Slovakia, Greece, 2011–2013
			Sprouts	Sweden, 2007
			Soft cheese	Switzerland, 2006–2007
			Unknown	Australia, 2004
Salmonella Thompson	GSCefCfxCafANCpTCSuSxt	Chicken	Papayas	USA, 2017
			Cilantro	USA, 1999

Serovar	Resistance type[b]	Ecology or host[c]	Vehicle of outbreak	Country and year(s)[d]
			Chicken scraps	USA, 1984, 2004
			Unknown	USA, 2015
			Smoked salmon	Netherlands 2012
			Bread	USA, 2000
			Unknown	USA, 2014
			Rucola lettuce	Norway, 2004
			Tomatoes	USA, 2000, 2004
			Beef	USA, 1999
			Peanuts	USA, 2006
Salmonella Typhi	ASxtCNAziCfxCafFoxAkGTobNitCpNoILMe	Humans	Frozen fruit pulp, 1999	USA, 2010
			Drinking water	Nepal, 2002
	CepSxtGAmcKCpT	Human	USA, 2000	
	CepAmcT		Chicken rice	France, 199
			Pork/human	France, 199
Salmonella Typhimurium	ATNSSuSxtCFSKGCefCafCfx	Bovine, chicken, swine	Chocolate	Finland and Norway, 19
	CpAgBlSuTSxtCepKAmcGCtx		Peanut butter	USA, 2008–2009
			Tomatoes	USA, 2004, 2006–2007
	AmcFoxTob		Chicken jerky pet treats	USA, 2013
			Dried coconut	USA, 2018
			Chicken salad	USA, 2018
			Ground beef	USA, 2003, 2004, 2011–2013; Canada, 201
			Live poultry	USA, 2013, 2017
			Pet hedgehogs	USA, 2012
			Cantaloupe	USA, 2012

Serovar	Resistance type[b]	Ecology or host[c]	Vehicle of outbreak	Country and year(s)[d]
			Pork, dried pork sausage	USA, 1998, 2000–2002 2007; England, 2011; Spain, 2011
			Duck eggs	Ireland, 201
			Frozen feeder rodents	USA, 2014
			Small turtles	USA, 2013
			Pet/environmental frogs	USA, 2009–2011
			Cheese	USA, 1999
			Sprouts	USA, 1999, 2008
			Eggs	USA, 2000, 2002, 2004
			Chicken	USA, 2000–2001, 2003-2004, 2006
			Fish (smoked)	USA, 1998
			Milk	USA, 1998, 2002, 2004, 2006–2007
			Turkey	USA, 2004, 2006
			Leafy greens	USA, 2007
			Orange juice	USA, 2005
Salmonella Urbana	ND	Unk	Papayas	USA, 2017
			Wash basin	USA, 1991
			Unknown	Finland, 2010; Czech Republic, 2010
Salmonella Virchow	*AgBlSuTSxt*	Avian, dogs	Organic shake/meal	USA, 2015–2016
	TNSxtFrSu		Unknown	USA, 2012
	AkCpAAmcCfxCafNT		Spit roasted chicken	England,196
			Infant formula	Spain, 1994
			Tomatoes	USA, 2003

Serovar	Resistance type[b]	Ecology or host[c]	Vehicle of outbreak	Country and year(s)[d]
Salmonella Weltevreden	GSCefCfxCafANTSuSxt	Unk	Frozen raw tuna	USA, 2004, 2015
			Unknown	India, 2010
			Sprouts	France, 200
			Drinking water	India, 2014
			Hostel food	India, 2010
			Mutton-ghugni	India, 2013
			Fish	India, 2009

[a] Listed are serovars with known ecologies and two or more documented outbreaks.

[b] Antibiotic resistance types represent all antibiotics for which, at some point in time, resistance has been detected in one or more isolates from humans (bold), animals only (not bold), food (italics), or the environment (underlined). A, ampicillin; Ag, aminoglycosides; Ak, amikacin; Amc, amoxicillin-clavulanic acid; Apr, apramycin; Atm, aztreonam; Azi, azithromycin; Bl, β-lactams; C, chloramphenicol; Caf, ceftazidime; Car, carbenicillin; Cb, carbapenems; Cef, ceftiofur; Cep, cephalothin; Cfx, cefotaxime; Cfz, cefazolin; Cin, cinoxacin; Co, colistin; Cp, ciprofloxacin; Cpm, cefepime; Ctx, ceftriaxone, D, doxycycline; E, enrofloxacin; Eo, enoxacin; Ert, ertapenem; Ery, erythromycin; F, florfenicol; Fox, cefoxitin; Fr, furazolidone; Fs, fosfomycin; G, gentamicin; I, imipenem; K, kanamycin; L, levofloxacin; M, minocycline; Ma, macrolides; Me, meropenem; N, nalidixic acid; ND, no data; Neo, neomycin; Net, netilmicin; Nit, nitrofuran; No, norfloxacin; O, ofloxacin; P, polymyxin B; Pef, pefloxacin; Pip, piperacillin; R, rifampin; S, streptomycin; Sp, spectinomycin; Su, sulfonamide compounds; Sxt, trimethoprim/sulfamethoxazole; T, tetracycline (including derivatives); Tic, ticarcillin; Tob, tobramycin; Tzp, piperacillin-tazobactam; V, vancomycin.

[c] Unk, unknown.

[d] Ranges indicate several outbreaks occurring within each year in the range, not a single outbreak lasting the duration of the range.

[e] Shares the antigenic formula 6,7:c:1,5 with *Salmonella* Choleraesuis and *Salmonella* Typhisuis, so outbreaks may be identified under a different serovar.

[f] Kratom (also known as thang, kakuam, thom, ketum, and biak) is a plant consumed as a stimulant and can be used as a substitute for opioids.

[g] Inferred from the presence of SGI-1; no resistant isolates detected to date.

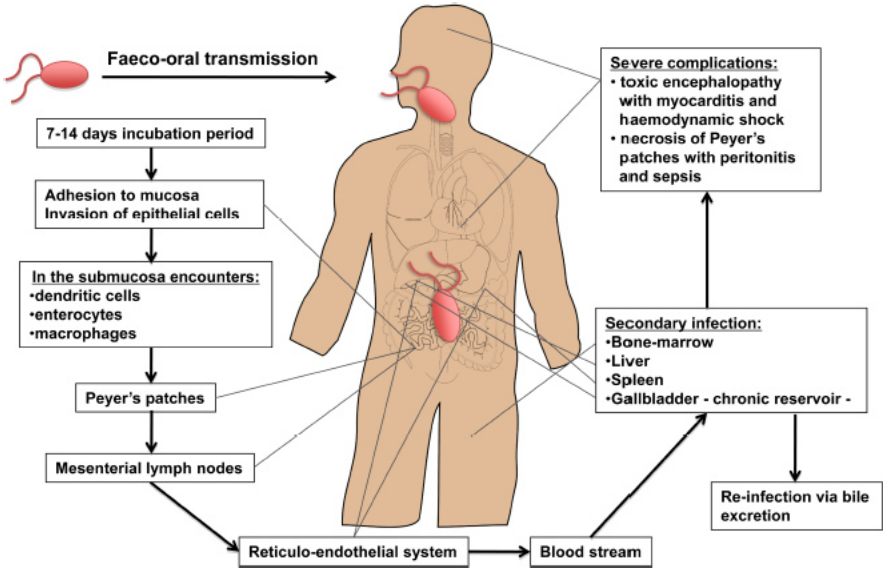


Figure 9.3. Diagram showing how *Salmonella* Typhi moves through the body during systemic infection.

After ingestion of *Salmonella* Typhi and *Salmonella* Paratyphi, which cause typhoid fever, an asymptomatic period follows lasting 7 to 14 days, and fecal shedding of the bacterium can occur during this time. Symptoms include fevers ranging from 39 to 40°C (103 to 104°F), weakness, abdominal pain, headache, loss of appetite, and in some cases a rash or flat, rose-colored spots. Additional symptoms may include a slightly distended abdomen with a "doughy" consistency or pain and tenderness in the right iliac fossa, suggestive of appendicitis or peritonitis (147). If the illness is left untreated, patients can experience complications including gastrointestinal bleeding, intestinal perforation, and encephalopathy. While definitive diagnosis is made through culturing of blood, bone marrow, or stool samples for *Salmonella* Typhi or *Salmonella* Paratyphi, assays have been developed for detection and diagnosis of typhoid fever using PCR, antigen-and antibody-based techniques, tube tests, and test strips, which are less invasive and purported to be as accurate as traditional culturing techniques (149). Once infection has occurred, treatment includes supportive therapy and antibiotics. For prevention, currently, there are two typhoid vaccines available within the United States, Ty21a (live-attenuated, oral vaccine) and the parenteral Vi vaccine. There are also several conjugate vaccines in development and in various stages of testing around the world, including TyVac, Typbar TCV, Vi-rEPA, and Vi-CRM.

Foodborne Outbreaks

NTS serotypes infect 1 in 10 people globally per year, with an estimated 33 million "healthy life years" lost and with inflammatory gastroenteritis being the most common result of *Salmonella* infection (150). For *Salmonella* Typhi and *Salmonella* Paratyphi, which cause typhoid fever, an estimated 11 to 22 million people are infected, resulting in 128,000 to 161,000 deaths per year (150). In 2016, a survey of 10 U.S. states found 7,554 confirmed *Salmonella* cases, and the most common serovars were *Salmonella* Enteritidis, *Salmonella* Newport, and *Salmonella* Typhimurium. In the same year, 94,530 confirmed human cases of salmonellosis were reported in the European Union (151, 152). In Asia, incidence data are limited for salmonellosis across the continent, but estimates from studies conducted in Southeast Asia and India suggest an incidence rate ranging from 7.2 to 525 cases per 100,000 persons per year within the continent (153, 154). In Africa, information on the incidence of *Salmonella* disease is also limited; however a study conducted between 2010 and 2014 for 13 sites across Africa which analyzed 13,431 blood samples found that of the noncontaminant bacteria isolated from febrile patients, 24% were *Salmonella* Typhi and 17% were NTS. The estimated burden from the 2010–2014 study in Africa for typhoidal and nontyphoidal fever was 100 cases per 100,000 person-years of observation (155).

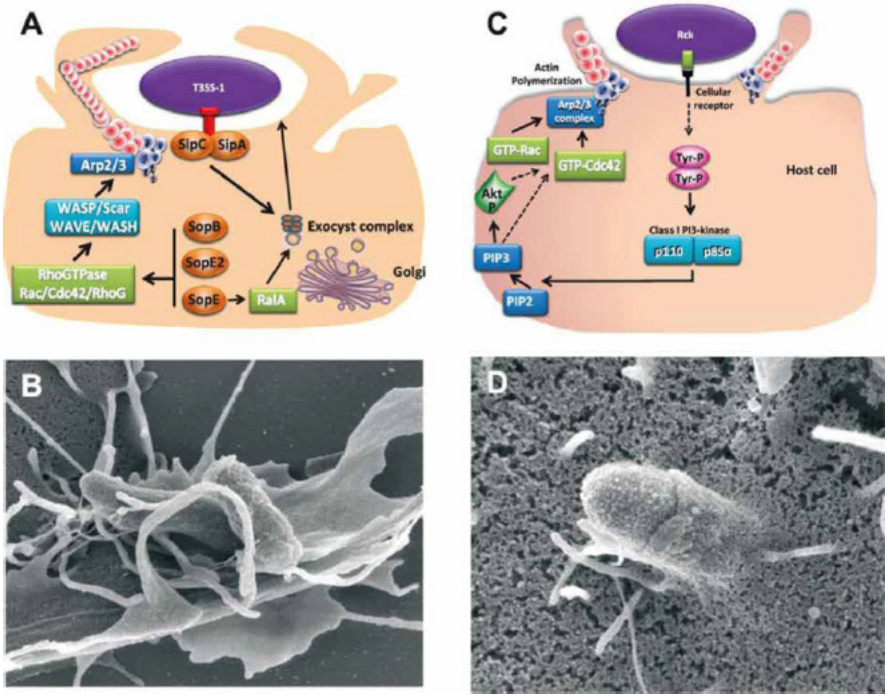


Figure 9.4. Diagrams and micrographs showing how *Salmonella* uses the trigger (A and B) and zipper (C and D) mechanisms to enter cells. (A) Diagram of the trigger mechanism. Using a TTSS, *Salmonella* effector proteins (SipA, SipC, SopB, SopE, and SopE2) are injected into the cell. SopE, SopE2, and SopB activate the Rho GTPases Rac, Cdc42, and RhoG to allow rearrangement of the actin cytoskeleton using the cellular proteins WASP, Scar, WAVE, and WASH, which activate the Arp2/3 complex. SipA and SipC bind to the actin. SipC and SopE, in concert with the Ras-related protein RalA, mediate formation of membrane ruffles and the recruitment of the exocyst complex. (B and D) Scanning electron microscopy images of *Salmonella* entering cells using the trigger and zipper mechanisms. Large membrane ruffles can be seen at the entry site. (C) Diagram of the zipper mechanism. Phosphorylation of tyrosine kinase is mediated by the Rck invasin protein expressed on the *Salmonella* outer membrane when it interacts with its receptor on the host cell membrane. The class I phosphatidylinositol 3-kinase is activated and induces phosphatidylinositol(3,4,5)-trisphosphate formation, using Akt activation. GTPase Rac1 and GTPase Cdc42 trigger actin polymerization via the Arp2/3 nucleator complex. The mechanism controlling Cdc42 during Rck-induced signaling is still unknown. Dotted arrows represent possible signaling events and/or interactions.

Approximately, 85% of all illnesses ascribed to *Salmonella* are foodborne, leading to ~155,000 deaths/year (3). Table 9.2 lists many of the notable foodborne outbreaks by serovar for *S. enterica* as investigated or described from 1967 to 2018. *Salmonella*'s unique ability to survive on substrates with high and low water activities facilitates its colonization and its correlation with outbreaks associated with dry products, wet products, and ecological hosts.

Salmonella serovars activate several mechanisms to combat desiccation, cold and heat shock, chemicals used for sanitation, and drops in pH. *Salmonella* Senftenberg, *Salmonella* Typhimurium, *Salmonella* Enteritidis, and *Salmonella* Bedford have all been shown to be heat resistant or to be able to adapt to heat shock, sometimes upregulating virulence genes in the process, thereby lowering infectious dose required for illness. *Salmonella* Tennessee, *Salmonella* Enteritidis, and *Salmonella* Typhimurium activate cold shock proteins to facilitate survival in cold environments, including refrigerated food. *Salmonella* serovars survive desiccation by forming biofilms and activating genes involved in fatty acid modification that facilitate survival by increasing osmotolerance or facilitating osmoprotection. Phenotypic changes in response to desiccation include *rdar* morphology (red, dry and rough), filament production, and cell shrinkage. *Salmonella* Senftenberg, *Salmonella* Enteritidis,

Salmonella Typhimurium, and *Salmonella* Mbandaka have shown very high survival in dry conditions, surviving for up to 30 months (156), and *Salmonella* Tennessee was found to survive for 182 days in cookie and cracker filling (157). A summary of *Salmonella* cellular responses to low-moisture environments is illustrated in Fig. 9.5.

Of final note is the cross-tolerance effect. Many virulence factors in *Salmonella* are regulated by the PhoPPhoQ system. Interactions or differences in regulation of genes associated with this system, such as *rpoS* and *fur*, have shown that induction of one stress (e.g., heat) can confer resistance to other stresses, such as low pH (*Salmonella* Enteritidis, *Salmonella* Mbandaka, and *Salmonella* Typhimurium), organic and inorganic acids, desiccation, UV, and chemical disinfectants (*Salmonella* Enteritidis, *Salmonella* Newport, *Salmonella* Infantis, and *Salmonella* Typhimurium) and/or can result in upregulation of virulence if pH decreases (*hilA*; *Salmonella* Senftenberg, *Salmonella* Heidelberg, *Salmonella* Mbandaka, *Salmonella* Montevideo, and *Salmonella* Infantis) (156).

Impact on the Host Microbiome

Since the advent and increased accessibility of high-throughput sequencing, there has been a surge in human and animal gut microbiome studies. The human gut contains 500 to 1,000 different species of bacteria, primarily belonging to three bacterial groups: *Firmicutes*, *Bacteroidetes*, and *Actinobacteria*. The gut microbiome plays a role in protecting the epithelia from invasion by producing toxins and by cross talk between different regions of the gut, which can allow the gut microbes to suppress growth of *Salmonella*, optimize the immune response, compete with *Salmonella* for nutrients, and mediate clearance of pathogens. *Salmonella* serovars and their associated virulence factors, in turn, have been shown to exploit aspects of the human gut to support their growth and compete with other microbes, such as using the gut inflammatory response to create a novel nutrient niche (158) or harnessing respiration from fermenting microbes via the generation of new electron acceptors (159). Characterization of the gut microbiome during infection is opening up a new area of research in microbiota-based therapies to remedy *Salmonella* infections in both humans and food animals. Currently, the main treatment for *Salmonella* infection is antibiotic administration. However, if infection is not completely cleared or there is antibiotic resistance, this treatment can reduce the bacteria within the gut, resulting in *Salmonella* expansion and causing prolonged disease, fecal shedding, and carrier status (159).

Antibiotic Resistance

As discussed above, the main prevention for typhoidal *Salmonella* infection is vaccination; however, once illness is acquired, supportive therapy and antibiotics are the main treatments offered. The WHO states that through the overuse and misuse of antibiotics as well as poor infection prevention and control, antimicrobial resistance is fast becoming one of the "biggest threats to global health, food security and development today" (160). Prior to the 1970s, chloramphenicol was the major treatment prescribed for enteric fever (typhoid) (161–163); however, isolates resistant to chloramphenicol were appearing before 1970 (163, 164). By the early 2000s, multi-drug (ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole)-resistant *Salmonella* Typhi and *Salmonella* Paratyphi were common globally. *Salmonella* Typhi is considered endemic in developing countries now, especially in Southeast Asia (147). With the rise in multi-drug-resistant *Salmonella* Typhi and *Salmonella* Paratyphi, antibiotic treatment has included the use of fluoroquinolones (e.g., ciprofloxacin), azithromycin (macrolide antibiotic), and extended-spectrum cephalosporins (e.g., ceftriaxone and β-lactams); however, within a couple of years, resistant strains were already emerging globally, and a concurrent resistance to nalidixic acid was also observed (147, 165, 166). Figure 9.6 shows a map of the resistance profile of *Salmonella* Typhi.

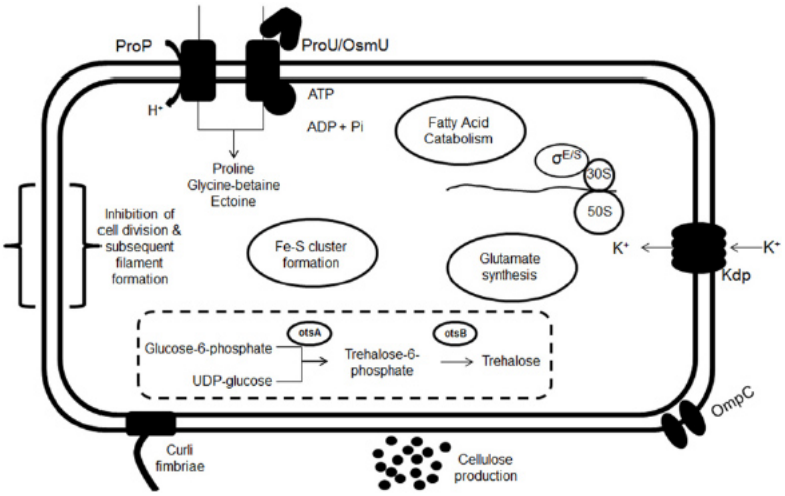


Figure 9.5 Diagram of proposed cellular responses to transition to a low-moisture environment. Responses include K^+ uptake by the Kdp transporter, osmoprotectant transport (ProU, ProP, and OsmU), glutamate synthesis, trehalose biosynthesis, upregulation of fatty acid catabolism and RpoE and RpoS regulators, Fe-S cluster formation and filament formation, and an increase in the number of OmpC porins. There may also be a role for cellulose and curli fimbriae in survival in low-moisture environments.

Vaccination is not an option for NTS, so, when supportive therapy fails, one of the most effective treatments for NTS infection has been (for the past 70 years) and continues to be the use of antibiotics (167, 168). In recent years, NTS has been showing increasing resistance to fluoroquinolones, β-lactams, chloramphenicol, penicillins, and trimethoprim-sulfamethoxazole; much of this resistance has been linked to agricultural use of these antibiotics in food animals (147, 165, 168–170). As of 2016, one of every four *Salmonella* infections was caused by bacteria resistant to three or more antimicrobials commonly used in human and animal medicine, and four countries reported for the first time extendedspectrum-β-lactamase-producing *Salmonella* Kentucky with high resistance to ciprofloxacin in humans (171).

As with *Salmonella* Typhi and *Salmonella* Paratyphi, the spread of resistant phenotypes has occurred globally, with ampicillin, chloramphenicol, streptomycin, sulfonamide, and tetracycline (ACSSuT) (Table 9.2) resistance having been detected in the United States, Europe, Canada, Israel, Turkey, and Japan, primarily associated with *Salmonella* Typhimurium (172–175). Quinolone (nalidixic acid) and fluoroquinolone (ciprofloxacin) resistance has been detected in serovars, including *Salmonella* Enteritidis, *Salmonella* Typhimurium, *Salmonella* Virchow, and *Salmonella* Hadar, found in Denmark and Southeast Asia. Serovars resistant to β-lactam drugs (e.g., cephalosporins; isolates harboring β-lactamases or AmpC-type enzymes) were first detected in North Africa in the mid-to late 1980s (176–178). Since then, cephalosporin resistance has been reported in Southeast Asia, Europe, Canada (associated with *Salmonella* Typhimurium and *Salmonella* Newport), and the United States (*Salmonella* Typhimurium, *Salmonella* Newport, and *Salmonella* Heidelberg). Serovars documented as having extensive drug resistance (resistance to more than six antimicrobial agents) include *Salmonella* Typhimurium and *Salmonella* Kentucky. These isolates have mostly been detected in Southeast Asia (China, Malaysia, Vietnam, and Thailand) (147, 179).

Resistant phenotypes are encoded by specific genes found on plasmids or genomic islands prone to horizontal gene transfer via transposons or conjugation between isolates and serovars. Antibiotic resistance genes on plasmids or other pieces of genetic material can also be transferred via uptake from the environment (transformation) or via phage (transduction). For example, *Salmonella* Typhimurium has been associated with definitive phage type 104 (DT104) (147), which confers resistance to the first-line antimicrobials ACSSuT (Table 9.2). For the ACSSuT resistance type, many of the genes encoding resistance are located on a genomic island (SGI-1) which was first identified in *Salmonella*

Typhimurium (180) but since has been detected in *Salmonella* Agona, *Salmonella* Albany, *Salmonella* Cerro, *Salmonella* Derby, *Salmonella* Dusseldorf, *Salmonella* Emek, *Salmonella* Haifa, *Salmonella* Infantis, *Salmonella* Kentucky, *Salmonella* Kiambu, *Salmonella* Kingston, *Salmonella* Meleagridis, *Salmonella* Newport, *Salmonella* Paratyphi B, and *Salmonella* Tallahassee (181–183). SGI-1 is a 14-kbp region bracketed by two integron structures which contain the antimicrobial resistance genes bla_{PSE-1}, *floR*, *aadA2* (streptomycin), *sul1*, and *tetG* (tetracycline) (Fig. 9.7) (147).

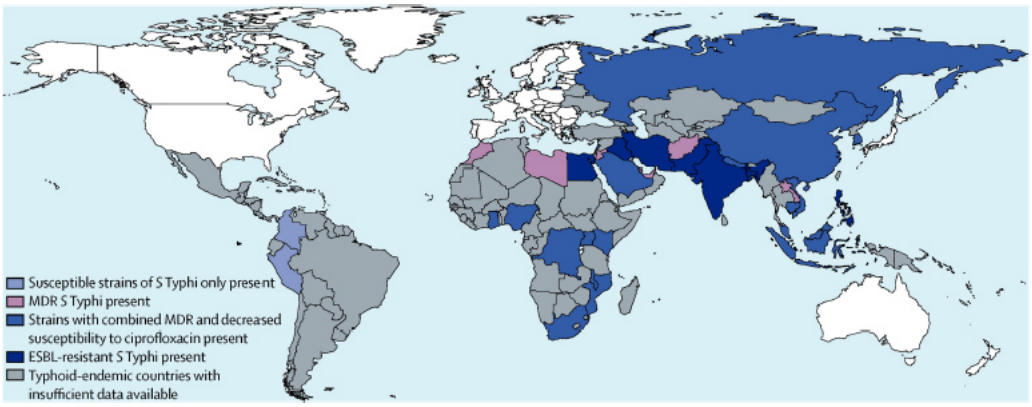


Figure 9.6 Global distribution of antimicrobial drug resistance in *Salmonella* Typhi. MDR, multi-drug resistant; ESBL, extended-spectrum β-lactamase. Reprinted from reference 147.

Resistance to penicillins and cephalosporins can be mediated by β-lactamase enzymes such as Bla_{PSE} and Bla_{TEM} (extended-spectrum β-lactamases), carbapenemases, AmpC-type enzymes, and β-lactamases, which are the most common mode of cephalosporin resistance in NTS (i.e., cephamycinases, encoded by bla_{CMY} genes). Cephalosporins typically target penicillin-binding proteins, disrupting cell wall synthesis. Genes conferring resistance are usually found on mobile genetic elements, such as plasmids, transposons, and integrons; thus, horizontal gene transfer is common between isolates and serovars.

Resistance to chloramphenicol can occur via chloramphenicol acetyltransferases (types I and III) or the nonenzymatic resistance genes *cmlA* or *floR*. Trimethoprim and sulfamethoxazole inhibit folate pathways, preventing synthesis of DNA, so any gene acquired that encodes folate pathway enzymes that do not bind to these drugs can provide resistance. Additionally, dihydrofolate reductase (*dfr*) facilitates resistance to trimethoprim, and the *sul* genes (*sul1* or *sul2*) mediate resistance to sulfamethoxazole.

Fluoroquinolones target DNA gyrase and topoisomerase IV, encoded by *gyrA*, *gyrB*, *parC*, and *parE*. Mutations in the quinolone resistance-determining regions of any of these genes have the potential to confer resistance. For instance, a single point mutation in *gyrA* corresponding to amino acid position 83 (serine to phenylalanine or tyrosine) or 87 (aspartic acid to asparagine or glycine) will result in resistance to nalidixic acid and decreased susceptibility to ciprofloxacin. Resistance to fluoroquinolones can also be acquired via a plasmid-mediated quinolone resistance mechanism [*qnrA*, *qnrB2*, *qnrS1*, and variants *aac(6′)-lb-cr*, *qepA*, and *oqxAB*].

Resistance to azithromycin (macrolide antibiotic) was first documented in 2010 in *Salmonella* Paratyphi A and has been spreading since (152, 184). Macrolides prevent protein synthesis by binding to the 50S ribosomal subunit. The plasmid-borne gene *mphA* (macrolide-2′-phosphotransferase) has been implicated in conferring resistance to azithromycin. Typically found in *E. coli*, the *mphA* gene has also emerged in *Shigella sonnei*, demonstrating intergenus transfer and validating concerns of further transfer to other *Shigella* and *Salmonella* spp. (147, 185).

Antimicrobial resistance has been exacerbated by the overprescription or misuse of antibiotics by medical professionals. Additionally, in many developing countries, antibiotics are available over the counter without a prescription or in substandard versions (expired or counterfeit) and are readily

consumed. In agriculture, antibiotics are used prophylactically to promote growth and reduce illness in food animals, often promoting the spread of antibiotic resistance into the food supply via human-animal interactions or environmental contamination. Efforts at control need to be focused on education of medical professionals and the public about the misuse or overuse of antibiotic therapies. Available antibiotics need to be managed at all levels to mitigate continued spread of resistant bacterial phenotypes. Finally, research in the area of alternative therapies, such as immunity-modulating agents, bacteriophage lysins, antimicrobial peptides, pro-, pre-, and synbiotics, plant extracts, and inhibitors targeting pathogenicity, need to be explored more fully in both animals and humans (186–188).

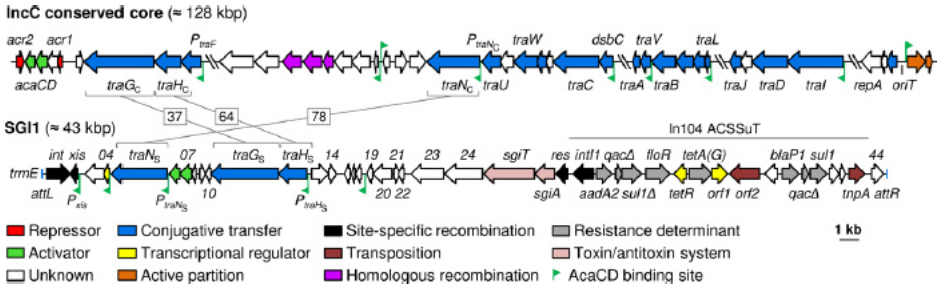


Figure 9.7 Diagram of the core sequence of IncC plasmids and SGI-1. The positions and orientations of open reading frames are indicated by arrowed boxes. Function was determined by BLAST comparisons and is indicated by colors. AcaCD binding sites are indicated by green flags. SGI-1 is flanked by the *attL* and *attR* attachment sites for integration into the 3′ end of the *trmE* gene in the chromosome of *Salmonella* Typhimurium DT104.

Interventions and Preventative

Controls in Foods

Salmonella is ubiquitous in our environment. Indeed, Table 9.2 lists the variety of ecological animal host reservoirs from which serovars have been isolated; however, some of the outbreaks listed there have also been due to consumption of, or exposure to, contaminated water (i.e., *Salmonella* Chester, *Salmonella* Typhi, and *Salmonella* Weltevreden). Additionally, many *S. enterica* serovars involved in prominent foodborne outbreaks, such as *Salmonella* Baildon, *Salmonella* Chester, *Salmonella* Hartford, *Salmonella* Manhattan, *Salmonella* Panama, *Salmonella* Rubislaw, *Salmonella* Weltevreden, and *Salmonella* Urbana, have no reservoir currently identified. As previously mentioned, *S. enterica* serovars have been detected in surface water, water storage tanks, piped water systems, irrigation water, and soil and in hospital environments within the water supply or on equipment in developed and developing countries alike. *S. enterica* serovars that are not mentioned in Table 9.2 but have been implicated in rare outbreaks in humans with environmental or nonfood animal reservoirs include *Salmonella* Agbeni, *Salmonella* Coeln, *Salmonella* Haifa, and *Salmonella* Potsdam (178, 189–192). Still other serovars interfere with agricultural practices by being carried by wild birds or waterfowl, whose excrement can contaminate crops and produce (192–194). The diversity of *Salmonella*'s hosts, ecologies, survival, and virulence strategies has contributed to its continued persistence in the food chain and given rise to the need for multipronged approaches to prevention and control.

The main source of foodborne disease caused by *Salmonella* continues to be poultry-derived products and eggs (195). Programs for the control of *Salmonella* in poultry and eggs date back to 1935 with the National Poultry Improvement Plan, which was established by an act of Congress and based on recommendations developed from meetings between the government, the International Baby Chick Association, and industry partners. It also established statewide breeding programs with uniform terminology. In the early 1990s, the outbreak traceback program dictated that laying flocks would be tested for *Salmonella* Enteritidis and the eggs would be processed at pasteurization plants (196). In 1996, the U.S. Department of Agriculture (USDA) Food Safety Inspection Service (FSIS) issued the Hazard Analysis and Critical Control Point (HACCP) Systems Final Rule. The HACCP Final Rule, with the goal of reducing the occurrence of pathogenic microorganisms on meat and poultry products,

required that all establishments (i) develop and implement standard operating protocols, (ii) require regular microbiological testing to validate prevention and removal protocols during slaughtering, (iii) establish pathogen reduction standards for *Salmonella*, and (iv) design and implement preventive controls to improve the safety of their products (197). The USDA also provides documents and guidelines for food defense planning and food safety assessments, as well as meat, poultry, and processed-egg-product inspection videos. In 2001, the USDA released a report highlighting the effectiveness of these programs, showing a 50% reduction of *Salmonella* presence in samples compared to pre-HACCP implementation (198). By 2010, the prevalence of *Salmonella* contamination in U.S. broiler chickens was 6.7% (198), and in 2014 this percentage dropped to 3.7% (199), highlighting the continued effectiveness of programs such as the HACCP system for controlling *Salmonella* in broiler chickens.

Control efforts have also extended to standardizing practices for the control of *Salmonella* on shell eggs. The risk assessment for *Salmonella* Enteritidis was initiated in 1996 and was followed by the Egg Safety Action Plan. By 1999, the number of cases per 100,000 people had dropped to 1.98, and FSIS continues to guide the development of standards for egg and egg products. In 2010, a large outbreak of *Salmonella* Enteritidis resulting in 1,470 illnesses and the recall of ~500 million eggs occurred during the FDA's establishment of the Egg Safety Final Rule (200). The Egg Safety Final Rule established (i) monitoring conditions and testing for eggs; (ii) limitations on visitors and establishment of practices to reduce cross-contamination; (iii) monitoring and reduction of pests and debris; (iv) cleaning and disinfection, including the removal of visible manure, dust, feathers, and old feed, followed by fumigation or another disinfection method; and (v) refrigeration at or below an ambient temperature of 45°F beginning 36 hours after laying.

Despite these policies, however, controlling *Salmonella* contamination on shell eggs continues to be a complex problem due to the number of variables that have to be considered, such as flock size, flock age, stress, feed, vaccination, and cleaning routines. Egg washing combined with pasteurization and/or irradiation continues to be the most effective method for *Salmonella* control. Pasteurization by heat treatment using a water bath at 57°C for 25 minutes followed by hot air at 55°C for 57 minutes resulted in a 1.9-log reduction in *Salmonella* (201). The minimum level of radiation required to inactivate *Salmonella* is 1.5 kGy (201). Recently, the merits of chlorine dioxide gas in combination with mild wet heat were explored, and this study demonstrated that 5.9 log/CFU/egg of *S. enterica* could be inactivated on the surfaces of egg shells within 4 hours (202). Standard guidelines for consumers include cooking eggs to an internal temperature of 160°F (70°C), use of pasteurized eggs whenever possible, and not storing eggs or foods made with eggs at room temperature for more than 2 hours. Recently, an outbreak occurred, resulting in 45 illnesses and the recall of over 200 million shell eggs from a farm in Indiana (200, 203); however, since the implementation of the Egg Safety Final Rule in concert with state-implemented egg quality assurance programs, the overall incidence of *Salmonella* on shell eggs has gone down significantly. As of 2017, the U.S. prevalence of *Salmonella* in pasteurized egg products was 0.14% (204–206).

Chlorine and ionizing radiation have been evaluated in the control of *Salmonella* in seafood. Studies found that the interferences of organic matter, the potential for carcinogenic by-products, and incomplete inactivation (leaving cells viable and infectious though not culturable) have resulted in limited use of chlorine as a bactericidal wash (207, 208). The use of ionizing radiation has been more promising in seafood, as it has been found that 0.43 to 0.71 kGy could inactivate 1 log of bacterial pathogens such as *Salmonella* in a variety of frozen seafood products, compared to frozen meat products, which required 0.98 to 1.26 kGy (209, 210).

For fresh produce, good manufacturing practices are important in the control of bacterial pathogens. Of the food imported into the United States, ~50% is fruits and vegetables, and the amount of food imported is rising. A survey of outbreaks of *Salmonella* disease associated with foods imported into the United States from 1996 to 2014 found that although the total number of outbreaks associated with imported food was small, the number has increased (211). Multicountry outbreaks have also occurred in Europe due to food imports (212–214). Many of the exporting countries may not use harvesting or processing practices that meet minimum standards, thereby facilitating contamination. Practices such

as irrigation with wastewater, fertilization with sludge or sewage, washing produce with contaminated water, handling of produce by local workers, and cross-contamination during drying and packaging in unregulated facilities all contribute to the movement of *Salmonella* contamination globally in our imported food supply. Establishment of global partnerships in the control of *Salmonella* is of great importance, considering that the amount of food imported, particularly in the United States, has risen over the past 20 years. Globally, systematic *Salmonella* control programs have yet to gain ground in Southeast Asia, Latin America, Africa, and some parts of Eastern Europe (215–218). In Brazil and Japan, there are widespread vaccination programs to reduce *Salmonella* impacts on poultry (219, 220) but, to date, no specific initiatives targeting *Salmonella* reduction in produce or food exports. *Salmonella* control programs, however, have been implemented in the United States (Food Safety Enhancement Act [2009] and Food Safety Modernization Act [2011]) and Europe (EU directive 2160/2003), and they have been integral in reducing *Salmonella* disease within the population as well as *Salmonella* contamination of foods both grown and manufactured in-country and exported (212, 221–223).

As discussed above, *Salmonella*'s success as a foodborne pathogen and its global ubiquity are due to its genomic and physiological plasticity, conferring the ability to survive in different hosts and environments. The potential for illness outbreaks associated with food animals, aquaculture, or produce is not altogether unexpected, since the vehicles of transmission have been shown to be animals and water and since cross-contamination even after sanitization is not uncommon. However, there have been a number of outbreaks involving low-moisture food, such as nuts, peanut butter, spices, and most recently tahini and dried coconut (Table 9.2) (224–226). It was mentioned above that as few as 10 to 36 CFU can cause a productive infection and that *Salmonella* can survive desiccation through putative stress response networks triggered by chemophysical signals (e.g., osmotic, thermal, and oxidative stress; see "Infectious Dose" and "Desiccation Resistance" above). Laboratory experiments that have shown inactivation of *Salmonella* on dry foods include the use of dry-and moist-air convection heating, propylene oxide or ethylene oxide fumigation, low-energy X-ray irradiation, freeze-drying or supercritical CO_2 drying, and cold plasma (227, 228). Misconceptions play a role in the persistence of bacterial pathogen contamination in food products as well. For instance, typical thermal treatments are 165°F and 158°F for turkey and beef, respectively, to ensure reductions in numbers of *Salmonella*. The public may assume that these temperatures are sufficient for nuts as well, when in fact, according to the Almond Board of California's Technical Expert Review Panel, oil roasting temperatures of 260°F for 2 minutes and blanching temperatures of 190°F are required for adequate reduction of *Salmonella*. The efficacy of each technique is impacted by the food matrix, specific water activity, and *Salmonella* strain used; control processes may not be effective across types of food products (i.e., dry food products versus fish or fresh produce).

Outreach, education, proper training tools, and industry and government initiatives such as HACCP or current good manufacturing practices are the best line of defense in the control of bacterial pathogen dissemination in our food supply. This is the foundation for the manufacturing of safe food, covering proper facility conditions, control and storage of food stuffs, processing protocols, personal hygiene, routine testing, and cleaning and sanitation practices. It is a complex and challenging issue, especially in developing nations, where regional cultural practices or unavailability of financial or training resources can interfere with compliance with food safety regulations. On farms, vaccination, pest control, adequate ventilation, disinfection, limiting animal contact, control of feed handling, hatchery management, and use of antibiotics can help control levels of *Salmonella*. In food processing plants, control has to be initiated at several levels: (i) evaluation and testing of the initial ingredients sourced from different suppliers within the processing chain, (ii) proper training of personnel in good microbiological and decontamination procedures, (iii) proper disinfection of equipment and treatment of water, (iv) proper thermal treatment of foods, (v) ensuring effective pest control, and (vi) consideration of adopting a positive-pressure air system in plants processing food items to reduce bacterial contamination in the air of processing plants. Continued end-point sampling and establishment of an effective environmental monitoring program encompassing checkpoints for all variables listed above should reduce *Salmonella* contamination on food products (147).

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10
Eleven *Campylobacter* Species

Campylobacter is regarded as a leading cause of bacterial foodborne infection in many areas of the world. *Campylobacter jejuni* and, to a lesser extent, *Campylobacter coli* are important causes of human diarrheal illnesses, even surpassing *Salmonella* in importance in many countries (1, 2). Although human illnesses are usually self-limiting, the associated morbidity and cost are significant. In Europe, the burden of human campylobacteriosis is estimated to be between 8 and 100 times higher than the annually reported number of cases, which has approximated 200,000 in recent years (3). Such a high incidence of *Campylobacter*-related diarrhea has significant socioeconomic impacts, making this pathogen a priority for public health and food safety researchers. This chapter highlights the important bacteriological and epidemiologic issues related to contamination by thermotolerant *Campylobacter* spp. (*C. jejuni* and *C. coli*) in the human food supply chain.

Since no single review can be comprehensive, the reader is encouraged to refer to several excellent recent books and reviews for additional information (1, 2, 4, 5).

THE GENUS *CAMPYLOBACTER*

The genus *Campylobacter* belongs to the family *Campylobacteraceae* together with the genera *Arcobacter* and *Sulfurospirillum*. The genus was established in 1963, and its taxonomic structure has been revised over the years (6, 7). In 2017, the genus *Campylobacter* contained 29 species, 5 of which have been further divided into 11 subspecies (8). These species can be traced to a variety of animal and environmental sources. In veterinary medicine, *Campylobacter fetus* subsp. *venerealis* is an important cause of epizootic bovine infertility, and *C. fetus* subsp. *fetus* is most often associated with spontaneous abortion in cattle and sheep. However, limited information can be found regarding isolation of *Campylobacter* species from wildlife. There is a recent report of isolation of *Campylobacter lari* subsp. *concheus* and *Campylobacter volucri*s from penguins in the Antarctic region (9). In human oral medicine, the species *Campylobacter concisus*, *C. curvus*, *C. hominis*, *C. sputorum*, *C. rectus*, *C. showae*, and *C. gracilis* are predominantly important (7, 10). However, from a wider public health and food safety perspective, *C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis* are important species causing foodborne and zoonotic illnesses, of which *C. jejuni* and *C. coli* are the most significant. These four species are often referred to as thermotolerant campylobacters, as most strains of these species exhibit optimal growth at 41 to 43°C. Relevant biochemical properties of these thermotolerant *Campylobacter* species are summarized in Table 10.1.

The species *C. jejuni* comprises two subspecies: the first, *C. jejuni* subsp. *jejuni*, is one of the most common causes of human gastroenteritis worldwide (7, 11), whereas the second, *C. jejuni* subsp. *doylei*, is less well defined as a human pathogen. It is isolated infrequently from human clinical samples and has been associated with infants' bacteremia and pediatric diarrhea in developing countries (12). The distinction between the two subspecies is rarely considered in public health and food microbiology laboratories. However, a multiplex PCR method has been described for the differentiation between the two *C. jejuni* subspecies (13). For simplicity, throughout this chapter, *C. jejuni* refers mainly to *C. jejuni* subsp. *jejuni*.

C. coli is regarded as the *Campylobacter* species isolated second most frequently from human diarrheal samples. It was first isolated from pigs with infectious dysentery and is still the *Campylobacter* species most frequently isolated from pork and swine farms (2). Nevertheless, *C. coli* can also be found in poultry, cattle, and dogs (10). This species is closely related to *C. jejuni*, having a large set of genes in common with *C. jejuni*. This likely explains the problematic differentiation between *C. jejuni* and *C. coli* that occurs in biochemical and phenotypic tests (14). To circumvent this problem, a number of PCR tests have been developed that can differentiate *C. jejuni* and *C. coli* (15, 16). Despite their being closely related, genetic evidence indicates that *C. coli* strains are less diverse and more clonal than *C. jejuni* strains (17–19).

C. lari strains have been isolated from seagulls, wild birds, poultry and products thereof, cattle, and shellfish and from untreated water (20–22). In humans, *C. lari* has been occasionally isolated from individuals with diarrhea (23). *C. lari* strains are primarily distinguished from *C. jejuni* and *C. coli* by their resistance to nalidixic acid (2); however, Endtz et al. (20) have described nalidixic-susceptible variant groups. *C. upsaliensis* is a catalase-negative thermotolerant *Campylobacter* species (7, 10), and its natural hosts appear to be pet dogs and cats (24), although it has been isolated from sporadic and outbreak cases of human enteric illness (25).

Table 10.1 Biochemical and growth characteristics of thermotolerant *Campylobacter* spp. causing human foodborne campylobacteriosis[a]

Characteristic	C. *jejuni*	C. *coli*	C. *lari*	C. *upsaliensis*
Growth at 25°C	–	–	–	–
Growth at 35-3JDC	+	+	+	+
Growth at 42°C	+	+	+	+
Nitrate reduction	+	+	+	+
3.5% NaCl	–	–	–	–
H2S, lead acetate strip	+	+	+	+
H2S, TSI	–	D	–	–
Catalase	+	+	+	–
Oxidase	+	+	+	+
MacConkey's agar	+	+	+	–
Motility (wet mount)	+	+	+	+
Growth in 1% glycine	+	+	+	+
Glucose utilization	–	–	–	–
Hippurate hydrolysis	+	–	–	–
Resistance to nalidixic acid	s[b]	s	R	s
Resistance to cephalothin	R	R	R	s

[a] +, 90% or more of strains are positive; –, 90% or more of strains are negative; D, 11 to 89% of strains are positive. R, resistant; S, susceptible; TSI, triple sugar iron.

[b] Nalidixic acid-resistant *C. jejuni* strains have been reported.

BACTERIOLOGICAL ASPECTS OF *CAMPYLOBACTER*

Characteristics and Growth Conditions

Campylobacters are Gram-negative, oxidase-positive, non-spore-forming bacteria. The name *Campylobacter* is derived from the Greek word "*kampylos*," which means curved, reflecting to a certain extent its morphology under the microscope. Log-phase cells have a characteristic spiral, curved, or occasionally straight rod shape, with pleomorphic size ranging from 0.2 to 0.8 μm in width and 0.5 to 8 μm in length. As *Campylobacter* cells begin to age, they tend to be coccoid. The cells are highly motile by means of a single flagellum or occasionally multiple flagella at one or both ends, which gives them a very characteristic darting, corkscrew-like motility and may serve as an important feature in pathogenesis. Campylobacters are relatively inactive biochemically, obtaining their energy from amino acids or tricarboxylic acid cycle intermediates (rather than carbohydrates) (2, 7, 26).

These microaerobic organisms grow best in an atmosphere containing 5 to 10% carbon dioxide and 3 to 5% oxygen. Most *Campylobacter* strains grow at 37°C; the optimum growth temperature for *C. jejuni* and *C. coli* ranges from 41 to 43°C, and the organisms do not grow at <30°C. These two characteristics restrict the ability of campylobacters to grow outside the host, and consequently, unlike most bacterial foodborne pathogens, campylobacters are not normally capable of multiplication in food during processing or storage (2, 7).

Responses to Unfavorable Environments

There is debate as to the extent to which *Campylobacter* is sensitive to unfavorable environmental stresses. Despite being widely prevalent in the human food chain and diarrheal cases, numerous well-established bacterial stress response genes are noticeably absent from the sequenced *C. jejuni*, *C. coli*, and *C. lari* genomes, for example, those encoding the stationary-phase and stress response sigma factor RpoS, the oxidative stress response regulatory elements SoxRS and OxyR, and osmotic shock regulators BetAB (27). *Campylobacter* spp. survive poorly under dry or acidic conditions and in sodium chloride concentrations above 2%. Survival in foods is better at chill (4°C) than ambient (25°C) temperatures; for instance, survival has been recorded in milk and water at 4°C after several weeks. Freezing reduces the cell numbers of *Campylobacter*, but even after freezing (−20°C) for several weeks, low levels of *Campylobacter* can be still detected on contaminated poultry (2, 28, 29). Adding to that, *Campylobacter* species are relatively sensitive to heat (the time for 1 log10 reduction [90% inactivation of the population] at 55°C is 2.5 to 6.6 min) and irradiation and can readily be inactivated during proper cooking (30, 31).

Valuable insights on mechanisms by which *C. jejuni* overcomes stressful environments are based on findings from laboratory stress models. The tolerance response of *C. jejuni* to several stressors, such as aerobic exposure and acid stresses, after mild laboratory adaptation has been reported (32, 33). Adding to that, it has been determined that challenging *C. jejuni* strains under aerobic conditions can enhance their acid tolerance response (34). Such a cross-protection response from one stress to another has also been determined, as genes involved in heat shock response in *C. jejuni* NCTC 11168 were upregulated in response to acid stress (35). Additionally, it has been revealed that starved *C. jejuni* cells are able to withstand heat stress (36). Some studies also showed that strain-to-strain differences in stress response may be a reflection of the genotypic diversity within the *Campylobacter* population (37, 38).

CAMPYLOBACTER DIVERSITY AND SUBTYPING

Insight into Diversity via Genomic Analysis

According to the literature, the genetic diversity of *C. jejuni* is greater than that of *C. coli*, which is responsible for a more stable population within a broiler flock (39, 40). In other words, the high frequency of genomic variation can be due to the high diversity of *C. jejuni* (41) or to the subsequent introduction of the bacterium (42). The

publication of the complete genome sequence of *C. jejuni* strain NCTC 11168 was crucial in understanding the molecular basis for the genotypic diversity of this pathogen (43). This opened the opportunity for in-depth molecular investigations. For instance, comparative genomic hybridization analysis of 11 *C. jejuni* clinical isolates (of various heat-stable serotypes) revealed low levels of genome plasticity among these strains; approximately 84% of the 1,654 genes analyzed were common to all strains tested. These "core genes" appeared to encode housekeeping functions, such as biosynthesis, metabolic, cellular, and regulatory processes. On the other hand, strain-specific gene differences were involved in the biosynthesis and modification of cell surface structures, including flagella, lipooligosaccharide (LOS), and capsular polysaccharide (44).

In line with this study, Leonard et al. (45) utilized *C. jejuni* DNA microarray technology to investigate genetic diversity among five clusters of epidemiologically related isolates. The authors observed a similar variability in genes involved in surface modification, such as the LOS, capsule, and flagellin loci. Hence, the presence of several dozen phase-variable genes in surface antigen biosynthesis loci provides *C. jejuni* with exceptional diversity for interactions with different niches, including immune evasion in the human host and survival in other reservoirs (46). This can be further elaborated from the study of On et al. (47), in which a whole-genome microarray was used to detect *C. jejuni* cytolethal distending toxin and hemolysin activities, along with survival under aerobic conditions. The strains tested were grouped into two clusters, of which one showed better viability during aerobic incubation. Sixty-seven genes were present in this cluster but were missing from the other one. Most of these variable genes were localized within the gene associated with surface structures, including flagella, LOS, and membrane transport protein. These studies provide a molecular basis for the correlation between *C. jejuni* genomic content, particularly in surface-coding regions, and its diversity and environmental survival potential.

Other elements that contribute to *C. jejuni* diversity are its natural competence and recombination ability. It has been determined that *C. jejuni* is naturally competent, as it can take up DNA from the environment. This leads to recombination between strains, which allows the generation of even more genetic diversity. *In vitro*, *C. jejuni* has a marked preference for DNA from *C. jejuni* strains, as opposed to DNA from other species (48, 49). Frequent recombination and intrachromosomal rearrangements in *C. jejuni* can affect the entire chromosome as well as individual loci, such as flagellin genes (43). Another feature of the *C. jejuni* genome that contributes to its diversity is the presence of homopolymeric tracts (short DNA intervals in which the same base is tandemly repeated) that can provide a window to genes that slip in and out of frame (44). According to van Belkum et al. (50), such phase-variable genes can enable key determinants to switch on and off depending on the environmental selective pressure to which *C. jejuni* is exposed.

Overview of Selected Molecular Subtyping Methods

As discussed in the section above, *C. jejuni* has considerable genetic diversity. According to Wassenaar and Newell (51), a combination of subtyping methods might

be required for studying *C. jejuni*, because diversity and recombination levels might render the use of a single typing method of limited value when applied in an epidemiologic context. Since the 1980s, subtyping of *C. jejuni* for public health and food safety purposes has benefited from the emergence of various genotypic typing methods. Table 10.2 highlights the main features of some of the commonly used genotypic typing methods for *C. jejuni*.

Contribution of Subtyping to Understanding *Campylobacter* Epidemiology

Over the past 2 decades, molecular subtyping of *Campylobacter* spp. has provided valuable clinical and epidemiologic insights, some of which are described below.

Discriminatory Power of Typing Methods for *C. jejuni* and *C. coli*

Overall, pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) generated typing results that had a better correlation with the geographic location of the isolates in comparison to other typing methods summarized in Table 10.2. PFGE and MLST exhibited a better discriminatory power for *C. coli* isolates than repetitive extragenic palindromic PCR or restriction fragment length polymorphism of the *flaA* gene (*flaA*-RFLP) (52). Duarte et al. (53) compared five different typing methods, i.e., MLST, *flaA*-RFLP, antimicrobial resistance profiling, determination of the presence or absence of five putative virulence genes, and, exclusively for *C. jejuni*, determination of LOS class for use in the monitoring of *C. jejuni* and *C. coli*. It was shown that MLST was individually the most discriminative typing method for both *C. jejuni* and *C. coli*, whereas the most discriminative method for both *C. jejuni* and *C. coli* was obtained by combining MLST with *flaA*-RFLP. This approach of combining MLST with variable gene sequence typing had been described before (17). Another study demonstrated that although multiple-locus variable-number tandem-repeat analysis has slightly higher discriminatory power than MLST, it is better to use this method as a prescreening method in epidemiology before employment of MLST for analyzing a large population of *C. jejuni* (54).

Recently, multiplex ligation-dependent probe amplification–binary typing (MBiT) was used as a novel method for subtyping of *C. jejuni* and *C. coli*. This study revealed that MBiT not only is a time-saving method (results produced within 6 h) but also is more rapid and cost-effective than both PFGE and MLST. Moreover, it has been found that MBiT is more discriminatory than MLST and requires only basic molecular biology equipment and skills (55). Instead of using fragment-based typing methods (such as PFGE or *flaA*-RFLP) or sequence-based typing methods (such as MLST), nowadays whole-genome sequencing (WGS) and subsequent bioinformatic analysis are being introduced into molecular epidemiological studies. The WGS approach has the ability to provide nearly complete genotype information for the isolates analyzed. The data are not limited to revealing the presence or absence of virulence factors or antimicrobial resistance genes but can provide insights into the molecular evolutionary forces behind these very important genotypes.

Table 10.2 Molecular subtyping assays for *Campylobacter*

Method	Typeability	Discriminatory power	Convenience	Practical considerations
flaA-RFLP	Almost 100%	Fair; can be enhanced by combining patterns of restriction enzymes (e.g., DdeI with HinfI).	Quick (<24 h) and simple PCR-based (equipment is becoming widely available) High-throughput analysis	Targets one polymorphic gene; thus, the technique is widely criticized for being vulnerable to genetic instability. Of important value in outbreak investigations, and could be used to predict MLST clonal complexes Internationally standardized protocol is available.
PFGE	95–100%; DNA methylation and DNase activity in some isolates can limit the action of restriction enzymes.	Good, and better than *flaA*-RFLP Using KpnI in addition to SmaI enhances PFGE discriminatory power.	Time-consuming (3 to 5 days; however, a 24-h protocol has been published) Low-throughput analysis Need specialized and expensive (initial setup) equipment	Analysis of results is difficult and includes the use of specialized software in combination with visual examination of bands on gel. Some reports indicate that PFGE is vulnerable to *C. jejuni* genetic instability, including point mutation, transformation, and plasmid acquisition or loss, which can lead to minor changes in band profile. Internationally standardized protocols are available.

Method	Typeability	Discriminatory power	Convenience	Practical considerations
MLST	100%	High	Automatable and high throughput Needs highly skilled operator and sequencing facility (failure on first run is frequently encountered) Costly; however, the price is rapidly declining.	Involves analysis of the DNA sequence for each isolate on a standardized panel of genes (seven for *Campylobacter*) that are chosen because they have an essential function (housekeeping genes) and are present in the vast majority of isolates Web-based free software is available for ease of result analysis and interpretation. Freely accessible online database is available. Direct comparison between different laboratories and different isolate collections is possible, which emphasizes its epidemiological importance.
MBiT	100%	Higher than MLST	Results produced within 6 h, more rapid and cost-effective than both MLST and PFGE	

Outbreak Investigations

PFGE has been useful in including or excluding cases of *Campylobacter* enteritis from outbreaks (56, 57). The most extensive use of PFGE to support outbreak investigation of *Campylobacter* enteritis has been within PulseNet, in which more than 2,500 profiles have been identified using standardized methods (58). Applying MLST to *Campylobacter* isolates from several outbreaks has been less discriminatory than PFGE in identifying which cases are outbreak related and which are not (59, 60). As of ca. 2015, next-generation WGS has also been shown to be a powerful tool for determining the relatedness of bacterial isolates in foodborne illness detection and outbreak investigations. However, WGS has rarely been used in smaller local outbreaks, such as those that often occur with *Campylobacter* spp. There was one study comparing PFGE, which since the inception of the CDC PulseNet program is the method of choice for bacterial “fingerprinting,” to WGS and a reference-free

bioinformatic analysis for retrospectively analyzing a specific outbreak of *C. jejuni* associated with raw milk in Utah (61).

The results of WGS and subsequent bioinformatic analysis correlated well with the PFGE and epidemiological outbreak investigation results. However, it was shown that PFGE has limitations compared to WGS. PFGE cannot indicate the phylogenetic relationships of isolates. PFGE lacked the resolution to determine the outbreak relatedness of all isolates, whereas the genomic approaches in the latter study indicate the phylogenetic relationships of the isolates, as well as providing nearly complete genotypic information for each isolate. A significant benefit of the selected WGS bioinformatic analysis in the latter study was that a reference genome sequence was not needed. This reference-free analysis removes the critical step of selecting the genome sequence of a closely related organism, which can dramatically affect the outcome of an analysis (62). Additionally, the bioinformatic analysis used is not reliant on curated MLST databases. As such, the selected reference-free approach not only allowed the resolution of relationships between highly similar bacterial isolates but also allowed the analysis of isolates that may not have representation in an MLST database or highly divergent strains that may not have an available reference genome sequence. The reference-free analysis allows the addition of historical sequence data and other known sequences to provide context for the analysis. The addition of these historical or known sequences allows the surveillance of pathogens associated with foodborne illnesses, the detection of possible outbreak clusters, and the surveillance of outbreaks over long periods and in relation to known sources of contamination. The genotypic information generated by WGS can be further exploited to gain insights into virulence factors, antimicrobial resistance, and molecular evolution for outbreak-associated isolates (61, 62).

Host Association

Recent findings from MLST-based studies have provided evidence for possible association of certain *C. jejuni* subtypes with specific hosts. Interestingly, the association between *Campylobacter* subtypes and certain hosts has shown a robustness to even large geographic distances. For example, relationships have been observed between clonal complex 61 (CC-61) and cattle, CC-257 and poultry, and CC-403 and pigs and also, perhaps less stringently, between CC-45 and poultry and wild birds and between CC-42 and sheep (2, 63–65). Despite the fact that some *C. jejuni* clonal complexes show consistent associations with certain hosts, others, such as CC-21, are widely distributed in multiple hosts (e.g., farm animals, humans, pets, wild birds, and environmental samples). Differences in the composition of intestinal mucin in various hosts play a significant role in the result of *Campylobacter* infection of these hosts (66).

Source Attribution

The philosophy behind the source attribution approach is that control of *Campylobacter* in the reservoir will prevent subsequent human exposure, regardless of the transmission route or vehicle identity. DNA-based subtyping studies, especially

those considering regional and temporal aspects in the study design, provided important insight into sources of clinical strains of *C. jejuni*. For example, in a study in New Zealand it was observed that *C. jejuni* PFGE subtypes of human origin closely matched subtypes from chicken meat, compared to isolates from other veterinary and environmental sources (67). Also, in Finland, Hänninen et al. (68) concluded that, based on PFGE typing, chicken meat was the most important source of human infection over 3 consecutive years in the Helsinki region. In the same study, certain PFGE types persisted throughout the 3-year period, suggesting that common Finnish *C. jejuni* genotypes from selected lineages are able to colonize both humans and chickens and that these genotypes persist from one year to another. Furthermore, findings of other DNA-based typing studies suggest a role of farm animals and wildlife hosts in *Campylobacter* infection of humans and colonization of broiler flocks (69–71).

Despite the value of DNA-based subtyping studies, conclusions from these studies are limited to a crude and qualitative indication of an overlap between clinical cases and infection source strains of *Campylobacter*. Some model-based approaches are increasingly being considered to quantify source attributions of *C. jejuni* clinical strains based on MLST data. For example, Wilson et al. (72) developed an evolutionary probability model to trace the sources of 1,231 human campylobacteriosis cases in England based on MLST data. The assignment probability estimated that chicken is the source of infection in most (56.5%) of the cases, followed by cattle (35.0%) and sheep (4.3%). Additionally, Mullner et al. (73) used MLST data as prior information in a Bayesian modeling approach to estimate the contribution of *C. jejuni* from different food animals to human illness in New Zealand. They estimated that of the 474 human cases, 379 (80%) were attributable to poultry, 48 cases (10%) to bovines, 44 cases (9%) to ovines, and 4 cases (1%) to the environment. These models highlight the fact that source contribution can vary widely depending on the geographical location and modeling approach. In addition, these models reveal that MLST provides a good degree of subtyping resolution that can be used in source attribution of *Campylobacter* enteritis.

Reservoir attribution of human *Campylobacter* enteritis can provide guidance to risk managers and policy makers on the implementation and evaluation of control strategies for major reservoirs of *Campylobacter* (74). It has been estimated that cecal contents of domestic and wild birds, as the main reservoir of *Campylobacter*, carry up to 10^{10} CFU *Campylobacter* per g, which can be transmitted from the intestines of poultry to meat during processing (75–77). Moreover, water is one of the main sources of *Campylobacter*. Although this pathogen can survive in water for long periods, especially at 4°C, finding modeling data to predict the survival characteristics of these organisms in water seems necessary (78).

CAMPYLOBACTER ASSOCIATION WITH FOODS

Campylobacter enteritis in humans is characterized by a large number of sporadic cases rather than single-source outbreaks. Infection can be acquired by a number of routes. However, *Campylobacter* enteritis in humans is considered to be mainly

foodborne. Campylobacteriosis has been attributed to the consumption of several kinds of food, including raw or undercooked meat, especially poultry meat, raw milk, contaminated water, and seafood. Additionally, other risk factors, including cross-contamination during preparation of ready-to-eat foods like vegetables, as well as direct contact with feces from infected animals, including domestic pets, have been reported as the sources for human campylobacteriosis (2, 79, 80).

As a common inhabitant of the gastrointestinal tract of warm-blooded animals, *Campylobacter* can contaminate foods in different ways. *Campylobacter* contaminates meat when carcasses (e.g., poultry and beef) come into contact with intestinal contents during slaughter and evisceration (11, 81). According to the European Food Safety Authority (82), the proportion of human campylobacteriosis cases attributed by microbial subtyping to chicken as a reservoir ranges between 50 and 80%. In comparison, attribution of human cases during case-control studies suggests that a lower proportion, 24 to 29%, can be attributed to the consumption of chicken meat. The apparent discrepancy between the results of these different attribution methods could be associated with several factors. Case-control studies assign attribution at the point of exposure, while subtyping studies assign it at the reservoir level. Molecular subtyping studies (often based upon MLST genotyping) may overestimate the importance of chicken due to incomplete data on other reservoirs, such as pets and wild birds. If more data on the diversity of strains in such reservoirs became available, a larger proportion of human cases might be linked to these reservoirs. Furthermore, strains from the chicken reservoir may reach humans by pathways other than via meat (e.g., via the environment or direct contact), and poultry-associated strains can also colonize other hosts, such as cattle, pigs, and pets. Conversely, case-control studies may underestimate the true attributable risks due to inaccurate exposure assessment and to confounding by acquired immunity. Still, among food sources, chicken and other poultry (in particular undercooked meat and meat in restaurants) constitute an important risk factor.

In the European Union, in 2016, few member states reported monitoring the presence of *Campylobacter* in food; if analysis was performed, it considered mainly fresh meat from broilers and turkeys and their meat products. In these foods, the occurrence was, respectively, 36.7% and 11% in fresh meat from broilers (n = 11,495) and fresh meat from turkeys (n = 1,505). The occurrence of *Campylobacter* was comparable between milk products and cheeses and was reported to be around 1% (n = 1,616) (83). Milk can be contaminated with *Campylobacter* as a result of fecal contamination on the farm. Most cases of *Campylobacter* enteritis attributed to milk or cheese have involved raw milk or homemade unpasteurized fresh or soft cheeses, because *Campylobacter* cannot survive proper pasteurization (84). Thoroughly processed dairy products (e.g., hard cheese and powdered milk) pose a limited threat due to the low resistance of *Campylobacter* to conditions of reduced pH and water activity.

Eggs do not appear to be an important source of *Campylobacter*, despite its frequent occurrence in poultry (85). The dry surface of the egg does not favor *Campylobacter* survival, and egg albumen has been shown to exhibit bactericidal properties. Results of some surveys on the presence of thermotolerant *Campylobacter* strains in different

food categories are presented in Table 10.3. In a European Union baseline survey carried out in 2008, *Campylobacter* was found in the intestines of 71% of chickens, indicating that they were already colonized when alive, and on 76% of sampled carcasses, which suggests some further contamination during slaughtering (86). But many studies have shown that much of the world's poultry meat is often contaminated with campylobacters.

Table 10.3 Examples of surveys on the prevalence of thermotolerant *Campylobacter* in different foods

Food source	Country (reference)	Sampling	No. positive/no. tested (%)
Poultry meat	Belgium (156)	Chicken meat preparation, processing	315/656 (48.02)
	England (203)	Chicken carcass, retail	199/241 (83.0)
	Ethiopia (204)	Chicken carcass, retail	160/220 (72.7)
	France (15)	Poultry meat, retail	53/70 (75.7)
	Germany (105)	Chicken fillet, retail	87/100 (87.0)
	Iran (205)	Chicken carcass, slaughter	186/336 (55.4)
	Japan (206)	Chicken meat and products, retail	110/170 (64.7)
	New Zealand (207)	Poultry meat, retail	204/230 (89.1)
	Poland (139)	Poultry carcass, slaughter	464/625 (74.2)
	The Netherlands (208)	Broiler breast skin, slaughter	1,447/3,680 (39.6)
	Turkey (209)	Chicken products, retail	106/127 (83.4)
	United Kingdom (210)	Chicken carcass, retail	616/877 (70.2)
	United States (211)	Broiler carcass, slaughter	1,094/4,200 (26)
	Estonia (151)	Chicken carcass, retail	98/517 (18.9)
	Philippines (212)	Chicken carcass, retail	57/120 (47.5)
	Italy (213)	Poultry meat, slaughter and retail	161/472 (34.1)
	Brazil (214)	Chicken carcasses, retail	18/92 (19.56)
	United States (215)	Chicken livers and gizzards, retail	136/202 (67)
	Iran (205)	Chicken carcass, processing	186 of 336 (55.4)
	Iran (216)	Poultry meat, retail	106/260 (40.8)
Raw milk	Ireland (217)	Raw cow's milk, bulk tank sampling	1/62 (1.6)
	Pakistan (218)	Raw cow's milk, bulk tank sampling	13/127 (10.2)
	Spain (219)	Raw cow's milk, bulk tank sampling	35/98 (36.0)
	Switzerland (220)	Raw goat's milk, bulk tank sampling	0/344 (0.0)

Food source	Country (reference)	Sampling	No. positive/no. tested (%)
	United States (221)	Raw cow's milk, bulk tank sampling	5/248 (2.2)
	Iran (216)	Raw cow's milk	7/80 (8.7)
	United States (222)	Raw cow's milk, bulk tank sampling	27/234 (11.5)
	Tanzania (223)	Raw cow's milk, vendors selling milk and bulk tanks	38/284 (13.4)
	Poland (224)	Raw cow's milk, bulk tanks	7/150 (4.6)
Red meat	Italy (225)	Beef, retail	20/142 (14)
		Pork, retail	6/106 (5.7)
	New Zealand (207)	Beef, retail	8/230 (3.5)
		Pork, retail	21/230 (9.1)
		Lamb and mutton, retail	16/231 (6.9)
	South Korea (226)	Beef, retail	3/250 (1.2)
		Pork, retail	4/250 (1.6)
	United Kingdom (227)	Lamb (liver), retail	70/96 (72.9)
	United States (228, 229)	Beef, retail	1/182 (0.5)
		Pork, retail	3/181 (1.7)
		Minced pork, retail	5/348 (1.3)
	Iran (216)	Beef, retail	28/200 (14)
	Tanzania (223)	Beef carcass, retail	24/253 (9.5)
Seafood	Ireland (217)	Raw oyster, retail	3/129 (2.3)
	The Netherlands (20)	Raw oyster, retail	11/41 (27)
		Raw mussel, retail	41/59 (69)
	Thailand (230)	Raw oyster, coastal farms	24/30 (80)
		Raw mussel, coastal farms	22/30 (73.3)
Fresh produce	Canada (231)	Ready-to-eat vegetables	0/361 (0.0)
	Ireland (217)	Vegetables	2/279 (0.72)
	Malaysia (232)	Vegetables	28/309 (9.1)

Food source	Country (reference)	Sampling	No. positive/no. tested (%)
	United Kingdom (233)	Ready-to-eat vegetables	0/3852 (0.0)

The concept of poultry meat being the prime source of human campylobacteriosis has been only partially supported by indirect epidemiologic evidence, because most human cases are sporadic and difficult to trace to a specific source. In Belgium, poultry was withdrawn from the market during the summer of 1999 in response to dioxin contamination in the preharvest sector. Subsequently, there was a 40% reduction in human cases of campylobacteriosis enteritis (87). Furthermore, in The Netherlands, during an avian influenza outbreak in 2003, 1,300 commercial and >17,000 noncommercial poultry flocks (more than 30 million birds) were culled. Concurrently, there was a 40% reduction in human campylobacteriosis enteritis cases during the months of culling compared to the same period in prior years (88).

Campylobacters extensively colonize the poultry cecum, large intestine, and cloaca but are generally restricted to the mucus layer in the crypts of the intestinal epithelium at these locations (89). The nature of the association between *Campylobacter* and poultry can be attributed to certain adaptation mechanisms. First, it has been suggested that the microaerobic nature of *Campylobacter* is probably a reflection of the limited oxygen concentration encountered in the avian gut (11). Second, the optimal growth temperature of the thermotolerant campylobacters (42°C) mirrors that of the avian gastrointestinal tract, which differs considerably from that of the mammalian gut (37°C) (11, 49). Third, the motility and chemotactic behavior of *C. jejuni* play a role in colonization of the chick gastrointestinal tract, because the organisms are preferentially attracted to fucose components of the chick's intestinal mucin. Fourth, campylobacters might have evolved mechanisms to overcome nutritional limitations while colonizing the avian gastrointestinal system. One of these mechanisms is the ability to acquire iron from both the avian host and the gastrointestinal flora. *C. jejuni* is able to utilize host-derived hemin and hemoglobin, and some *C. jejuni* strains have transport systems that enable them to scavenge siderophores (high-affinity iron compounds) generated by other members of the gastrointestinal flora (90, 91). Although there are several reports indicating a common presence of *C. jejuni* in *Campylobacter*-positive broilers (92, 93), recent investigations have revealed that *C. coli* can also be common in these birds (94, 95).

Campylobacter is primarily a surface contaminant and can be retained with water on the poultry skin, eventually entering openings and crevices in the skin (96). Campylobacters that enter skin crevices and feather follicles may be difficult to remove because of capillary action or irreversible attachment to the skin tissue (97). These sites can provide a suitable microenvironment for the survival of *Campylobacter* in chicken skin. Moreover, some components within the surface structure of chicken skin, such as proteins, fatty acids, and oils, can enhance the survival of campylobacters by hindering the formation of ice crystals in frozen poultry meat (98, 99). The reported *Campylobacter* counts on fresh poultry carcasses and products (e.g., fillets) can vary

from none or less than 1 to 6 $\log_{10}$ CFU/g, depending on the study settings and testing methods (2, 38, 100–103). Hence, once poultry contaminated with *Campylobacter* is introduced into the kitchen, it can serve as a focal point for cross-contamination (104). In addition, irrespective of the transfer route, *Campylobacter* cells initially present on chicken breast fillets can be transferred efficiently to prepared foods (2, 104, 105). This efficient transfer potential emphasizes the importance of taking measures to prevent cross-contamination during food handling as long as the incidence of *Campylobacter* on chicken products remains high.

CLINICAL ASPECTS OF HUMAN ILLNESSES

High rates of campylobacteriosis translate into substantial social and economic costs. Gellynck et al. (106) estimated that the cost for 2004 of *Campylobacter* enteritis and sequelae in Belgium was €27.3 million. In The Netherlands, the incidence of *Campylobacter* enteritis for 2000 was estimated to be 500 cases per 100,000 people, resulting in a total social cost of €21 million (107). In the United States, the economic burden from health losses due to this pathogen was estimated at $1.56 billion (108). In the United Kingdom, estimated costs to patients and the health care system were £50 million for *Campylobacter* enteritis, while the cost of hospitalization for *Campylobacter*-related Guillain-Barré syndrome was £1.26 million (109). A careful estimation of the *Campylobacter* enteritis disease burden should consider the impact of both intestinal illness and extraintestinal illness and its complications.

Intestinal Illness

More than 90% of human cases of campylobacteriosis cases can be attributed to *C. jejuni* and, to a lesser extent, *C. coli* (11, 81). However, enteric illnesses due to *C. jejuni* and *C. coli* are clinically indistinguishable. Gastroenteritis is the most common clinical presentation seen in humans infected with *C. jejuni*. Following an incubation period typically on the order of 24 to 72 hours, an acute diarrheal illness develops and is commonly followed by a nonspecific syndrome of fever, chills, myalgia, and headache, in addition to abdominal cramping. Patients typically have 8 to 10 bowel movements a day at the peak of illness. The diarrhea can range from loose and watery to bloody, and leukocytes and erythrocytes are present in most cases, whether or not blood is voided in the stool (110–112). A few reports are available on the infective dose of *C. jejuni*, which is believed to be as low as a few hundred bacteria. In two studies of experimental infections in humans, *C. jejuni* caused illness with oral doses of 500 CFU (113) and 800 CFU (114). The enteric illness was self-limiting and resolved within a week in most individuals. However, extended illness can occur, particularly in individuals with underlying illness (111, 112).

In many areas of the world, human campylobacteriosis cases are reported throughout the year but peak during the summer. The mechanisms behind this phenomenon are poorly understood; however, ambient temperature is hypothesized to play a role in this seasonal trend. A recent investigation in Japan (115) showed effects of climatic elements on *Campylobacter* colonization in broiler flocks. There was a positive

correlation between *Campylobacter* colonization in chickens and increasing temperature, which can be associated with the effect on the *Campylobacter* environmental sources and/or vectors. Additionally, humidity also exhibited a positive correlation with *Campylobacter* colonization. It was shown that during the period of decreasing temperature, high humidity can be a fundamental factor for *Campylobacter* infection. Furthermore, similar to humidity, solar radiation, like other climatic elements, showed a negative correlation with temperature, which can promote *Campylobacter* colonization during the period of reducing temperature (115).

A study in England revealed a linear relationship between mean weekly temperature and reported *Campylobacter* enteritis cases, with a 1°C increase corresponding to a 5% increase in the number of reports up to a threshold of 14°C (116). In addition, some studies attribute the seasonality of *Campylobacter* enteritis cases to variation in human behavior during the summer months, such as increased outdoor activities (e.g., barbecuing and swimming). Seasonal variation in the occurrence of *Campylobacter* in poultry flocks is also suggested to play a role in the seasonality of human cases (116). The numbers of *Campylobacter* in the small intestines and ceca of broilers have a seasonal pattern, with a distinct peak in the summer (117, 118). Also, it is hypothesized that temporal distribution of human cases of campylobacteriosis could be a function of the growth kinetics of one or more fly species that become more active in the summer months (119, 120).

The development of protective immunity has been documented in volunteer infection studies in which individuals challenged with a particular *C. jejuni* strain were protected from disease (but not reinfection) when rechallenged with the same strain (114). Studies of poultry abattoir workers have revealed that they develop antibody responses during their employment. Long-term workers may asymptomatically shed *C. jejuni* in their feces, unlike new workers, who develop clinical signs of *C. jejuni*-associated gastroenteritis (121). Recently, Tam et al. (122) determined in a case-control study that the risk for illness associated with recent chicken consumption was much lower for persons who regularly ate chicken than for those who did not, suggesting that partial immunological protection against enteric illness may follow regular chicken preparation or consumption. Hence, it may be necessary to assess the relative importance of immunity and behavioral factors when one is determining the risk of acquiring a *Campylobacter* infection (123).

Extraintestinal Illness

Occasionally, individuals infected with *C. jejuni* present with a pseudoappendicitis syndrome and do not have diarrhea. Furthermore, *Campylobacter* enteritis may play a role in the induction of irritable bowel syndrome and inflammatory bowel disease (124). Several reports indicated the positive relation between *Campylobacter* spp. and inflammatory bowel diseases such as Crohn's disease and ulcerative colitis (125). Other organ systems can also be affected following *C. jejuni* infection; however, the incidence of extraintestinal manifestations associated with *C. jejuni* infection is very low compared to the incidence of the enteric disease (111, 126). Furthermore, HIV/AIDS patients have exhibited symptoms including persistent diarrhea and severe bacteremia

when infected by *Campylobacter* spp. (127). This can be one of the main reasons of increasing morbidity and mortality caused by *Campylobacter* among HIV-positive individuals, particularly in the developing world (128).

To date, Guillain-Barré syndrome (GBS) is the most extensively studied extraintestinal manifestation associated with *C. jejuni* infection. GBS is the most common form of acute neuromuscular paralysis since the near-worldwide eradication of poliomyelitis, with an annual incidence of 1 to 2 per 100,000 population in many parts of the world (129). Symptoms generally begin with motor and sensory deficits of the lower extremities, which can subsequently spread to upper extremities and the trunk; this can lead to the need for ventilatory support. Most GBS patients recover completely, but others can be left with severe neurological impairment (130, 131). It is now known that in most cases in which a specific pathogen is identified, *C. jejuni* is the most frequently reported etiologic agent associated with GBS cases (Table 10.4).

Table 10.4 Prevalence of production of antibodies to *Campylobacter jejuni* in patients with GBS

Study	No. positive/no. tested(%)	Detection procedure
Islam et al. (234)	54/97 (56)	Serology testing
Barzegar et al. (235)	23/48 (48)	Serology testing
Nachamkin et al. (236)	32/78 (41)	Serology testing
Takahashi et al. (237)	113/1,049 (11)	Stool culture testing
Sinha et al. (26)	19/80 (26)	Serology testing
Hadden et al. (238)	53/229 (23)	Serology testing

It has been hypothesized that patients who suffer from GBS following *C. jejuni* infection develop antibodies against certain classes of the bacterial LOS that cross-react with peripheral nerve cell surface gangliosides (110, 132).

ANTIMICROBIAL-RESISTANT *CAMPYLOBACTER*

Antimicrobial intervention is recommended only in campylobacteriosis cases with severe and/or prolonged enteritis, septicemia, or extraintestinal complications (133). Ternhag et al. (134) determined through a meta-analysis of clinical trials that antimicrobial therapy shortened the carrier state of *Campylobacter* species in the human intestinal flora. In cases where *Campylobacter* infections need to be treated, macrolide antibiotics such as erythromycin are the first choice of treatment. Fluoroquinolones such as ciprofloxacin are also recommended, but the treatment is challenged by a higher prevalence of resistance against this antibiotic than against erythromycin (Table 10.5). Moreover, the rate of resistance to erythromycin and to an lesser extent to ciprofloxacin is higher within the species *C. coli* than *C. jejuni*. Also, the country or region has a clear correlation with the prevalence of resistance to both antibiotics (Table 10.5).

Development of resistance to macrolides in *C. jejuni* and *C. coli* may be due to different events, such as target mutations in 23S rRNA genes and in ribosomal proteins encoded by *rpID* and *rpIV*, ribosomal methylation encoded by *erm*(B), and multidrug efflux pumps, which can confer on *Campylobacter* strains high-level resistance (>512 μg/ml) to erythromycin. More details about those events can be found in the review by Bolinger and Kathariou (135). The effects of macrolide-resistant *Campylobacter* on human health have been determined in only a small number of studies. For instance, a Danish cohort study estimated an increase in the risk of invasive illness and death following infection with macrolide-resistant *Campylobacter* strains compared to susceptible strains (136). Development of resistance to macrolides in *Campylobacter* during therapy has not been documented in humans, and the origin of erythromycin-resistant strains has been linked to veterinary use of the macrolide-lincosamide group (137, 138).

Ciprofloxacin is widely regarded as a valuable antimicrobial therapy in severe cases of community-acquired bacterial diarrhea (133). Mutations in the gyrase gene (*gyrA*), most commonly at position 86 (threonine to isoleucine; ACT → ATT), are the principal mechanism conferring resistance in *Campylobacter*. Luo et al. (139) determined that *C. jejuni* strains gain increased fitness after acquiring this resistance-conferring mutation. The CmeABC multidrug efflux pump works in synergy with GyrA mutations. As a consequence, changes in the activation of this pump also have an effect on the fluoroquinolone resistance level (140).

Table 10.5 Prevalence[a] of erythromycin-and ciprofloxacin-resistant *Campylobacter* isolated from humans and broilers

Source of isolates	Country	Prevalence[a] of strains resistant to:	Erythromycin	Ciprofloxacin		
		C. *jejuni*	C. *coli*	C. *jejuni*	C. *coli*	Reference
Humans	EU countries[b]	1.5 (0-8.7)	14.4 (0-53.5)	60.8 (42.1-96.6)	70.6 (36.5-100)	239
	u.s.	2.7	12.7	25.3	39.8	240
Broilers	Ecuador	4.2	25.9	97.9	100	95
	China	6.0	84.0	98.8	100	241
		18.8	92.0	100	100	242
	Japan	0.0	0.0	29.5	41.3	243
Broiler carcasses	China	13.0	97.0	95.7	100.0	242
Broiler meat	Belgium	0.7		53.1		244
	EU countries[b]	0.9 (0-3.7)	10.9 (0-72.7)	53.0 (20.0-75.0)	76.2 (36.4-100)	239

[a] Prevalence is reported as number of antibiotic-resistant *Campylobacter* strains/number of *Campylobacter* strains tested.

[b] Numbers in parentheses are the ranges found in European Union countries.

There is growing concern regarding the increase in resistance of *C. jejuni* to ciprofloxacin and other fluoroquinolones (141–143). Several studies have revealed that infections with fluoroquinolone-resistant *Campylobacter* in humans are associated with prolonged diarrhea (144–146). Hence, in areas of high incidence of quinolone-resistant organisms, fluoroquinolones are not recommended for treating community-acquired bacterial diarrhea, because campylobacters are often among the predominant causes (147). The use of fluoroquinolones (mainly enrofloxacin) in veterinary medicine is correlated with an increase in quinolone resistance in food animals, especially in poultry, and most importantly in human *Campylobacter* infections (142). A temporal association between the emergence of quinolone resistance and its increase in animals and humans following the introduction of enrofloxacin in animal production has been determined by several investigators (148, 149). These findings, along with risk assessment modeling, prompted the U.S. Food and Drug Administration to propose the withdrawal of fluoroquinolone use in poultry in 2000; a lengthy legal hearing

concluded with an order to withdraw enrofloxacin from use in poultry (effective in September 2005). However, it is important to note that the veterinary use of fluoroquinolones is not the only selective pressure that acts on *C. jejuni* to select for quinolone resistance. A number of studies have revealed that fluoroquinolone use in humans can lead to the emergence of quinolone-resistant campylobacters (24, 144, 145). In addition, some studies revealed significantly higher rates of quinolone resistance in travel-related *Campylobacter* infections than in domestically acquired infections (144).

Additionally, it has been revealed that the *tet*(O) gene is responsible for tetracycline resistance in *Campylobacter* isolates (150). The result of an investigation in Baltic countries indicated that resistance to tetracycline in *Campylobacter* isolates of human-origin was significantly higher than that in animal-origin isolates (151).

DETECTION OF *CAMPYLOBACTER* IN FOODS

Since it became clear that *Campylobacter* is a foodborne pathogen, methods to detect the occurrence of this bacterium in different types of samples were developed and used. Based on the fact that the risk for human infections is related to the number of ingested *Campylobacter* cells (and the number in food products), more and more attention is being given to the determination of the numbers of *Campylobacter* in samples, and direct enumeration (quantitative) methods are increasingly being used in monitoring and surveillance instead of presence/absence (qualitative) testing after enrichment.

To grow *Campylobacter* in the laboratory, preventive measures have to be taken to avoid the inhibitory effect of oxygen and its reactive derivatives. Therefore, most of the selective media used today include ingredients that quench the toxic effect of oxygen derivatives on campylobacters, such as lysed blood, charcoal, hemin, or hematin and a combination of ferrous sulfate, sodium metabisulfite, and sodium pyruvate; the generation of a microaerobic atmosphere, containing 5 to 10% carbon dioxide and 3 to 5% oxygen, for culturing is also required (85).

Enumeration Methods

For enumeration of campylobacters, direct plating on modified charcoal cefoperazone deoxycholate agar (mCCDA) is a reliable alternative to the most-probable-number method (152). In addition, Rosenquist et al. (153) determined through a collaborative study that direct plating on mCCDA is an acceptable protocol for enumeration of thermotolerant *Campylobacter* on chicken meat. Oyarzabal et al. (154) determined that mCCDA and modified Campy-Cefex agar yielded comparable results for enumerating *Campylobacter* spp. from poultry carcass rinses. mCCDA is currently the medium recommended by the International Organization for Standardization (ISO) for enumeration of *Campylobacter* spp. in the food chain (155). A disadvantage of mCCDA is the partial or total swarming of bacteria, including *Campylobacter*, making counting impossible or difficult (156, 157).

As for other pathogens, chromogenic selective agar media are available on the market, such as CampyFood agar (bioMérieux, Marcy l'Étoile, France), RAPID 'Campylobacter agar (Bio-Rad, Marnes-la-Coquette, France), Brilliance CampyCount Agar (Oxoid, Basingstoke, United Kingdom), and CHROMagar *Campylobacter* (CHROMagar, Paris, France). For broiler meat, counts obtained using CampyFood Agar and RAPID 'Campylobacter agar were similar to those obtained with mCCDA (156, 157), whereas Ahmed et al. (158) found significantly lower *Campylobacter* counts on CampyFood agar than on mCCDA for artificially contaminated samples, and they also found that the recovery rate on different media differed with *Campylobacter* species. Only RAPID'Campylobacter agar totally inhibited the growth of background microflora (157). Swarming on those two media could be prevented through a long drying period (more than 30 min) before inoculation.

Detection Methods

Detection after enrichment is required for recovery of a low number of *Campylobacter* organisms. The formulation of enrichment broth media has been modified to avoid inhibitory effects on *Campylobacter* by components in the broth formula. Corry et al. (85) reviewed the historical developments in culture-based procedures for detection of campylobacters.

The new ISO 10272-1:2017 standard (155) describes methods for the detection of *Campylobacter* not only in food and feed samples but also in the primary production stage (Fig. 10.1). This ISO method encompasses three procedures: procedure A is enrichment in Bolton broth; procedure B is enrichment in Preston broth, and procedure C is plating on mCCDA without a previous enrichment step. Enrichment in Bolton broth is the standard isolation procedure. When problems with background flora resistant to third-generation beta-lactam antibiotics are suspected, procedure B can be included. After enrichment, plating on selective agar media is carried out, followed by identification of presumptive colonies. Bolton broth is also recommended by the U.S. Food and Drug Administration for the isolation of *Campylobacter* species from food and water (159).

It is likely that no single method is ideal for the entire range of types of samples to be tested. Various studies focused on the effect of enrichment media or selective agar media on the detection efficiency of *Campylobacter* in samples collected from broiler production chain. Baylis et al. (160) compared Bolton broth, Preston broth, and *Campylobacter* enrichment broth. This study revealed that Bolton broth was the best compromise between recovery of *Campylobacter* from naturally contaminated food samples and inhibition of competitors. However, the same study also revealed that *Escherichia coli* and *Pseudomonas* spp. were frequently isolated from Bolton broth. The efficiency of detection methods is clearly influenced by the combination of enrichment broths and selective agar media used for the detection of *Campylobacter* in broiler meat samples (161). In this study, the number of positive samples increased considerably through the replacement of mCCDA by CampyFood agar as selective agar medium after enrichment in Bolton broth, while after enrichment in Preston broth, the type of selective medium had no influence on the detection rate. Moreover, this study

demonstrated that for samples containing fewer than 10 CFU/g, the use of CampyFood agar instead of mCCDA after enrichment in Bolton broth made it possible to double the number of positive samples.

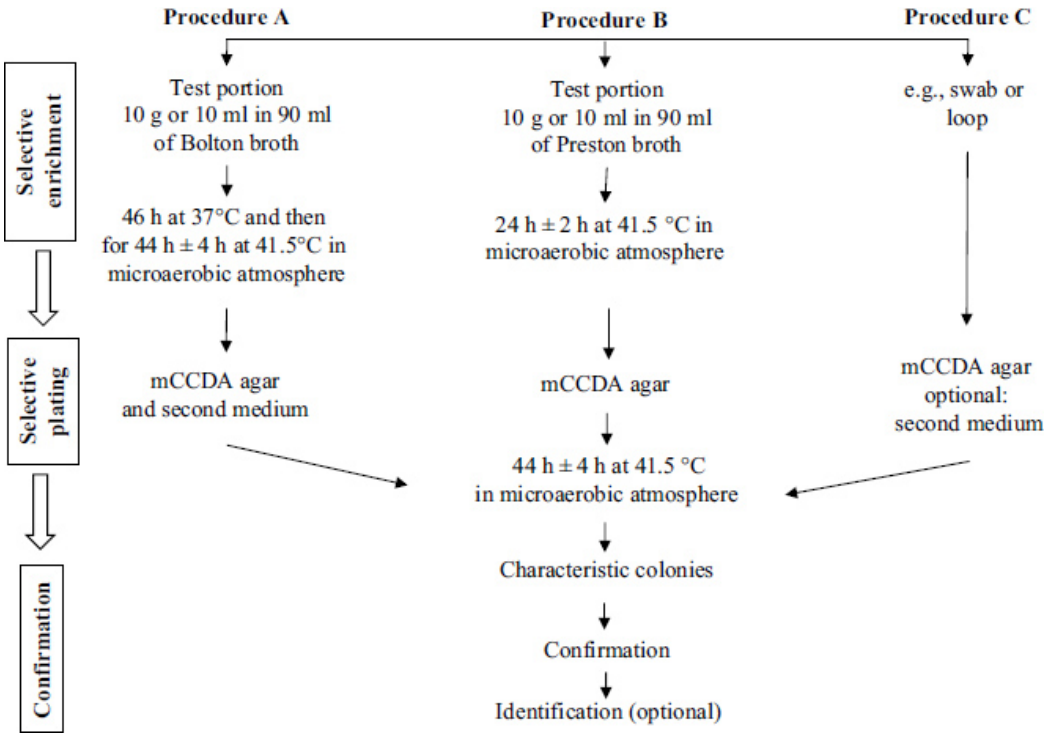


Figure 10.1 ISO 10272-2:2017 method for the detection of *Campylobacter* spp. in the food chain.

Jasson et al. (162) demonstrated that extended-spectrum-beta-lactamase-producing *E. coli* was able to grow in Bolton broth. Since the same antibiotic (sodium cefoperazone) is used in mCCDA as in Bolton broth, such *E. coli* strains may be able to crowd the mCCDA plates, resulting in the inability to discover suspected *Campylobacter* colonies on medium. Therefore, replacement of mCCDA by a selective agar medium containing other inhibitory substances is recommended. It has also been suggested to adapt the Bolton supplement so that the growth of extended-spectrum-beta-lactamase-producing *E. coli* can be prevented (163, 164). Assessment of the effect of Bolton broth supplemented with potassium clavulanate, triclosan, or polymyxin on the isolation of *Campylobacter* from poultry meat indicated that higher numbers of positive samples were obtained when the enrichment broth was supplemented with potassium clavulanate or triclosan, whereas addition of polymyxin led to an extensive reduction of the number of positive samples (165). Smith et al. (166) restored the selectivity of mCCDA successfully by the addition of tazobactam to the medium in an experimental setup.

PCR-Based Methods

In recent years, alternative methods have been developed for detection of *Campylobacter* spp. in foods. Conventional PCR is now routinely used for the detection

and identification of *Campylobacter*, and numerous methods with various levels of accuracy and sensitivity, depending on the specificity of the targeted regions of the genome, have been published (14, 16). For detection of the genus *Campylobacter*, a highly conserved region of the 16S rRNA is the target for the PCR, whereas more specific loci are used for the detection of particular *Campylobacter* species (167–169).

Real-time PCR (RT-PCR) has been developed for qualitative and quantitative detection of campylobacters in food samples, and some of these methods have been commercialized. The quantitative dimension of RT-PCR is based on correlating the number of cycles required to reach the threshold value with the initial number of campylobacters in the sample. However, the threshold limit of quantification of these RT-PCR methods ranges from 2 to 3 log CFU/g, which could result in false-negative results (170). Another drawback of PCR-based detection methods is that many research groups have developed specific protocols that work well in their own laboratories but do not allow comparison of results between laboratories using different protocols. Hence, interlaboratory proficiency tests, collaborative trials, and standardized protocols are urgently needed for these alternative methods.

In contrast to classical culture methods that detect living bacteria that can grow, genome-based methods detect DNA from live and dead bacteria. This feature might present interpretation difficulties when the method is used for analysis of food and environmental samples. It has been proposed that treating samples with propidium monoazide (PMA) prior to quantitative PCR (qPCR) could ensure the quantification of only viable cells with intact membranes. PMA can intercalate into the double-helical DNA available from dead cells with compromised membranes, and upon extensive visible light exposure, cross-linking of the two strands of DNA occurs, leaving it unavailable for PCR amplification (171). However, it has been demonstrated that the PMA-qPCR does not fully reduce the signal from dead cells, although if the remaining PMA-qPCR signal was associated with viable cells, this related to unacceptably high numbers of campylobacters being present in the initial sample (172, 173).

Optimization of molecular methods to the level at which they would enable reliable *Campylobacter* quantification reduces both the time and cost requirements, as well as facilitating *Campylobacter* surveillance throughout all stages in the food production chain (174).

CAMPYLOBACTER CONTROL IN THE FOOD CHAIN

Control of *Campylobacter* at the Farm Level

As described previously, direct and indirect epidemiologic data indicate that poultry meat is the most important source of human cases of *Campylobacter* enteritis (175). This evidence provides the justification for focusing control measures primarily along the poultry meat processing chain. Poultry-related interventions at the primary production stage have been proposed. Two types of interventions at the farm level can be identified, namely, interventions intended to avoid the entry of *Campylobacter* into

poultry houses, leading to colonization of birds, and interventions meant to decrease the number of *Campylobacter* organisms in the intestines of colonized birds (176).

A wide range of risk factors contributing to the status of flocks have been identified (177, 178). Therefore, biosecurity (e.g., hand washing, fly screens, pest-proof buildings, and foot dipping) plays an important role. However, applying biosecurity interventions at poultry production sites has resulted in variable levels of success in different countries (179, 180). Such variation may be attributed to differences in the *Campylobacter* loads in the poultry chain and environment. Hence, the effectiveness of biosecurity-related interventions in primary production should be based on a good understanding of the regional risk factors at the farm level (181).

The use of antimicrobials to eliminate *Campylobacter* infection in flocks is not acceptable, since this may stimulate the development of resistant microflora, including *Campylobacter*, in the treated birds, with possible consequences for the treatment of patients. A wide range of approaches to reduce *Campylobacter* counts in colonized birds have been screened in recent decades. A first group of approaches consists of using products known to have an antimicrobial effect against *Campylobacter in vitro*, such as organic acids, medium-chain fatty acids, herb extracts, essential oils, and probiotics (182). A large number of these studies found significant reductions compared to the control groups, but in most of those cases, the reduction was limited to a 1-log-CFU reduction of the numbers in the ceca (183–186).

The effect of bacteriophages is still variable (no reduction of more than 2 log CFU). Moreover, the development of phage resistance in *Campylobacter* can reduce the effect of the treatment (187). Passive immunization by the addition of hyperimmune egg yolk to feed could reduce the *Campylobacter* counts in ceca by about 4 log CFU/g (188). Also, vaccination experiments are still not very successful, since only a 1-to 2-log-CFU reduction is obtained in the ceca. Recently preliminary tests with vaccine candidates demonstrated a reduction between 2 and 4 log CFU (77). Additional studies are needed to find treatments which result in a significant reduction of *Campylobacter* in the ceca not only under experimental conditions but also under field conditions. It has been estimated that a 3-log-CFU reduction of *Campylobacter* in the ceca would result in a more than 90% reduction of human infections related to poultry meat consumption (189).

Control of *Campylobacter* at the Slaughterhouse Level

In most cases, carcasses from *Campylobacter*-colonized flocks become contaminated during slaughter. Colonized birds carry *Campylobacter* not only in the intestines but also on feathers and skin, sometimes in high numbers (up to 6 log CFU/g and 5 log CFU/g, respectively) (190), when entering the slaughter process. Plucking and evisceration are steps potentially leading to an increase of the contamination level of carcasses, whereas final washing and air chilling can reduce the contamination (76, 190). Skin contamination after air chilling has been linked to the numbers present in the ceca, the transport and holding time before slaughter, and the frequency of rupture of the intestines (191). Slaughtering of positive flocks leads to contamination of the slaughter equipment. When a negative flock is slaughtered after a positive one,

carcasses of the former flock become contaminated, especially at the start of slaughter, and contamination decreases over time (192).

A European Union study performed in 2008 found that 53% of the tested broiler carcasses contained at least 10 CFU/g of neck skin, and 5.8% of the carcasses contained more than 10,000 CFU/g (86). Highly contaminated samples have been associated with a higher probability of causing human illness (105, 193). In the EU risk assessment, based on the collected baseline data of 2008, it was estimated that microbiological criteria with critical limits of 1,000 and 500 CFU/g of skin would lead to reductions of 50% and 90% of the health risk caused by poultry meat (176).

Therefore, in addition to primary interventions at the farm level, there is a need to apply interventions at the processing level in order to reduce the contamination of poultry meat meant for human consumption.

Freezing of contaminated poultry carcasses is a reliable intervention to achieve a 1-to 2-log-CFU reduction of *Campylobacter* counts. Compulsory freezing of processed broilers from *Campylobacter*-positive broiler flocks in Iceland resulted in substantial reductions in the number of human cases of *Campylobacter* enteritis and is currently being used on a voluntary basis in Norway, Sweden, and Denmark (153, 194, 195). However, worldwide, many consumers prefer to buy fresh poultry meat with no change in product quality. In addition, freezing meat from all *Campylobacter*-positive broiler flocks might not be a feasible option in many countries, as it would limit the marketing of domestically produced chilled meat and increase dependence on imported product. This dilemma highlights the need to apply multiple hurdles during postharvest processing in order to achieve low counts of *Campylobacter* on chicken meat (30, 31).

Some alternative physical decontamination technologies may also achieve a reduction in *Campylobacter* numbers. For example, Corry et al. (196) determined that crust freezing of chicken carcasses (based on rapid ice crystallization on the “meat surface” that results in a thin frozen crust, followed by temperature equalization) could reduce *Campylobacter* numbers by 2 log CFU. However, Boysen and Rosenquist (197) reported that crust freezing of broiler carcasses provided only a 0.42-log-CFU reduction in *Campylobacter* counts. Hence, the application of crust freezing needs to be optimized before it is widely adopted as a *Campylobacter* intervention. Another temperature-related intervention is the application of a steamultrasound treatment. A study in Denmark revealed that this technology could reduce *Campylobacter* counts by 2.5 log CFU on broiler carcasses (197). However, Musavian et al. (198) attained a reduction of only about 1 log CFU when treating naturally contaminated broiler skin. Irradiation allows a high reduction of bacteria, including *Campylobacter*, in food. However, this treatment is not accepted by consumers all over the world. Therefore, treatment of poultry meat with this method is not an option.

Chemical decontamination can also be an effective intervention for reducing the microbial load on carcasses. However, in the European Union, decontamination of poultry carcasses and meat using chemicals is not allowed. Chlorine, chlorine dioxide, acidified sodium chlorite, trisodium phosphate, and peroxyacid are typically used in poultry processing in the United States, either as sprays or washes for online

reprocessing or as additions to the chill water tank to reduce microbial contamination and to limit the potential for microbial cross-contamination. Trisodium phosphate solutions of 8 to 12% can reduce *Campylobacter* counts on chicken carcasses by 1.0 to 2.0 log CFU (199, 200). Treatment of chicken carcasses with chlorine compounds has also been extensively studied, but with varying results, depending on the compound and treatment regimen used in the processing plant. The use of electrolyzed water, of which hypochlorous acid is the principal active antimicrobial agent, has shown some potential under experimental conditions for reducing numbers of *Campylobacter* on broiler carcasses but needs additional evaluation under processing facility conditions (106, 201).

Application of lactic acid (2.5%) was highlighted as a cost-effective intervention strategy in a Dutch risk assessment study (193). However, lower concentrations are required, as the use of 2.5% lactic acid causes a yellow discoloration of the skin of chicken carcasses. Detailed research on appropriate treatment time and temperature and the effects of the food matrix on the antimicrobial activity of chemicals is still needed. In addition, more research may be needed on the toxicological, environmental, and food-sensory effects of chemical applications to carcasses.

Results of current risk assessment models are in agreement in showing that reducing the numbers of *Campylobacter* on broiler meat is highly effective in reducing the burden of illness (193). Because *Campylobacter* enteritis is a leading foodborne bacterial infection in many parts of the world, there is a need for setting targets (e.g., process hygiene criteria) for *Campylobacter* in the broiler meat chain. In 2017, the European Commission issued a regulation fixing process hygiene criteria for *Campylobacter* on broiler carcasses after chilling, which went into effect 1 January 2018 (202). The limit is set at 1,000 CFU/g of neck skin; in a first implementation step, 20 of 50 tested samples may contain more than the stated limit, but over time, the number of samples permitted to exceed the limit will be reduced to reach 10 samples in 2025. This legislation will force the poultry production industry to take actions. Therefore, more scientific research is needed so that appropriate and feasible interventions can be applied in the field.

CONCLUSIONS

Campylobacter species, and notably *C. jejuni*, are among the most common causes of human bacterial enteric illnesses worldwide. In the past decade, and after completion of the genome-sequencing project for multiple *Campylobacter* strains, more fundamental information became available regarding the diversity and pathogenesis of these intriguing bacteria. In addition, the application of discriminatory molecular subtyping tools (e.g., MLST and PFGE) was useful in clarifying some aspects of host association and source attribution. Evidence from epidemiological studies and molecular subtyping investigations has identified poultry meat as a major vehicle for foodborne transmission of *Campylobacter* enteritis. Future research and scientific collaborations among the medical, food, and veterinary professions are needed to substantially reduce *Campylobacter* contamination in the poultry meat chain.

Research directions should focus on practical control options that would be appealing to stakeholders in the farm, slaughterhouse, and processing sectors. In addition, there are opportunities for the development of enhanced *Campylobacter* detection and quantification methods. Methods for identifying highly contaminated samples through online detection would be very useful, as this could help in identifying and excluding highly contaminated samples from the human food supply chain.

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11
Shiga Toxin-Producing *Escherichia coli*

Escherichia coli is a facultatively anaerobic, Gram-negative bacterium that is primarily present in the gastrointestinal tract of humans and warm-blooded animals. Although most commensal *E. coli* strains are harmless, many are pathogenic and cause a variety of diseases in humans and animals (1). Specific virulence attributes that have been acquired by such strains enable them to cause three principal types of infections in humans: intestinal gastroenteritis, urinary tract infections, and neonatal sepsis/meningitis.

E. coli isolates can be serologically or genetically differentiated based on three major surface antigens or their encoding genes, which enable serotyping: the O (somatic), H (flagellar), and K (capsule) antigens (2, 3). At present, more than 700 serotypes of *E. coli* have been identified based on O, H, and K antigens (4). It is considered necessary to determine only the O and the H antigens, not the K antigens, to serotype strains of *E. coli* associated with diarrheal disease. The O antigen identifies the serogroup of a strain, and the H antigen identifies its serotype. The application of serotyping to isolates associated with diarrheal disease has revealed that particular serogroups often fall into one category of pathogenic *E. coli*. However, some serogroups, such as O55, O111, O126, and O128, are in more than one category.

Pathogenic *E. coli* strains are categorized into specific groups (pathotypes) based on their virulence determinants. These virulence determinants include those controlling adhesions (CFAI/CFAII, type 1 fimbriae, P fimbriae, S fimbriae, and intimin), invasions (hemolysins, siderophores, siderophore uptake systems, and *Shigella*-like invasins), motility (flagella), toxins (heat-stable enterotoxin [ST] and heat-labile enterotoxin [LT], Shiga toxins [Stxs], cytotoxins, and endotoxins), antiphagocytic surface structures (capsules, K antigens, lipopolysaccharides), and genetic characteristics (genetic exchange through transduction or conjugation, transmissible plasmids, R factors, and drug resistance and virulence plasmids). The five categories of gastrointestinal pathogenic *E. coli* include enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and Shiga toxinproducing *E. coli* (STEC). This chapter focuses largely on the STEC group, which among the *E. coli* strains that cause foodborne illness is the most significant group based on the frequency of foodborne illness in the United States and the severity of illness. More information on other types of diarrheagenic *E. coli* is available in several review articles (5, 6).

EPEC

EPEC was the first pathotype of *E. coli* described and can cause watery diarrhea like ETEC, but these organisms do not possess the same colonization factors as ETEC and do not produce LT or ST. The major O serogroups associated with illness include O55, O86, O111ab, O119, O125ac, O126, O127, O128ab, and O142. Humans are an important reservoir. The original definition of EPEC is “diarrheagenic *E. coli* belonging to serogroups epidemiologically incriminated as pathogens but whose pathogenic mechanisms have not been proven to be

related to either enterotoxins, or *Shigella*-like invasiveness" (6). However, EPEC strains have been determined to induce attaching-and-effacing (A/E) lesions in cells to which they adhere and can invade epithelial cells. Some types of EPEC are referred to as enteroadherent *E. coli*, based on specific patterns of adherence. EPEC is an important cause of traveler's diarrhea in Mexico and in North Africa.

ETEC

ETEC is a major cause of infantile diarrhea in developing countries and regions with poor sanitation (5, 6). It is also the agent most frequently responsible for traveler's diarrhea but does not cause disease in the local adults because of developed immunity. ETEC colonizes the proximal small intestine by fimbrial colonization factors (e.g., CFAI and CFAII) and produces LT or ST, which elicits fluid accumulation and a diarrheal response. LT is similar to cholera toxin in both structure and mode of action, and ST is a peptide that causes an increase in cyclic GMP in host cell cytoplasm, leading to the same effects as those that occur with an increase in cyclic AMP. The most frequently isolated ETEC serogroups include O6, O8, O15, O20, O25, O27, O63, O78, O85, O115, O128ac, O148, O159, and O167. Humans are the principal reservoir of ETEC strains that cause human illness.

EIEC

EIEC causes nonbloody diarrhea and dysentery similar to that caused by *Shigella* spp. by invading and multiplying within colonic epithelial cells (5, 6). As with *Shigella*, the invasive capacity of EIEC is associated with the presence of a large plasmid (ca. 140 MDa) that encodes Several outer membrane proteins involved in invasiveness. The antigenicity of these outer membrane proteins and that of the O antigens of EIEC are closely related. EIEC strains do not produce LT, ST, or Stx. The principal site of bacterial localization is the colon, where EIEC invades and proliferates in epithelial cells, causing widespread cell death. Humans are a major reservoir, and the serogroups most frequently associated with illness include O28ac, O29, O112, O124, O136, O143, O144, O152, O164, and O167. Among these serogroups, O124 is the most commonly encountered.

EAEC

EAEC has recently been associated with persistent diarrhea in infants and children in several countries worldwide (5, 6). These *E. coli* strains are uniquely different from the other types of pathogenic *E. coli* because of their ability to produce a characteristic pattern of aggregative adherence on HEp-2 cells. EAEC organisms adhere to the surface of HEp-2 cells in a formation with the appearance of stacked bricks. Serogroups associated with EAEC include O3, O15, O44, O77, O86, O92, O111, and O127. A distinctive heat-stable, plasmid-encoded toxin has been isolated from these strains, called the EAST (enteroaggregative ST). EAEC also produces a hemolysin related to that produced by uropathogenic strains of *E. coli*. However, the roles of the toxin and the hemolysin in virulence have not been proven. More epidemiologic information is needed to elucidate the significance of EAEC as an agent of diarrheal disease.

Although EAEC has seldom been implicated in major foodborne disease incidents, there was a large outbreak in 2011 that was centered in Germany but affected various other countries in the European Union (7). This outbreak, suspected to have been caused by contaminated

sprouts, infected over 4,000 people, had a high hemolytic-uremic syndrome (HUS) rate (~23%), and resulted in 50 fatalities (8). The causative agent was identified as *E. coli* O104:H4 belonging to sequence type 678 (ST678), which produced Stx2a and therefore was considered a STEC strain (8–10). However, wholegenome sequencing (WGS) of the pathogen revealed that it shared 93% genomic homology with EAEC strain 55589 and also carried the *aggR* gene, which is a transcriptional activator essential for the expression of the aggregative adherence fimbriae (AAF) I and is found on an EAEC virulence plasmid. Hence, genetic analyses revealed that the causative pathogen was a multiantibioticresistant EAEC strain that had acquired the ability to produce Stx via phage conversion.

STEC

STEC was first recognized as a human pathogen in 1982, when *E. coli* O157:H7 was identified as the cause of two outbreaks of hemorrhagic colitis. Since then, many other serogroups of *E. coli*, such as O26, O111, O145, O45, O113, O121 (also known as the "big six") (11–13) and sorbitol-fermenting O157:NM, also have been associated with cases of hemorrhagic colitis and have been classified as STEC. However, serotype O157:H7 is the predominant cause of STEC-associated disease in the United States and many other countries. All STEC strains produce factors cytotoxic to African green monkey kidney (Vero) cells, factors which are hence named verotoxins or Stxs because of their similarity to the Shiga toxin produced by *Shigella dysenteriae* type 1 (14). Production of Stxs by *E. coli* O157:H7 was first reported in 1983 (15) and was subsequently associated with a severe and sometimes fatal condition, HUS (16). *E. coli* organisms of many different serotypes are capable of producing Stxs and hence are named Shiga toxin-producing *E. coli* (STEC). More than 600 serotypes of STEC have been identified, including approximately 160 O serogroups and 50 H types, and the list is still increasing (17). However, only strains that cause hemorrhagic colitis are considered enterohemorrhagic *E. coli* (EHEC), and at least 130 EHEC serotypes have been recovered from human patients.

Major non-O157 EHEC serogroups identified in the United States include O26, O45, O103, O111, O121, and O145 (18). In 2011, the U.S. Department of Agriculture Food Safety and Inspection Service (USDA FSIS) declared O26 and these five non-O157 STEC serogroups adulterants in ground beef and nonintact beef products, beginning testing for these pathogens in June 2012 in domestic and imported beef trimmings (19). From 2010 to 2012, the incidence rate of human STECs in the United States, as reported by the CDC, showed an increasing trend, with fewer O157 and more non-O157 STEC strains (https://www.cdc.gov/nationalsurveillance/pdfs/STEC-2014-REPORT_508c.pdf). Even though the total incidence of human STEC infections in the United States from 2012 to 2014 has decreased, with O157 cases trending down, cases caused by non-O157 strains are increasing, reinforcing the importance of these pathogens. The annual report of human STEC infections published by CDC for 2014 identifies the 10 most frequently culture-confirmed human STEC serogroups causing infections in the United States as O157 (43.2%), O26 (16.3%), O103 (14.2%), O121 (3.7%), O45 (2.6%), O145 (2.5%), O186 (0.9%), and O69 (0.4%) (https://www.cdc.gov/nationalsurveillance/pdfs/STEC-2014-REPORT_508c.pdf).

CHARACTERISTICS OF *E. COLI* O157:H7 AND NON-O157 STEC

E. coli O157:H7 was first identified as a foodborne pathogen in 1982. There had been prior isolation of the organism, which was identified retrospectively among isolates at the CDC; the

isolate was obtained from a California woman with bloody diarrhea in 1975 (20). In addition to production of Stxs, most strains of *E. coli* O157:H7 also possess several characteristics uncommon to most other *E. coli* strains: inability to grow well at temperatures of ≥44.5°C in *E. coli* broth, inability to ferment sorbitol within 24 hours, inability to produce β-glucuronidase (i.e., inability to hydrolyze 4-methylumbelliferyl-D-glucuronide [MUG]), possession of a pathogenicity island known as the locus of enterocyte effacement (LEE), and carriage of a 60-MDa (92-kbp) plasmid. Non-O157 STEC strains do not share the growth and metabolic characteristics described above, although they allproduce Stxs and many contain LEE and the large plasmid. EHEC can produce pediatric diarrhea, copious bloody discharge, i.e., hemorrhagic colitis, and intense inflammatory response, and the illness may be complicated by HUS.

There is tremendous genetic diversity among STEC isolates. O157 STEC infections are more likely than non-O157 STEC infections to result in bloody diarrhea (80% versus 45%), hospitalization (34% versus 8%), and HUS (6% versus <2%) (21). Of the cases of non-O157 STEC illness reported by the CDC in 2014 in the United States, 96% were represented by just six serotypes, including O26 (31%), O103 (27%), O111 (21%), O121 (7%), O145 (5%), and O45 (5%) (https://www.cdc.gov/nationalsurveillance/pdfs/STEC-2014-REPORT_508c.pdf). At present, *E. coli* O157:H7 is the dominant STEC isolate in the United States (43% of reported human STEC infections), Canada, the United Kingdom, and Japan, and non-O157 STEC strains are dominant among isolates in Europe, Argentina, Australia, Chile, and South Africa.

Acid Resistance

Foodborne pathogens must pass through an acidic gastric barrier with pH values as low as 1.5 to 2.5 to cause infections in humans. Some enteric pathogens, such as *Vibrio cholerae*, use an "assault tactic" that involves large numbers of infecting cells, in the hope that a few will survive and gain entrance into the intestine. *E. coli* O157:H7, however, has effective mechanisms for tolerating extreme acid stress. Three systems in STEC are involved in acid resistance, including an acid-induced oxidative system, an acid-induced arginine-dependent system, and a glutamate-dependent system (22). The oxidative system is less effective in protecting the organism from acid stress than the arginine-dependent and glutamate-dependent systems. The alternate sigma factor RpoS is required for oxidative acid tolerance but is only partially involved with the other two systems. Once induced, the acid resistance state can persist for a prolonged time (ca. 28 days) at refrigeration temperature. More detailed information on acid resistance can be found in a review article by Foster (23).

The minimum pH for *E. coli* O157:H7 growth is 4.0 to 4.5, but growth is dependent upon the interaction of pH with other factors. Studies on inactivation of *E. coli* O157:H7 with organic acid sprays on beef using acetic, citric, or lactic acid at concentrations of up to 1.5% revealed that *E. coli* O157:H7 populations were not appreciably affected by any of the treatments (24). *E. coli* O157:H7, when inoculated at high populations, survived fermentation, drying, and storage in fermented sausage (pH 4.5) for up to 2 months at 4°C (25), in mayonnaise (pH 3.6 to 3.9) for 5 to 7 weeks at 5°C and for 1 to 3 weeks at 20°C (26), and in apple cider (pH 3.6 to 4.0) for 10 to 31 days or 2 to 3 days at 8 or 25°C (27). Induction of acid resistance in *E. coli* O157:H7 also can increase tolerance to other environmental stresses, such as heat, radiation, and some antimicrobials.

Studies (28) have compared the survival characteristics of *E. coli* O157:H7 and other STEC types (O26:H11 and O111:NM) in chocolate and confectionery products during storage at different temperatures. Results revealed that all three serotypes survived storage at 38°C for

up to 43 days, but after 90 days, only *E. coli* O26:H11 and O111 were recovered. However, *E. coli* O157:H7 was recovered after O26 and O111 were no longer detected when a similar study was conducted with biscuit cream and mallow. The determination of the desiccation tolerance with 15 strains of *E. coli* O157:H7, 15 strains of *E. coli* O26:H11, and 5 strains of *E. coli* O111:NM revealed that all of them survived on paper disks after 24 h of drying at 35°C, showing no difference among serotypes (29).

Antimicrobial Resistance

Initially, when *E. coli* O157:H7 was first associated with human illness, the pathogen was susceptible to most antimicrobials affecting Gram-negative bacteria (30). Several studies revealed a trend toward increasing resistance to antimicrobials among *E. coli* O157:H7 isolates (31, 32). Overall, antimicrobial resistance among *E. coli* O157:H7 clinical isolates is low compared to that of other enteric pathogens and had no significant change from 2005 to 2014 (https://www.cdc.gov/narms/pdf/2014-Annual-Report-narms-508c.pdf). However, resistance to clinically important antimicrobials has been reported; in 2014, 5.1% (9/155) of *E. coli* O157 clinical isolates were resistant to streptomycin, 1.9% (3/155) were resistant to ampicillin, 5.81% (9/155) were resistant to nalidixic acid, 7.1% (11/155) were resistant to sulfisoxazole, 7.1% (11/155) were resistant to tetracycline, and two (1.3%) were resistant to trimethoprim-sulfamethoxazole. Some *E. coli* O157:H7 strains isolated from humans, animals, and food have developed resistance to multiple antimicrobials, with streptomycin-sulfisoxazole-tetracycline being the most common resistance profile. Approximately 6% (9/155) of the *E. coli* O157 clinical isolates in 2014 were resistant to three or more classes. Non-O157 STEC strains isolated from humans and animals also have acquired antimicrobial resistance, and some are resistant to multiple antimicrobials commonly used in human and veterinary medicine (33). However, antimicrobial resistance among STEC strains was low compared to that of non-STEC *E. coli* strains (34). The recent emergence of the *mcr-1* gene—a plasmid-borne gene that can make a bacterium resistant to colistin (a last-resort antimicrobial against multidrug-resistant bacteria)—has raised concerns about the ability of such genes to easily spread to other bacteria that are already multidrug resistant, which is a persistent threat to public health worldwide. Fortunately, the *mcr-1* gene has not yet been detected in STEC strains, although it has been found in many non-STEC strains (35–37).

Inactivation by Heat and Irradiation

Studies on the thermal sensitivity of *E. coli* O157:H7 in ground beef revealed that the pathogen has no unusual resistance to heat, with *D* values at 57.2, 60, 62.8, and 64.3°C of 270, 45, 24, and 9.6 seconds, respectively (38). Heating ground beef sufficiently to kill typical strains of *Salmonella* will also kill *E. coli* O157:H7 (Table 11.1). The presence of fat protects *E. coli* O157: H7 in ground beef, with *D* values for lean (2.0% fat) and fatty (30.5% fat) ground beef of 4.1 and 5.3 min, respectively, at 57.2°C and 0.3 and 0.5 min, respectively, at 62.8°C (39). Pasteurization of milk (at 72°C for 16.2 s) is an effective treatment that kills more than 10^4 *E. coli* O157:H7 cells per ml (40). Proper heating of foods of animal origin, e.g., heating foods to an internal temperature of at least 68.3°C for several seconds, is an important critical control point to ensure inactivation of *E. coli* O157:H7.

The use of irradiation to eliminate foodborne pathogens in food has been approved by many countries. Unlike many other processing technologies, irradiation at dosages that kill enteric foodborne pathogens still maintains the raw character of foods. In the United States, an irradiation dose of 4.5 kGy is approved for refrigerated and 7.5 kGy for frozen, raw ground

beef. D_{10} values for *E. coli* O157:H7 in raw ground beef patties range from 0.241 to 0.307 kGy, depending on temperature, with D_{10} values being significantly higher for patties irradiated at −16°C than at 4°C (41). Hence, an irradiation dose of 1.5 kGy should be sufficient to eliminate *E. coli* O157:H7 at the cell numbers likely to occur in ground beef. At present, there is no reason to believe that current interventions used in foods for mitigating *Salmonella* and *E. coli* O157:H7 contamination would not be effective against non-O157 STEC.

Table 11.1 Comparison of *D* values for *E. coli* O157:H7 and *Salmonella* spp. in ground beef and *Salmonella* spp. in ground beef

		D value (min)	
	E. coli O157:H7		
Temp (°C)	30.5% fat	17–20% fat	*Salmonella* spp.
51.7	115.5	ND[a]	54.3
57.2	5.3	4.5	5.43
62.8	0.47	0.40	0.54

[a] ND, not determined.

WGS OF STEC

Thanks to the recent decrease in the cost of sequencing, the instigation of numerous sequencing initiatives (GenomeTrakr at CFSAN FDA [https://www.fda.gov/food/foodscienceresearch/wholegenomesequencingprogramwgs/ucm363134.htm], global microbial identifier consortium-Global Effort [http://www.globalmicrobialidentifier.org/]; 100K genomes at UC Davis [42]), and the availability of quicker and benchtop sequencing platforms, such as Ion Torrent (Thermo Scientific) and MiSeq (Illumina), the number of sequenced STEC genomes has increased substantially. Overall, more than 59,000 genomes of *E. coli* and *Shigella* have been sequenced as of November 2017. There are two freely available databases that accumulate all *E. coli* genome data that are being released to the public: Pathogen Detection (https://www.ncbi.nlm.nih.gov/pathogens/) and EnteroBase (http://enterobase.warwick.ac.uk/species/index/ecoli) (Fig. 11.1A and B). These databases provide different tools for analysis of the genomes. The EnteroBase website also contains historical data for >77,000 *E. coli* and *Shigella* strains and hosts the most-used multilocus sequence typing (MLST) scheme database for *E. coli*. The number of STEC genomes available in the NCBI and EnteroBase databases will continue to increase, and these resources can be used for source tracking, detection of antimicrobial resistance genes, epidemiology, and determining evolution of different groups of strains or phylogroups. The availability of this wealth of data had resulted in a more comprehensive approach to comparative and functional genomics of STECs. Evidence of the use of these data is that researchers have built a data repository for the genes that cause pathogenicity in *E. coli* (http://www.mgc.ac.cn/cgi-bin/VFs/compvfs.cgi?Genus=Escherichia), and it has been used by open-source genomic web tools to perform *in silico* searches for the presence of virulence genes, serotype, antimicrobial resistance genes, and sequence type from *E. coli* draft genomes (https://cge.cbs.dtu.dk/services) (Fig. 11.1C). The combination of all these results can be used for predicting the virulence potential of *E. coli* strains and for fast genotyping of their genomes. When this tool was used together with phylogenetic analysis, it was shown that an

EPEC O26:H11 strain was also able to acquire an *stx*-carrying phage and that certain strains believed to be STEC O26:H11 were indeed EPEC strains (43).

Comparative Genomics of EHEC

Comparative analysis showed that the chromosome of *E. coli* O157:H7 consists of 4.1-Mb backbone sequences shared by *E. coli* K-12 (commensal strain) and 1.4-Mb O157-specific sequences encoding many virulence determinants, such as Stx genes (*stx*) and the LEE (44, 45). This comparative analysis revealed considerable genomic plasticity, since *E. coli* O157:H7 EDL933 contained 1,000 more genes than *E. coli* K-12. Besides differences in the sizes of their chromosomes, O157 EHEC strains carry other plasmids, with the most important being the EHEC virulence plasmid pO157 (46). This EHEC plasmid is described in more detail below. Genomic comparison between EHEC strains of serotypes O26, O111, and O103 reveals that, similar to O157:H7, EHECs have even larger genomes (5.37 Mb for O111:H$^-$ strain 11128 and 5.69 Mb for O26:H11 strain 11368) than *E. coli* K-12 (4.6 Mb) and contain a large number of mobile elements, such as prophages and integrative elements. However, the chromosomal backbone regions are highly conserved as well among non-O157 EHEC strains of O26, O111, and O103 (44). EHECs are divided into two main lineages: EHEC lineage 1 contains O157:H7/– (e.g., Sakai and EDL933) and O145:H28 (e.g., RM13514 and RM13516), whereas EHEC lineage 2 contains O26 (e.g., 11368), O111 (e.g., 11128), and O103 (e.g., 12009) (46, 47). The EHEC-like virulence plasmids in the EHEC lineage 2 strains are very different from pO157, with variation in gene content and organization, indicative of a different and independent evolutionary history for each individual plasmid. The availability of genomic information from many of these strains has enabled the construction of phylogenies, which showed that that some strains unrelated to either EHEC lineage carry *stx* genes and have the potential to become EHEC strains, revealing the plasticity of the *E. coli* genome (Fig. 11.2).

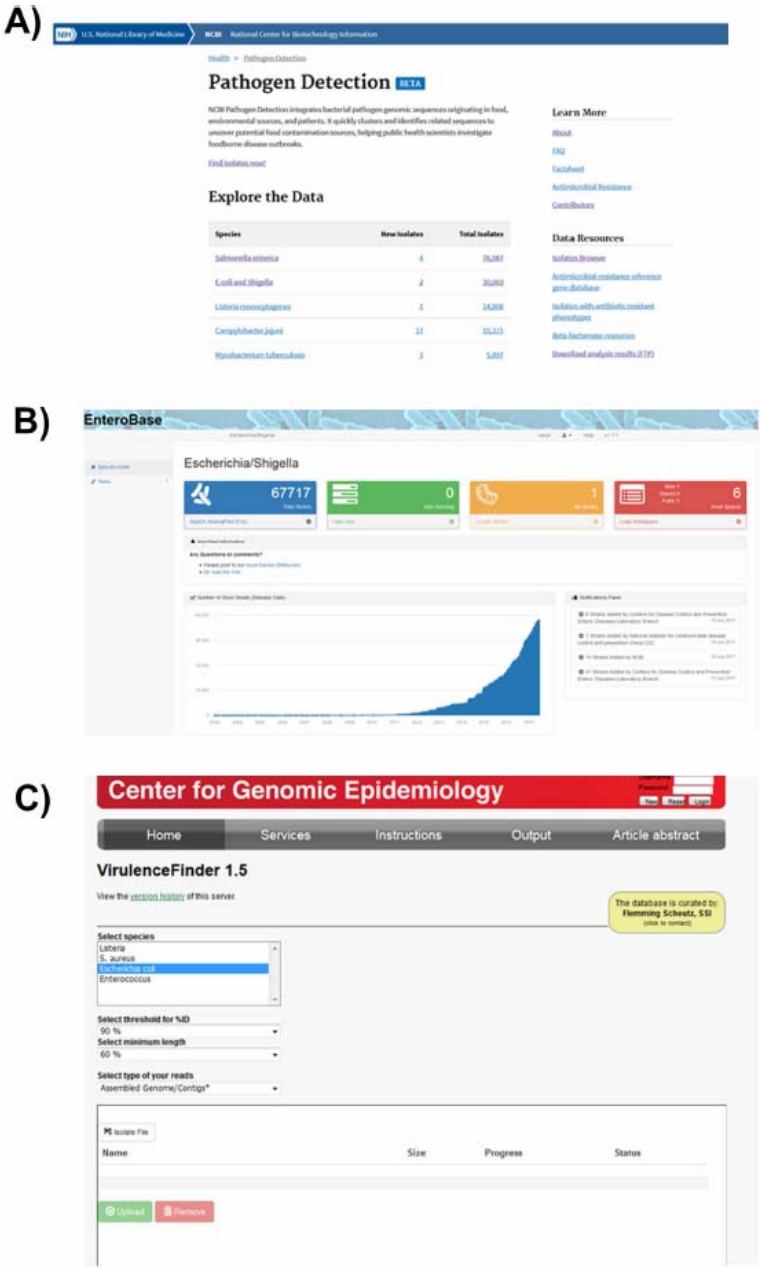


Figure 11.1 Snapshot of the two freely available databases containing all genomes as well as historical metadata (MLST) available for *E. coli* and *Shigella* and the web tool for *in silico* determination of virulence genes, serotyping, and MLST for *E. coli*. (A) NCBI pathogen detection; (B) EnteroBase; (C) DTU web tool.

Virulence genes, especially those for non-LEE effectors and nonfimbrial adhesins, are well conserved in non-O157 EHEC, in addition to the *stx* genes and LEE island. They have great similarity to their whole gene repertoire and share many genes that are specific to EHEC or rarely present in other pathotypes (45). These genes are directly or indirectly related to virulence, thus conferring a similar virulence potential among EHEC strains.

It is noteworthy that, despite carrying the same or similar virulence genes, mobile elements that are commonly present in EHEC (multiple lambdoid prophages, several types of

integrative elements, and virulence plasmids) have remarkably divergent genomic structures. This property suggests that EHEC strains have complex and independent evolutionary pathways and that mobile elements are the primary driving force for the parallel evolution of EHEC (45). However, the availability of thousands of genomic sequences has enabled better comparisons and the ability to determine the true phylogenetic history of strains that were considered to be atypical EHEC strains, such as O26:H11 (43) and O157:H7 (48). In both cases, it was revealed that these atypical strains either had lost their *stx* phage or had not yet acquired the phage. Comparative genomics have also revealed recently that an O111:H strain that caused an HUS outbreak in Australia in 1995 contained two copies of the stx_2 phage, explaining why the infections caused by these strains were more severe than those caused by an earlier clone of the same strain that contained only one copy of the stx_2 phage (21). A more detailed summary of the use of genomic information available for STEC analysis, determination of evolution, and control in global production systems can be found in a review article by Franz et al. (49).

RESERVOIRS OF *E. COLI* O157:H7 AND NON-O157 STECS

Cattle

Initially, foods of bovine origin (notably, undercooked ground beef, and, less often, unpasteurized milk) were the vehicles most frequently associated with outbreaks of *E. coli* O157:H7 infection (20). Subsequently, cattle were identified as important sources, or reservoirs, of this pathogen. Since that time, many studies have focused on their role as a reservoir of *E. coli* O157:H7. A number of other vehicles have since been implicated in *E. coli* O157 infections, including fresh produce, contaminated water, and direct contact with animals or their environment at livestock exhibitions and by petting (50, 51). The sources and reservoirs of non-O157 STEC infections are not as clearly defined. Unlike *E. coli* O157 illness, most major outbreaks of illness caused by non-O157 STEC have not been directly associated with beef products. Instead, outbreaks have been traced to a wider variety of sources, including vegetables, water, and unpasteurized milk (52). Given that non-O157 STEC also colonizes live cattle, it is probable that cattle play an important role in the epidemiology of human non-O157 STEC infection as well. However, epidemiologic data to pinpoint the primary routes of human exposure are lacking. Recently, meat or meat products have been implicated in human outbreaks (53–55). As isolation and detection methods for non-O157 STEC are improved, the understanding of the vehicles and routes of transmission of non-O157 STEC should also improve.

Detection of *E. coli* O157:H7 and STEC on Farms

The first reported isolation of *E. coli* O157:H7 from cattle was from a <3-week-old calf with colibacillosis in Argentina in 1977 (56). However, this presentation was atypical, as the clonal genotypic group of *E. coli* O157 most frequently associated with human disease rarely causes bovine illness. Instead, most cattle harbor these bacteria without outward signs of illness or loss of productivity (57). The prevalence of fecal excretion of *E. coli* O157:H7 varies by age, with higher prevalence values being reported for younger animals (2 to 24 months of age) than adults in field studies. The reasons for agerelated differences are unclear, but they may be due to ruminal development differences, differences in microbial flora in the gastrointestinal tract, or management differences, such as dietary factors (58). Nevertheless, older animals, including those at the time of harvest and during lactation, may also shed these

bacteria in their feces asymptomatically. The presence of more than one strain of *E. coli* O157:H7 on a single farm on a single sample date has been described (59).

Shedding of *E. coli* O157 at the individual-animal level typically lasts for a few days to several weeks following exposure (60). However, there is an association with the excretion of larger numbers of bacteria and for longer periods of time if the bacteria are intimately attached to the intestinal mucosa, a phenomenon that occurs predominantly, if not exclusively, at the rectoanal junction in cattle (61). Cattle may excrete *E. coli* O157 in the feces at cell numbers that are so low as to be detectable only through sensitive enrichment culture methods or are as high as 10^6 CFU/g. Cattle shedding high numbers of *E. coli* O157 (>10^3 CFU/g) can contribute substantially to contamination of carcasses at harvest, contamination of the environment, and cattle-to-cattle transmission and are considered to be "supershedding" (62, 63). The factors that govern bovine supershedding of *E. coli* O157 (and whether the phenomenon occurs for non-O157 STEC) are currently poorly understood and are under investigation.

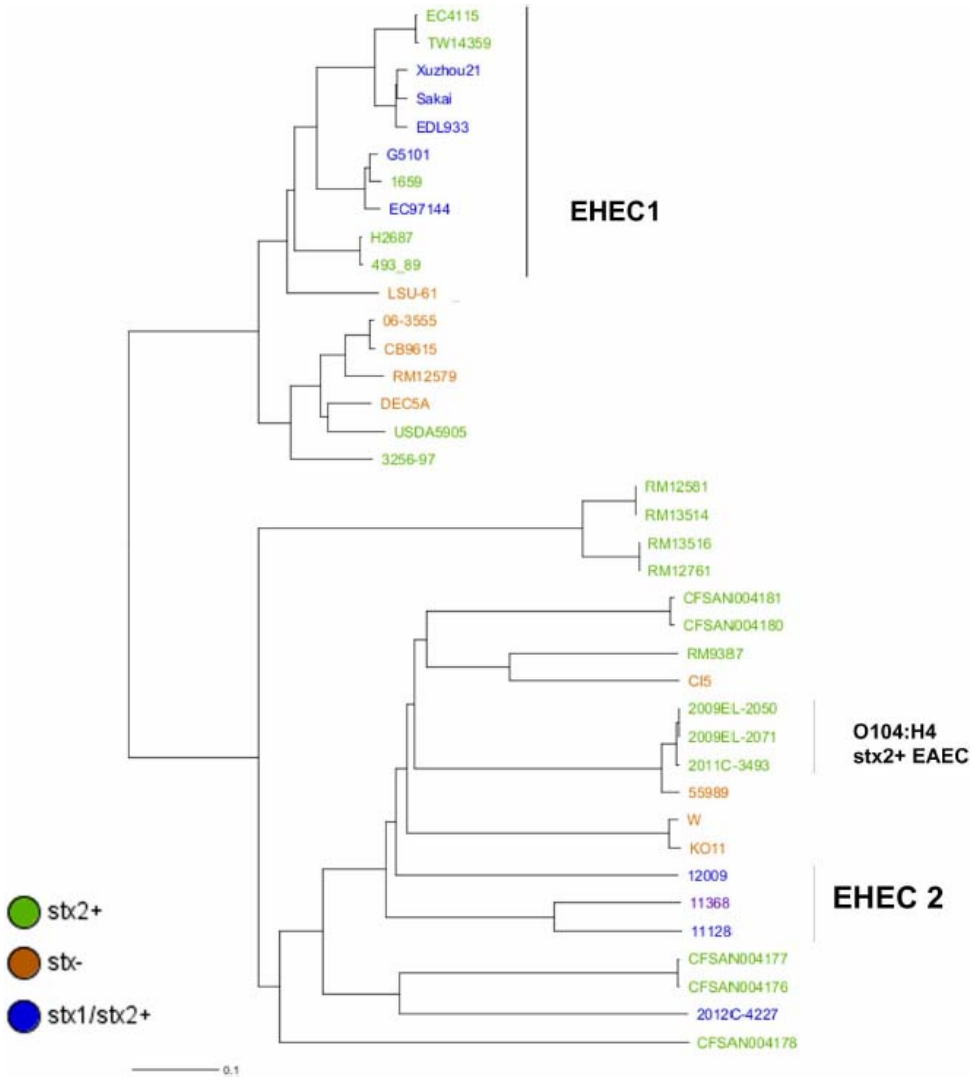


Figure 11.2 Neighbor-joining phylogenetic tree of 59 *E. coli* genomes available at NCBI using a custom-made core genome MLST analysis showing *stx* distribution on those genomes. The species-based phylogeny was inferred using 820 conserved core genome loci. The two EHEC lineages are shown as well as strains belonging to the German outbreak of *E. coli* O104:H4 in 2011 (stx_2 EAEC). The colors represent the presence and type of *stx* gene.

At the herd level, most bovine populations are positive for *E. coli* O157 and non-O157 STEC at some time or another (64). Prevalence, however, is variable, and peaks in prevalence are sporadic and currently unpredictable. Fecal excretion of *E. coli* O157:H7 by cattle occurs in a seasonal pattern, with higher prevalence occurring in the summer or early fall, which coincides with the seasonal variation in disease incidence seen in humans, where higher rates are also observed during the summer months. In nine herds sampled for approximately 1 year, the prevalence of *E. coli* O157:H7 during June through October was several times that observed in December through March. Observed seasonal effects of *E. coli* O157:H7 excretion in cattle could be due to confounding factors, such as differences in the microbial flora of the gastrointestinal tract in cattle during the summer and during the winter months, changes in diet, or the presence of conditions conducive to multiplication of the bacteria in

environmental niches. Non-O157 STEC strains are suspected to follow a similar seasonal pattern of colonization in cattle, but this has not been extensively documented (65).

Herd prevalence rates of STEC fluctuate between 0 and 100% (66). Although total STEC prevalence in a herd may average 60 to 70%, the fraction of these strains that actually pose a threat to public health is undetermined. Many STEC isolates from cattle carry only the *stx* gene and no ancillary virulence genes typically found in cases of human disease (67, 68). A study on STEC carriage by dairy cattle on farms in Canada revealed that 36% of cows and 57% of calves were STEC positive in all of the 80 herds tested (69). Of these, only seven animals (0.45%) on four farms (5%) were positive for *E. coli* O157:H7. A 2002 USDA national study revealed that 38.5% of dairy farms had at least one *E. coli* O157:H7-positive cow and that 4.3% of individual cows were *E. coli* O157 positive.

Factors Associated with Bovine Carriage of *E. coli* O157:H7

E. coli O157:H7 has been isolated from cattle feces from most regions of the world in which studies have been conducted (64). There are, however, some regions, such as Scandinavia, Africa, and Norway, which report lower prevalence rates than others. This may be due to climate factors or farm management practices that are less conducive to cattle's being colonized with *E. coli* O157:H7.

Domestic Animals and Wildlife

Although cattle contribute significantly to STEC contamination of the food chain, either directly through meat or milk or indirectly through contamination of water and the food production environment, STEC is also frequently isolated from many other domestic and wild ruminants, such as sheep, goats, deer, and water buffalo (57, 70). In addition, *E. coli* O157 and other non-O157 STEC types can colonize a number of other animals, including dogs, horses, swine, wild birds, and rodents (71), albeit less commonly than occurs in ruminants. Outbreaks of foodborne STEC infections have been associated with food products derived from sheep, goats, and deer. Moreover, nonruminant species may play a role in transmission of STEC between cattle farms and contamination of the environment (and crops) and may constitute a source of direct transmission. For a list of animal hosts of STECs, see the review article by Persad and LeJeune (72).

Possibility of Control of STEC in Food Animals

Despite 25 years of research on the topic and their potential for enhancing food safety, very few effective control measures for STEC in live animals have been identified. Several tentative associations between fecal shedding of *E. coli* O157:H7 and feed or environmental factors have been made from epidemiologic studies of dairy herds. For example, some calf starter feed regimens or environmental factors and feed components, such as whole cottonseed, were associated with reduced prevalence of *E. coli* O157:H7. Feeding of distillers' grains or barley can increase *E. coli* O157 shedding compared to that observed with corn-fed cattle, but the mechanisms driving these differences are unknown (73, 74).

Likewise, grouping calves before weaning is associated with increased carriage of *E. coli* O157:H7 (75). Given that *E. coli* O157 can survive for several months to years in environmental niches on the farm, food and water hygiene and manure handling have been researched (76). Several studies have revealed that *E. coli* O157:H7 can survive for weeks and months in bovine feces and water (66). The pathogen was frequently isolated from water troughs on farms. Commercial feeds often contained detectable *E. coli*, indicating widespread

fecal contamination, although *E. coli* O157:H7 was only infrequently detected (77, 78). Despite the gaps in understanding the factors influencing *E. coli* O157 carriage in cattle, preharvest interventions have been applied to live cattle with mixed results (79). Feeding of specific probiotic bacteria has repeatedly resulted in decreased prevalence of *E. coli* O157 in feedlot cattle (80, 81). Likewise, the administration of sodium chlorate in the feed or water may provide a control method immediately prior to harvest (80). Vaccination of cattle to reduce *E. coli* O157 colonization has also been studied (81, 82), with some promising results, but sufficient data demonstrating the efficacy of current vaccines are lacking. Other possible control measures include bacteriophage therapy and washing the hides of cattle. Achieving a better understanding of the factors influencing the exposure and colonization of cattle with *E. coli* O157 and other STEC will enhance the development of novel control strategies.

Many interventions have also been applied at the time of harvest to mitigate the potential negative impacts of cattle entering beef-processing facilities carrying STEC. These include strict attention to slaughter and processing hygiene, as well as postharvest interventions, such as surface steam pasteurization and application of acid rinses on carcasses (79). Although preliminary studies clearly identified a direct correlation between the prevalence of *E. coli* O157:H7 in the live animal and rates of contamination of carcasses, modern processing techniques can reduce and mitigate the impacts of carriage of *E. coli* O157 and other STEC types among animals presented for harvest (83). Presently, it is thought that most animals can be processed safely, and it is only when the prevalence is extremely high or the magnitude of shedding is large (supershedding cattle) that these factors overwhelm the current system and contamination persists on carcasses or in products.

Humans

Fecal shedding of *E. coli* O157:H7 by patients with hemorrhagic colitis or HUS usually lasts for no more than 21 days following the onset of symptoms (84). However, in some instances, the pathogen can be excreted in feces for many weeks. A child infected during a day care center-associated outbreak continued to excrete the pathogen for 62 days after the onset of diarrhea (85). In the 2011 outbreak of *E. coli* O104:H4, adults shed the microorganism for up to 90 days (86). Studies of persons living on dairy farms in Canada, aiming to determine carriage of *E. coli* O157:H7 by farm families, revealed elevated titers of antibody against the surface antigens of *E. coli* O157, and *stx*-positive strains were isolated from feces from 6.3% of the persons tested on 80 farms (87). Some of the *stx*-positive strains were O157:H7, whereas the rest belonged to eight other serotypes (87). The prevalence of STECs in employees working in slaughterhouses or dairy farms appears to be influenced by country and seasons (88–90). An asymptomatic long-term carrier state has not been identified. The significance of fecal carriage of *E. coli* O157:H7 by humans is the potential for person-to-person dissemination of the pathogen, a situation which has been observed repeatedly in outbreak settings. A factor contributing to person-to-person transmission of the pathogen is its extraordinarily low infectious dose; it is estimated at <100 cells, and it is possible that as few as 10 cells can produce illness in highly susceptible populations (91). Inadequate attention to personal hygiene, especially after using the restroom, can result in transfer of the pathogen to other persons through contaminated hands, resulting in secondary transmission.

CHARACTERISTICS OF DISEASE

The spectrum of human illness of STEC infection includes mild nonbloody diarrhea and life-threatening conditions, such as hemorrhagic colitis and HUS. The occurrence of these

conditions is relatively low in the United States (92); however, they are medically and epidemiologically actionable (93). Some persons may be infected but asymptomatic, but typically for a short time (<3 weeks). Ingestion of the bacteria is followed typically by a 3-to 4-day incubation period (range, 2 to 8 days), during which colonization of the large bowel occurs. Illness typically begins with severe abdominal cramps and nonbloody diarrhea for 1 to 2 days, which then progresses in the second or third day of illness to bloody diarrhea that lasts for 4 to 10 days (94, 95). Many outbreak investigations revealed that more than 80% of patients with microbiologically confirmed cases of diarrhea caused by *E. coli* O157:H7 had frank blood in the stools, but in some outbreaks, there have been reports of 30% of cases having nonbloody diarrhea. Symptoms usually resolve after a week, but about 6% of patients progress to HUS, one-half of whom require dialysis and 75% of whom require transfusions of erythrocytes and/or platelets. The case fatality rate from *E. coli* O157:H7 infection is about 1%. Similar but less severe symptoms have been observed in infections with non-O157 STEC: only 45% of patients develop bloody diarrhea, and fewer than 2% progress to HUS.

HUS largely affects children, among whom it is the leading cause of acute renal failure (96). The risk that a child younger than 10 years of age with a diagnosed *E. coli* O157:H7 infection will develop HUS is about 15% (95). The syndrome is characterized by a triad of features: acute renal insufficiency, microangiopathic hemolytic anemia, and thrombocytopenia. Significant pathological changes include swelling of endothelial cells, widened subendothelial regions, and hypertrophied mesangial cells between glomerular capillaries. These changes combine to narrow the lumina of the glomerular capillaries and afferent arterioles and result in thrombosis of the arteriolar and glomerular microcirculation. Complete obstruction of renal microvessels can produce glomerular and tubular necrosis, with an increased probability of subsequent hypertension or renal failure.

INFECTIOUS DOSE

Retrospective analyses of foods associated with outbreaks of EHEC infection revealed that the infectious dose is very low. For example, analyses of frozen ground beef patties associated with a 1993 multistate outbreak caused by *E. coli* O157:H7 in the western United States suggested that the infectious dose was below 700 organisms (97). Another study in 1994 revealed that 2 to 45 bacteria was the probable infectious dose for persons who consumed dry fermented salami contaminated with *E. coli* O157:H7 (98). These data suggest that the infectious dose of *E. coli* O157:H7 may be fewer than 100 cells. Additional evidence for a low infectious dose is the capability for person-to-person and waterborne transmission of EHEC infection.

DISEASE OUTBREAKS

Geographic Distribution

E. coli O157:H7 has been the cause of many major outbreaks of severe illness worldwide. At least 30 countries on six continents have reported *E. coli* O157:H7 infection in humans. In the United States, 107 outbreaks of *E. coli* O157:H7 infection associated with food were documented between 2011 and 2015 (Table 11.2). These outbreaks contributed to 1,460 illnesses during this time. More importantly, most cases of STEC infections occur as sporadic cases. Over the last 5 years, the number of actual cases reported each year in the United States

averaged about 4,000 (Fig. 11.3) (https://www.cdc.gov/foodsafety/outbreaks/multistate-outbreaks/outbreaks-list.html).

The precise incidence of foodborne *E. coli* O157:H7 illness in the United States is not known, because infected persons experiencing mild or no symptoms and persons with nonbloody diarrhea are less likely to seek medical attention than patients with bloody diarrhea; hence, such cases would not be reported. The CDC reports that the annual average numbers of laboratory-confirmed cases of *E. coli* O157:H7 and non-O157 infections are 3,704 and 1,579, respectively (99). It also estimates that *E. coli* O157:H7 causes 63,153 illnesses each year in the United States, and non-O157 STEC accounts for an additional 112,752 cases. Sixty-eight percent of O157 cases and 82% of non-O157 cases are attributed to foodborne transmission.

Large outbreaks of *E. coli* O157:H7 infections involving hundreds of cases also have been reported in Canada, Japan, and the United Kingdom. The largest outbreak occurred in May to December 1996 in Japan, involving more than 9,000 reported cases (100). In the same year, 21 elderly people died in a large outbreak involving 501 cases in central Scotland (101). Although *E. coli* O157:H7 is still the predominant serotype of STEC in the United States, Canada, the United Kingdom, and Japan, an increasing number of outbreaks and sporadic cases related to STEC of serotypes other than O157:H7 have been reported. In continental Europe, Australia, and Latin America, non-O157 STEC infections are more common than *E. coli* O157:H7 infections. Details of many reported foodborne and waterborne outbreaks of STEC infections are provided in Table 11.3. There are no distinguishing biochemical phenotypes for non-O157 STEC, making screening for these bacteria problematic and laborintensive, and for this reason, only a limited number of clinical laboratories test for it. Therefore, the prevalence of non-O157 STEC infections may be underestimated.

Table 11.2 Vehicles of foodborne outbreaks and associated cases of *E. coli* O157 infections in the United States between 2011 and 2015[a]

Transmission route	No. of outbreaks	% of total outbreaks	No. of cases associated with outbreaks	% of total cases
Ground beef	14	13.08	121	8.29
Unknown food vehicle	33	30.84	322	22.05
Produce	26	24.30	560	38.36
Other beef	2	1.87	6	0.41
Chicken	4	3.74	134	9.18
Dairy product	11	10.28	105	7.19
Other food vehicles: pizza, pork, apple cider, deli meat, etc.	17	15.89	212	14.52
Total	107	100	1,460	

[a] Data from the CDC (https://wwwn.cdc.gov/foodborneoutbreaks/). For data from 2000 to 2010, see previous versions of this chapter.

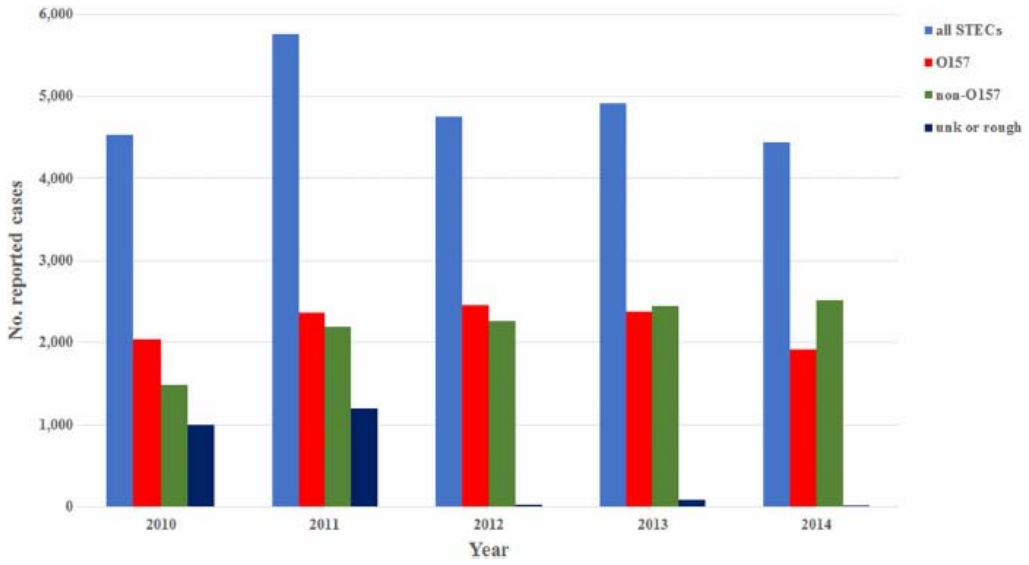


Figure 11.3 Number of cases of STEC disease in the United States by year, 2010 to 2014 (https://www.cdc.gov/ecoli/surveillance.html). Unk, unknown.

Seasonality of *E. coli* O157:H7

Outbreaks and clusters of *E. coli* O157:H7 infections peak during the warmest months of the year. Approximately 89% of the outbreaks reported in the United States occurred from May to November (102). FoodNet data indicate the same trend. The reasons for this seasonal pattern are unknown but may include (i) an increased prevalence of contaminated product on the market (due to increased prevalence in cattle), (ii) increased exposure to *E. coli* O157 from nonbeef sources during the summer (103), and (iii) increased exposure from consumption of vegetables or from recreational contact with the environment.

Age of Patients

All age groups can be infected by *E. coli* O157:H7, but the very young and the elderly most frequently experience severe illness with complications (104). HUS usually occurs in children. Population-based studies have suggested that the highest age-specific incidence of *E. coli* O157:H7 infection occurs in children 2 to 10 years of age. In addition to naïve or incompletely developed immune responses, the high rate of infection in this age group may be attributable to more frequent exposure to contaminated environments and infected animals and more opportunities for person-to-person spread between infected children with relatively undeveloped hygiene skills.

Transmission of *E. coli* O157:H7

Food remains the predominant transmission route, accounting for 52% of 350 outbreaks and 61% of 8,598 outbreak-related cases from 1982 to 2002 (102). A variety of foods have been identified as vehicles of *E. coli* O157:H7 infections, although ground beef is one of the most frequent food vehicles. Examples of other foods that have been implicated in outbreaks include roast beef, cooked meats, venison meat and jerky, salami, raw milk, pasteurized milk, yogurt, cheese, ice cream bars, lettuce, prepackaged spinach, unpasteurized apple cider/juice, cantaloupe, potatoes, radish sprouts, alfalfa sprouts, fruit and vegetable salads, cookie dough, flour, pepperoni pizzas, and cake (105).

The route of *E. coli* O157:H7 transmission for many outbreaks is unknown. Outbreaks attributed to transmission by person-to-person contact, water, animal contact, manure exposure, and laboratory exposure have also been reported. An outbreak investigation of a petting zoo-associated *E. coli* O157:H7 infection in 2003 in British Columbia, Canada, revealed that the main contributors to the propagation of illnesses among class-room and families were secondary transmission (of the 45 cases reported, 26 were reported as primary and 19 as secondary), asymptomatic infection (one infected individual was asymptomatic but transmitted the infection to his parents and siblings, and his stool sample was positive for *E. coli* O157:H7), and prolonged shedding (50). In contrast to *E. coli* O157:H7 outbreaks, in which a food is most often identified as a vehicle, the modes of transmission of most outbreaks caused by non-O157 STEC are unknown (18, 52). Only a few outbreaks of non-O157 STEC have been clearly associated with foods/water (Table 11.3).

Table 11.3 Representative foodborne and waterborne outbreaks of *E. coli* O157:H7 and other STEC infections[a]

Yr	Month	Location[b]	No. of cases/no. of deaths	Setting	Vehicle or transmission mode
1982	2; 5	Oregon; Michigan	26; 21	Community	Ground beef
1985		Canada	73/17	Nursing home	Sandwiches
1987	6	Utah	51	Custodial institution	Ground beef, person-to-person contact
1988	10	Minnesota	54	School	Precooked ground beef
1989	12	Missouri	243	Community	Water
1990	7	North Dakota	65	Community	Roast beef
1991	11; 7	Massachusetts; Oregon	23; 21	Community	Apple cider; swimming water
1992[c]		France	>4	Community	Goat cheese
1992	12	Oregon	9	Community	Raw milk
1993	1; 7; 8	California, Idaho, Nevada, and Washington; Washington; Oregon	732/4; 16; 27	Restaurant; church picnic; restaurant	Ground beef; pea salad; cantaloupe
1994[d]	2	Montana	18	Community	Milk
1994	11	Washington, California	19	Home	Salami
1995[e]	2	Adelaide, Australia	>200	Community	Semidry sausage
1995	10; 11; 7; 9	Kansas; Oregon; Montana; Maine	21; 11; 74; 37	Wedding, home, community, camp	Punch/fruit salad; venison jerky; leaf lettuce; lettuce
1996[f]		Komatsu, Japan	126	School	Luncheon
1996	5; 7; 10; 11	Connecticut, Illinois; Japan; California, Washington, Colorado; central Scotland, United Kingdom	47; 9,451/12; 71/1; 501/21	Community	Mesclun lettuce; white radish sprouts; apple juice; cooked meat
1997	5; 6; 11	Illinois; Michigan, Virginia; Wisconsin	3; 108; 13	School; community; church banquet	Ice cream bar; alfalfa sprouts; meatballs/coleslaw
1998	6; 6; 7; 7; 8; 9; 7	Wisconsin; Wyoming; North Carolina; California; New York; California; Texas	63; 114; 142; 28; 11; 20; 56	Community; community; restaurant; prison; deli; church; camp	Cheese curds; water; coleslaw; milk; macaroni salad; cake; salad bar
1999[g]	7	Connecticut	11	Community	Lake water
1999	8	New York	900/2	Fair	Well water

1999	10	Ohio, Indiana	47	Community	Lettuce
2002	8	Washington	32	Camp	Romaine lettuce
2005	9, 10	Minnesota	23	Community	Prepackaged lettuce
2006	8, 9	26 states	199/3	Community	Prepackaged spinach
2006[i]	2, 4	Norway	17/1	Community	Mutton
2007[j]	2, 5	Denmark	20	Community	Fermented beef sausage
2007	6	United Kingdom	12	Community	Ready-to-eat chicken wrap
2008[e]	8	Oklahoma	341/1	Community	Food handler
2009[k]	2	France	2	Home	Ground beef
2010[l]	3, 5	5 states	26	Community/food service	Romaine lettuce
2011[m]	5, 6	Germany	>3,700	Community/food service	Sprouts
2011	1	9 states	58	Community	Romaine lettuce
2012[j]	3	11 states	29	Restaurant	Clover sprouts
2013[j]	3	Italy	22	Apulia region	Bulk milk or curd samples
2016[h]	10	24 states	63	Community	Flour
2017	4	12 states	32	Community	Soy nut butter

[a] E. coli O157:H7 unless otherwise noted.

[b] State of the United States unless otherwise noted.

[c] E. coli O119.

[d] E. coli O104:H21.

[e] E. coli O111:NM.

[f] E. coli O118:H2.

[g] E. coli O111:H8.

[h] E. coli O121:H19.

[i] E. coli O103:H25.

[j] E. coli O26:H11.

[k] E. coli O123:H–.

[l] E. coli O145.

[m] E. coli O104:H4.

Examples of Foodborne and Waterborne Outbreaks

Original Outbreaks

The first documented outbreak of *E. coli* O157:H7 infection occurred in the state of Oregon in 1982, with 26 cases and 19 persons hospitalized (106). All patients had bloody diarrhea and severe abdominal pain. The median age was 28 years, with a range of 8 to 76 years. The duration of illness ranged from 2 to 9 days, with a median of 4 days. This outbreak was associated with eating undercooked hamburgers from fast-food restaurants of a specific chain. *E. coli* O157:H7 was recovered from stools of patients. A second outbreak followed 3 months later and was associated with the same fast-food restaurant chain in Michigan, with 21 cases and 14 persons hospitalized. The median age was 17 years, with a range of 4 to 58 years. Contaminated hamburgers again were implicated as the vehicle, and *E. coli* O157:H7 was isolated both from patients and from a frozen ground beef patty. That *E. coli* O157:H7, a heretofore-unknown human pathogen, was the causative agent was established by its association with the food and recovery of the bacterium with identical microbiologic characteristics from both the patients and the meat from the implicated supplier.

1993 Multistate Outbreak

A large multistate outbreak of *E. coli* O157:H7 infection in the United States occurred in Washington, Idaho, California, and Nevada in early 1993 (107). Approximately 90% of primary cases were associated with eating at a single fast-food restaurant chain (chain A), from which *E. coli* O157:H7 was isolated from hamburger patties. Transmission was amplified by secondary spread (48 patients in Washington alone) via person-to-person transmission. In total, 731 cases were identified, with 629 in Washington, 13 in Idaho, 57 in Las Vegas, NV, and 34 in southern California. The median age of patients was 11 years, with a range of 4 months to 88 years. One hundred seventy-eight persons were hospitalized, 56 developed HUS, and 4 children died. Because neither specific laboratory testing nor surveillance for *E. coli* O157:H7 was carried out for earlier cases in Nevada, Idaho, and California, the outbreak went unrecognized until a sharp increase in cases of HUS was identified and investigated in the state of Washington.

The outbreak resulted from insufficient cooking of hamburgers by chain A restaurants. Epidemiologic investigation revealed that 10 of 16 hamburgers cooked according to chain A's cooking procedures in Washington State had internal temperatures less than 60°C, which was substantially less than the minimum internal temperature of 68.3°C required by the state. Cooking patties to an internal temperature of 68.3°C would have been sufficient to kill the low populations of *E. coli* O157:H7 detected in the contaminated ground beef.

Outbreaks Associated with Produce

Produce-associated outbreaks of *E. coli* O157:H7 infection were first reported in 1991, and produce has since remained a prominent food vehicle of STEC infections. Raw vegetables, particularly lettuce and alfalfa and vegetable sprouts, have been implicated in several outbreaks of *E. coli* O157:H7 infection in North America, Europe, and Japan. In May 1996, a mesclun mix of organic lettuce was associated with a multistate outbreak in which 47 cases were identified in Illinois and Connecticut. A large multistate (26 states) outbreak of *E. coli* O157:H7 infection occurred in the summer of 2006 (108). A total of 199 persons were infected, with 102 (51%) patients hospitalized, 31 (16%) cases of HUS, and three deaths. *E. coli* O157 was isolated from 13 packages of spinach supplied by patients living in 10 states.

Between May and December 1996, multiple outbreaks of *E. coli* O157:H7 infection occurred in Japan, involving 9,451 cases and 12 deaths (100). The largest outbreak affected 7,470

schoolchildren, teachers, and staff in Osaka in July 1996. Epidemiologic investigations revealed that white radish sprouts were the vehicle of transmission.

Apple Cider and Juice Outbreaks

The first confirmed outbreak of *E. coli* O157:H7 infection associated with apple cider occurred in Massachusetts in 1991, involving 23 cases (109). In 1996, three outbreaks of *E. coli* O157:H7 infection associated with unpasteurized apple juice and/or cider were reported in the United States. The largest of the three occurred in three western states (California, Colorado, and Washington) and in British Columbia, Canada, with 71 confirmed cases and one death. *E. coli* O157:H7 was isolated from the implicated apple juice. An outbreak also occurred in Connecticut, with 14 confirmed cases. Manure contamination of apples was the suspected source of *E. coli* O157:H7 in several of the outbreaks. Using apple drops (i.e., apples picked up from the ground) for making apple cider is a common practice, and apples can become contaminated by resting on soil contaminated with manure. Apples can also become contaminated if they are transported or stored in areas that contain manure or are treated with contaminated water. Investigation of the 1991 outbreak in Massachusetts revealed that the implicated cider press processor also raised cattle that grazed in a field adjacent to the cider mill. Fecal droppings from deer also were found in the orchard where apples used to make the cider were harvested.

Waterborne Outbreaks

Reported waterborne outbreaks of *E. coli* O157:H7 infection have increased substantially in recent years, being associated with swimming water, drinking water, well water, and ice. Investigations of lake-associated outbreaks revealed that in some instances, the water was likely contaminated with *E. coli* O157:H7 by toddlers defecating while swimming, and swallowing lake water was subsequently identified as the risk factor. A 1995 outbreak in Illinois involved 12 children ranging in age from 2 to 12 years (110). Although *E. coli* O157:H7 was not recovered from water samples, high levels of *E. coli* were detected, indicating likely fecal contamination. A large waterborne outbreak of *E. coli* O157:H7 among attendees of a county fair in New York occurred in August 1999 (51, 111). More than 900 persons were infected, of which 65 were hospitalized. *Campylobacter jejuni* also was identified in some patients. Two persons died from HUS, including a 3-year-old girl and a 79-year-old man. Unchlorinated well water used to make beverages and ice was the vehicle. Recreational water exposure is responsible for many cases of *E. coli* O157:H7 infections (112).

Waterborne outbreaks of *E. coli* O157 infections have also been reported in other locations of the world. Drinking water, which was probably contaminated with bovine feces, was implicated in outbreaks in Scotland (51) and southern Africa (113). *E. coli* O157:NM was isolated from water associated with the latter outbreak. Walkerton, Ontario, Canada, was the site of one of the largest waterborne disease outbreaks associated with *E. coli* O157:H7 (114). In this community, 2,300 people were infected and 7 of them died.

Outbreaks of Non-O157 STEC Infections

Several outbreaks of non-O157 STEC infections have been reported worldwide. An outbreak in early 1995 in South Australia was associated with *E. coli* O111:NM and involved 23 cases of HUS after consumption of an uncooked, semidry fermented sausage product. In June 1999, an outbreak of *E. coli* O111:H8 infection occurred, involving 58 cases at a teenage cheerleading camp in Texas. Contaminated ice was the implicated vehicle. More recently, a restaurant-associated *E. coli* O111:NM outbreak occurred in Oklahoma during late August and early

September 2008 (115, 116). The outbreak caused 341 cases, 70 hospitalizations, and one death. The exact source of the contamination was undetermined, but contamination by a food handler was suspected.

Several other outbreaks caused by non-O157 STEC have also been reported. STEC O103:H25 was the cause of an outbreak associated with fermented sausage in Norway in 2006 (32). STEC O123:H$^-$ was identified as the causative agent in a family outbreak associated with eating undercooked ground beef in France in 2009 (104), whereas STEC O26 sickened several individuals in Maine and New York in 2010, leading to a large recall of ground beef. A multistate outbreak of STEC O145 infections occurred in May 2010 in the United States, with more than 30 cases reported from five states (http://www.cdc.gov/ecoli/2010/ecoli_o145/index.html). Shredded romaine lettuce from one processing facility was identified as a source of infection in this outbreak. An outbreak associated with multiple STEC serotypes (O26, O84, and O121) occurred in a Colorado prison in 2007, involving 135 cases and 10 hospitalizations. Pasteurized cheese and margarine were the food vehicles of the outbreak. More recently, an outbreak of STEC O121:H19 that began in December 2015 and ended in September 2016 in the United States was linked to flour (https://www.cdc.gov/ecoli/2016/o121-06-16/index.html). The outbreak caused 63 confirmed cases and 17 hospitalizations and resulted in the product's being recalled. The outbreak affected 24 states. WGS analyses revealed that the strains belonged to ST655 and carried stx_{2a} and *eae* genes, as well as other virulence genes described for EHECs. A similar O121 *E. coli* outbreak, also linked to flour, occurred in Canada from November 2016 to April 2017, with 30 confirmed cases occurring in six provinces.

A foodborne outbreak in Germany in May and June 2011 sickened more than 4,000 people and caused more than 50 deaths (7, 9, 10, 117). The causative agent was identified as an *E. coli* O104:H4 that produced Stx2a. Approximately 23% of the patients developed HUS, which was a much higher rate than those previously reported for patients infected with EHEC. The outbreak spread quickly over northern Germany, with some cases in other European countries, the United States, and Canada, and is one of the largest outbreaks of *E. coli* infection reported to date. Six confirmed cases of O104:H4 infections were identified in the United States. An Arizona resident who traveled to Germany before becoming ill died. Results of WGS analysis revealed the outbreak strain was genetically more related to EAEC, which is associated with cases of acute or persistent diarrhea worldwide in children and adults (see "EAEC" above), than with EHEC.

MECHANISMS OF PATHOGENICITY

Significant virulence factors associated with the pathogenicity of EHEC have been identified based on histopathology of tissues of HUS and hemorrhagic colitis patients, studies with tissue culture and animal models, and studies using cell biology and molecular genetics approaches. A general body of knowledge of the pathogenicity of EHEC has been developed and indicates that the bacteria cause disease by their ability to adhere to the host cell membrane, colonize the large intestine, and then produce one or more Stxs (118–120).

Attaching and Effacing

Numerous studies on the pathogenesis of EHEC have focused on elucidating the mechanisms of adherence and colonization. By adhering to intestinal epithelial cells, EHEC subverts cytoskeletal processes to produce a histopathological feature known as an A/E lesion (Fig.

11.4). *E. coli* O157:H7 produces an A/E lesion in the large intestine similar to that induced by EPEC, which in contrast occurs predominantly in the small intestine. The A/E lesion is characterized by intimate attachment of the bacteria to the plasma membranes of the host epithelial cells, localized destruction of the brush border microvilli, and assembly of highly organized pedestal-like actin structures (121). Most EHEC strains contain a ca. 43-kb pathogenicity island called the LEE and other phage genes encoding effector proteins (Fig. 11.5). The LEE is organized into five major operons, *LEE1* to *LEE5*, encoding a type III secretion system (T3SS), secreted proteins, chaperones, and regulators (5). The secreted proteins consist of effectors that are translocated into the host cell by the T3SS and translocators required for delivering the effectors.

Type III Secretion System

T3SS is associated with the virulence of many Gramnegative bacterial pathogens. The T3SS apparatus (see Fig. 11.6) is a complex "needle and syringe" structure that is assembled from the products of approximately 20 genes in the LEE (121, 122). The system is used by EHEC to directly translocate virulence factors from the bacteria into the targeted host cells in a single step.

The genes encoding structural proteins of the T3SS are largely conserved, whereas genes encoding effector proteins display substantial variability (123). The conserved T3SS gene cluster in the LEE is likely acquired by horizontal gene transfer, whereas genes encoding secreted proteins are more diverse and may have been obtained by distinct events.

Intimin

Intimin is a 94-kDa outer membrane protein encoded by *eae* (*E. coli* attaching and effacing). The *eae* genes of pathogenic *E. coli* present considerable heterogeneity in their 3′ ends, which encode the C-terminal 280 amino acids (Int280) involved in binding to the enterocytes and transmembrane intimin receptor (Tir) (see below), and the corresponding changes in the amino acid sequence also represent antigenic variations. Based on the sequence and antigenic differences, more than 10 distinct intimin types have been identified and classified, with α, β, ε, and γ being the main intimin types (124). Intimin α is typically found in EPEC, whereas ε and γ are closely associated with EHEC, and β is present in both EPEC and EHEC. *E. coli* O157:H7 produces intimin γ.

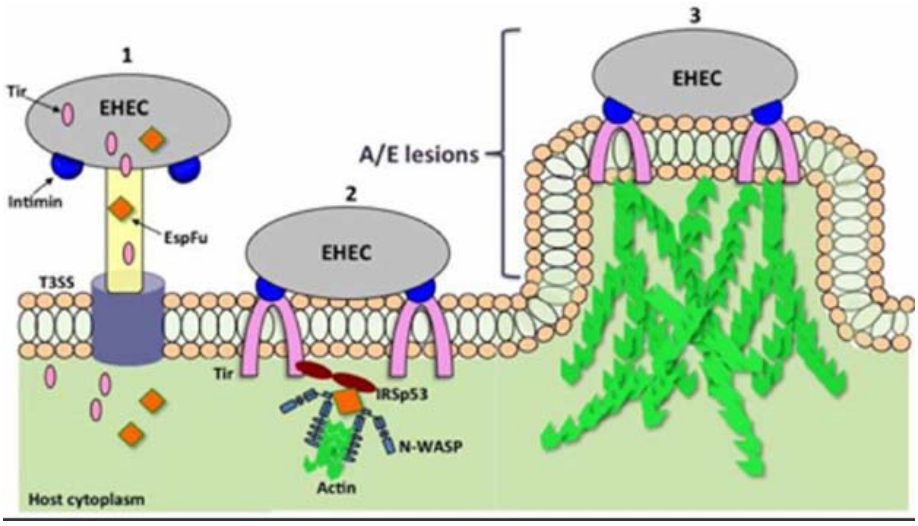


Figure 11.4 Electron microscopy image of an A/E lesion and schematic illustration of A/E lesion formation in EHEC. Effector proteins undergo A/E translocation through the T3SS, which forms a pore through the membranes of EHEC. EHEC translocates a number of proteins: EspB and EspD, which form a translocon in the plasma membrane; the cytoplasmic proteins EspF, EspG, and Map; the translocated intimin receptor Tir, which inserts into the plasma membrane; and other unidentified effectors. Formation of the EHEC pedestal is also shown. EHEC intimately attaches to the host cell through intimin-Tir binding. The binding triggers the formation of actin-rich pedestals beneath adherent bacteria after Wiskott-Aldrich syndrome protein and the heptameric actin-related protein Arp2/3 are recruited to the pedestal tip. Reproduced from reference 117 with permission.

Intimin is exported via the general secretory pathway to the periplasm, where it is inserted into the outer membrane. Intimin has two functional regions: the highly conserved N-terminal region is inserted into the bacterial outer membrane, forming a β-barrel-like structure, and mediates dimerization; the variable Int280 extends from the bacterium and interacts with Tir receptors in the host cell membrane, which were previously translocated into the host cell membrane. Interaction of intimin with host cells stimulates production of microvillus-like processes.

Autoagglutinating Adhesin (*saa*)

Most STECs causing diseases are LEE positive, but some STEC strains that cause diseases or HUS are LEE negative. Besides *eae*, there are other described adhesin genes in LEE-positive STEC strains, such as *iha*, *lpfA*, and *efa-1*, which influence bacterial adhesion and intestinal colonization (125). Paton et al. in 2001 described an autoagglutinating adhesin protein designated Saa (STEC autoagglutinating adhesin) that increased the adhesion of LEE-negative STEC O113:H21 to epithelial cells (125).

Effector Proteins

Numerous effector proteins have been identified in EHEC and are translocated into the host cell via the LEE-encoded T3SS (121), including Tir, Map (mitochondrionassociated protein), EspF, EspG, EspH, SepZ, and EspB, which are encoded by the LEE, whereas others, such as Cif (cycle inhibiting factor), EspI, EspJ, and TccP (Tir-cytoskeleton coupling protein) are encoded by prophages (Fig. 11.5). A more extensive analysis and description of these factors

can be found in a review article by Stevens and Frankel (126). Tir localizes to the host cell plasma membrane. It contains two membrane-spanning transmembrane domains and forms a hairpin-like structure, with both its C and N termini being located within the host cell and the region between the two transmembrane domains forming an extracellular loop, exposed on the surface of the cell, which interacts with intimin. Like intimin in the bacterial outer membrane, plasma membrane-bound Tir is a dimer. The intracellular amino and carboxy termini of Tir interact with a number of focal adhesion and cytoskeletal proteins, linking the extracellular bacterium to the host cell cytoskeleton. These interactions lead to the formation of actin-rich pedestals beneath adherent bacteria after Wiskott-Aldrich syndrome protein and the heptameric actin-related protein Arp2/3 are recruited to the pedestal tip (Fig. 11.4).

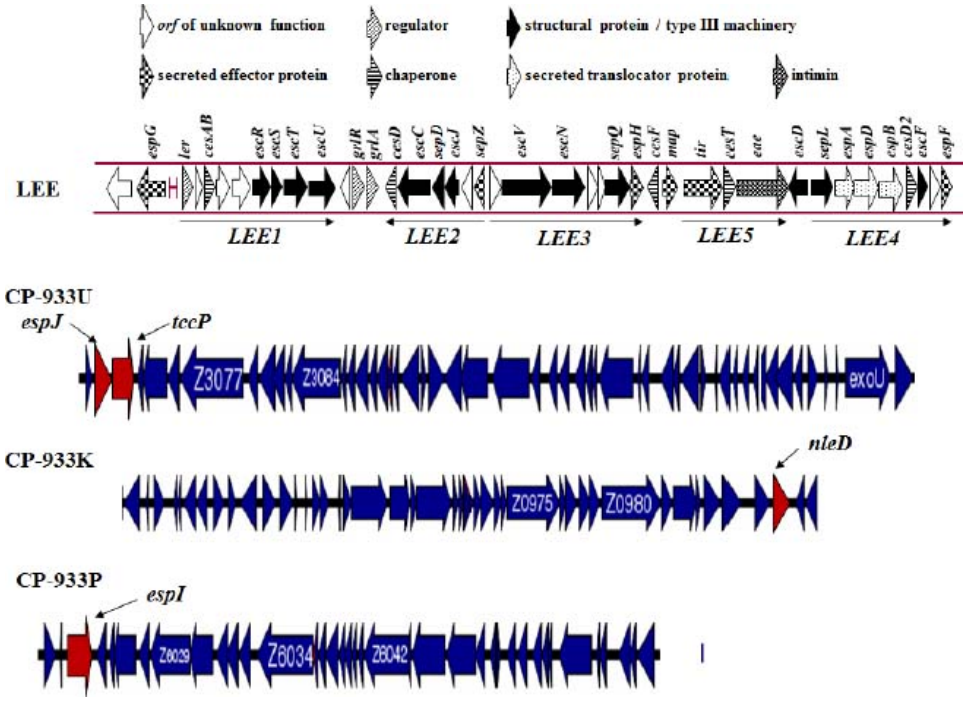


Figure 11.5 Genetic organization of the EHEC LEE and EHEC prophages CP-933U, CP-933K, and CP-933P. Reproduced from reference 121.

Effector proteins are delivered to the host cell cytoplasm from the extremity of the EspA filament through a translocation pore formed in the plasma membrane of the host cell by the translocator proteins EspB and EspD (Fig. 11.6) (121). Additional proteins, SepL ("Sep" is an acronym for "secretion of EPEC protein") and SepD, also play a role in the formation of the translocation apparatus. SepL is a soluble cytoplasmic protein that interacts with SepD. These proteins could be involved in the switch from secretion of translocator proteins to secretion of effector proteins through the type III machinery.

Virulence Plasmids

The primary virulence determinants of EHEC strains are chromosomally encoded. However, plasmids may play an important role in EHEC pathogenesis as well. An F-like 92-kb plasmid, pO157, found in most clinical isolates of *E. coli* O157:H7, shares sequence similarities with plasmids present in other EHEC serotypes. Based on DNA sequence analysis, pO157 contains 100 open reading frames (127). Genes coding for putative virulence factors in pO157 include those coding for enterohemolysin (*ehxA*), the general secretory pathway (*etpC* to *etpO*), serine

protease (*espP*), catalase-peroxidase (*katP*), a potential adhesin (*toxB*), a Cl esterase inhibitor (*stcE*), and A/E gene-positive conserved fragments (*ecf*). Nonetheless, the role of pO157 in bacterial virulence and survival is largely unknown. Toxicity results from the insertion of EhxA into the cytoplasmic membranes of target mammalian cells, thereby disrupting permeability. The EHEC catalase-peroxidase, a bifunctional periplasmic enzyme, protects the bacterium against oxidative stress, a possible defense strategy of mammalian cells during bacterial infection.

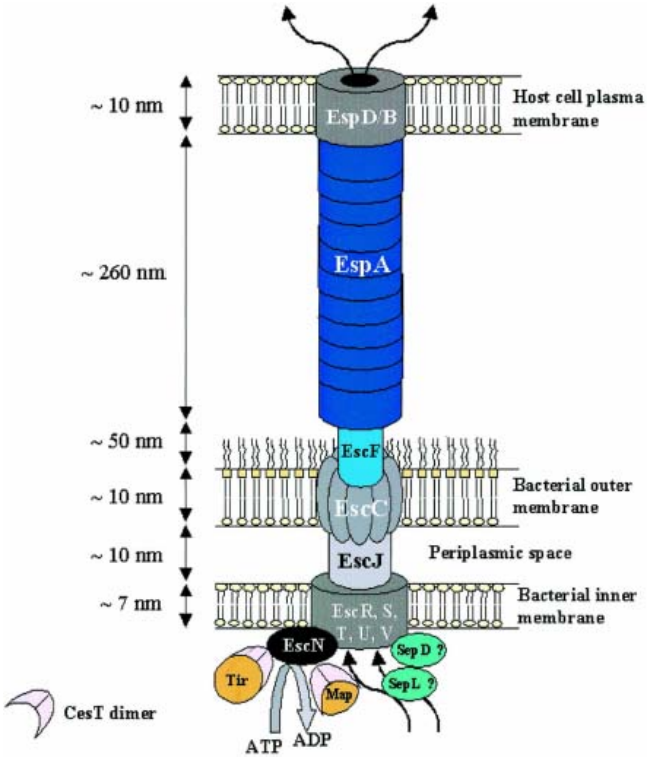


Figure 11.6 T3SS apparatus of EHEC. The basal body of the T3SS is composed of the secretin EscC, the inner membrane proteins EscR, EscS, EscT, EscU, and EscV, and the EscJ lipoprotein, which connects the inner and outer membrane ring structures. EscF constitutes the needle structure, whereas EspA subunits polymerize to form the EspA filament. EspB and EspD form the translocation pore in the host cell plasma membrane, connecting the bacteria with the eukaryotic cell via EspA filaments. The cytoplasmic ATPase EscN provides the energy to the system by hydrolyzing ATP molecules into ADP. SepD and SepL are represented as cytoplasmic components of the T3SS. Reproduced from reference 121.

Large hemolysin-encoding plasmids are also present in most non-O157 EHEC strains (128). A large plasmid of *E. coli* O113 (pO113) shares *ehx*, *espP*, and *iha* genes present in pO157. It also contains genes sharing similarity with the IncI1 transfer region and several genes for adhesins and toxins but lacks the *toxB* region found in pO157. Among those are Sab and SubA proteins. Sab is a 1,431-amino acid outer membrane autotransporter protein that enhances biofilm formation (129), whereas SubA (encoded by the *subA* gene) is a potent toxin that causes cytotoxicity in eukaryotic cells (130). These three genes (*saa*, *subA*, and *sab*) were all identified in pO113 from an *E. coli* O113:H21 strain that caused HUS (125). Analysis of *ehxA* and *repA* (a replication gene) of the RepFIB replicon revealed the evolutionary divergence of plasmids pO157 and pO113 from a common ancestor. Phylogenetic analyses of *ehxA* and *repA* were incongruent. These findings reveal differences in selective pressures between virulence

genes and constitutive genes and point to the difficulties in examining the phylogeny of plasmid genomes due to their high degree of plasticity and mobility.

Shiga Toxins

STEC strains produce one or two Stxs. The nomenclature of the Stx family and their important characteristics are listed in Table 11.4. Molecular studies on Stx1 from different *E. coli* strains revealed that Stx1a either is completely identical to the Stx of *S. dysenteriae* type 1 or differs by only one amino acid. However, during the last decade, several variants of Stx1 have been described. Some minor variants have 99% nucleotide sequence homology with stx_1 of phage 933J. A more substantial deviation from Stx1 was observed in an Stx variant from an ovine strain, *E. coli* OX3:H8 131/3, and subsequently among human isolates (131). It differs from Stx1a of phage 933J by 9 amino acids within the A subunit and 3 amino acids within the B subunit and is designated Stx1c. Another Stx1 variant, Stx1d, was identified in STEC ONT:H19, of bovine origin, with a difference from Stx1a of 20 amino acids in the A subunit and 7 amino acids in the B subunit (132).

Table 11.4 Nomenclature and biological characteristics of Stxs[a]

				Biological characteristics				
		% Nucleotide sequence homology to *stx*		% Nucleotide sequence homology to stx_2				
Nomenclature	Genetic loci	A subunit	B subunit	A subunit	B subunit	Receptor	Activation by intestinal mucus	Disease
Stx	Chromosome	NA	NA			Gb3	No	Human diarrhea, HC, HUS
Stx1a	Phage	99	100			Gb3	No	Human diarrhea, HC, HUS
Stx1c	Chromosome	97	96			Unknown	Unknown	Human and sheep?
Stx1d	Unknown	93	92			Unknown	Unknown	Cattle?
Stx2a	Phage			NA	NA	Gb3	No	Human diarrhea, HC, HUS
Stx2b	Phage			95	87	Gb3	No	Human diarrhea, HC, HUS
Stx2c	Phage			100	97	Gb3	No	Human diarrhea, HC, HUS
Stx2d	Phage			99	97	Gb3	Yes	Human diarrhea, HC, HUS
Stx2e	Chromosome			93	84	Gb4	No	Pig edema disease
Stx2f	Unknown			63	57	Unknown	Unknown	Pigeon
Stx2g	Unknown			94	91	Unknown	Unknown	Bovine

[a] From references 14, 113 and 143. Abbreviations: HC, hemorrhagic colitis; HUS, hemolytic-uremic syndrome; NA, not applicable.

Unlike Stx1, toxins of the Stx2 group are not neutralized by antiserum raised against Stx and do not cross-hybridize with Stx1-specific DNA probes. There is sequence and antigenic variation within toxins of the Stx2 family produced by *E. coli* O157:H7 and other STEC. At least seven variants of Stx2 have been identified, including Stx2a, Stx2b, Stx2c (Stx2vh-a and Stx2vh-b), Stx2d (Stx2d-OX3a and Stx2d-Ount), Stx2e, Stx2f, and Stx2g (133–135). The Stx2c subgroup is approximately 97% related to the amino acid sequence of the B subunits of Stx2a, whereas the A subunit of Stx2c shares 98 to 100% amino acid sequence homology with Stx2a. Stx2e is associated with edema disease, which occurs principally in piglets, and shares 93% and 84% amino acid sequence homology with the A and B subunits, respectively, of Stx2a. Hence, the Stx2-related toxins have only partial serological reactivity with anti-Stx2 serum. Stx2f and Stx2g of STEC strains isolated from feral pigeons and cattle wastewater have also been described (135, 136).

Structure of the Stx Family

Stxs are holotoxins composed of a single enzymatic A subunit of approximately 32 kDa in association with a pentamer of receptor-binding B subunits of 7.7 kDa (14). The Stx A subunit can be split by trypsin into an enzymatic A1 fragment (approximately 27 kDa) and a carboxyl-terminal A2 fragment (approximately 4 kDa) that links A1 to the B subunits. The A1 and A2 subunits remain linked by a single disulfide bond until the enzymatic fragment is released and enters the cytosol of a susceptible mammalian cell. Each B subunit is composed of six antiparallel strands forming a closed barrel capped by a single helix between strands 3 and 4. The A subunit lies on the side of the B subunit pentamer, nearest to the C-terminal end of the B-subunit helices. The A subunit interacts with the B-subunit pentamer through a hydrophobic helix that extends to half of the 2.0-nm length of the pore in the B pentamer. This pore is lined by the hydrophobic side chains of the B-subunit helices. The A subunit also interacts with the B subunit via a four-stranded mixed sheet composed of residues of both the A2 and A1 fragments.

Genetics

Whereas most stx_1 operons share a great deal of homology, there is considerable heterogeneity in the stx_2 family. Unlike the genes of other Stx2 proteins, which are located on bacteriophage that integrate into the chromosome, the Stxs of *S. dysenteriae* type 1, Stx1c and Stx2e, are encoded by chromosomal genes (131, 137). A sequence comparison of the growing stx_2 family reveals that genetic recombination among the B-subunit genes, rather than base substitutions, has given rise to the variants of Stx2 present in human and animal strains of *E. coli* (138). However, the operons for every member of the Stx subgroups are organized identically; the A and B subunit genes are arranged in tandem and separated by a 12-to 15-nucleotide gap. The operons are transcribed from a promoter that is located 5′ to the A-subunit gene, and each gene is preceded by a putative ribosome-binding site. The existence of an independent promoter for the B-subunit genes has been suggested. The holotoxin stoichiometry suggests that expression of the A-and B-subunit genes is differentially regulated, permitting overproduction of the B polypeptides.

Receptors

All members of the Stx family bind to globoseries glycolipids on the eukaryotic cell surface; Stx, Stx1a, Stx2a, Stx2b, Stx2c, and Stx2d bind to glycolipid globotriaosylceramide (Gb_3), whereas Stx2e primarily binds to glycolipid globotetraosylceramide (Gb_4) (139). The alteration of binding specificity between Stx2e and the rest of the Stx family is related to carbohydrate specificity of receptors (140). The amino acid composition of B subunits of Stx2a and Stx2e differs at only 11 positions, yet Stx2e binds primarily to Gb_4, whereas Stx2a binds only to Gb_3. High-affinity binding also depends on multivalent presentation of the carbohydrate, as would be provided by glycolipids in a membrane. The affinity of Stx1 for Gb_3 isoforms is influenced by the length of the fatty acyl chain and by its level of saturation. Stx1 binds preferentially to Gb_3 containing $C_{20:1}$ fatty acid, whereas Stx2c prefers Gb_3 containing $C_{18:1}$ fatty acid. The basis for these findings may be related to the ability of different Gb_3 isoforms to present multivalent sugar-binding sites in the optimal orientation and position at the membrane surface. It is also possible that different fatty acyl groups affect the conformation of individual receptor epitopes on the sugar. Bovine cells do not express high numbers of the Gb_3 receptors on their surface, and hence, cattle are not adversely affected by the toxin.

Mode of Action

Stxs act by inhibiting protein synthesis. Each of the B subunits is capable of binding with high affinity to an unusual disaccharide linkage (galactose-1,4-galactose) in the terminal trisaccharide sequence of Gb_3 (or Gb_4) (141). Following binding to the glycolipid receptor, the toxin is endocytosed from clathrin-coated pits and transferred first to the *trans*-Golgi network and subsequently to the endoplasmic reticulum and nuclear envelope. While it appears that transfer of the toxin to the Golgi apparatus is essential for intoxication, the mechanism of entry of the A subunit from the endosome to the cytosol and particularly the role of the B subunit in the process remain unclear. In the cytosol, the A subunit undergoes partial proteolysis and splits into a 27-kDa active intracellular enzyme (A1) and a 4-kDa fragment (A2) bridged by a disulfide bond. Although the entire toxin is necessary for its toxic effect on whole cells, the A1 subunit is capable of cleaving the *N*-glycoside bond in one adenosine position of the 28S rRNA that comprises 60S ribosomal subunits (142). This elimination of a single adenine nucleotide inhibits the elongation factor-dependent binding to ribosomes of aminoacyl-bound tRNA molecules. Peptide chain elongation is truncated, and overall protein synthesis is suppressed, resulting in cell death.

Role in Disease

The role of Stxs in mediating colonic disease, HUS, and neurological disorders has been investigated in numerous studies (14, 141, 143). However, there is no satisfactory animal model for studying hemorrhagic colitis or HUS, and the severity of disease precludes study of experimental infections in humans. Therefore, the present understanding of the role of Stxs in causing disease is obtained from a combination of studies, including histopathology of diseased human tissues, animal models, and endothelial tissue culture cells. Results of recent studies support the concept that Stxs contribute to pathogenesis by directly damaging vascular endothelial cells in certain organs, thereby disrupting the homeostatic properties of these cells.

The involvement of Stx in enterocolitis is demonstrated when fluid accumulation and histological damage occur after purified Stx is injected into ligated rabbit intestinal loops. The

fluid secretion may be due to the selective killing of absorptive villus tip intestinal epithelial cells by Stx. However, intravenous administration of Stx to rabbits can produce nonbloody diarrhea, suggesting that other mechanisms for triggering diarrhea are possible. Studies with genetically mutated STEC strains also reveal that Stx has a role in intestinal disease, but the significance of Stx in provoking a diarrheal response differs depending upon the animal model used.

Epidemiologic studies have revealed a correlation between enteric infection with *E. coli* O157:H7 and development of HUS in humans. Histopathologic examination of kidney tissue from HUS patients revealed profound structural alterations in the glomeruli, the basic filtration unit of the kidney (143). The damage caused by Stxs is often not limited to the glomeruli. Arteriolar damage, involving internal cell proliferation, fibrin thrombus deposition, and perivascular inflammation, occurs (144). Cortical necrosis also occurs in a small number of HUS cases. In addition, human glomerular endothelial cells are sensitive to the direct cytotoxic action of bacterial endotoxin. Endotoxin in the presence of Stxs can also activate macrophage and polymorphonuclear neutrophils to synthesize and release cytokines, superoxide radicals, or proteinases and amplify endothelial cell damage.

Neurological symptoms in patients and experimental animals infected with *E. coli* O157:H7 have also been described and may be caused by secondary neuron disturbances that result from endothelial cell damage by Stxs. Studies involving mice perorally administered an *E. coli* O157:H strain revealed that Stx2 impaired the blood-brain barrier and damaged neuron fibers, resulting in death. The presence of the toxin in neurons was verified by immunoelectron microscopy (145).

Epidemiologic and laboratory studies have revealed that *stx* genotype and host factors such as age, preexisting immunity, and the use of antibiotics are important in the development of HUS (134, 146, 147). Stx2a and Stx2c are associated with high virulence and the ability to cause HUS, whereas Stx2b, Stx2d, Stx2e, Stx1a, and Stx1c occur in milder or asymptomatic infections (134, 147). Cell culture studies revealed that Stx2a, Stx2d, and elastase-cleaved Stx2d were at least 25 times more potent than Stx2b and Stx2c. *In vivo* studies in mice revealed that the potency of Stx2b and Stx2c was similar to that of Stx1, whereas Stx2a, Stx2d, and elastase-cleaved Stx2d were 40 to 400 times more potent than Stx1 (7).

CONCLUDING REMARKS

The serious nature of the symptoms of hemorrhagic colitis and HUS caused by *E. coli* O157:H7 and EHEC places this group of pathogens in a category apart from other foodborne pathogens, which typically cause only mild symptoms. The severity of the illness it causes combined with its apparent low infectious dose (<100 cells) qualifies *E. coli* O157:H7 to be among the most serious of known foodborne pathogens. *E. coli* O157:H7 causes disease by its ability to adhere to the host cell membrane and colonize the large intestine, after which it produces one or more Stxs. Although the pathogen has been isolated from a variety of domestic animals and wildlife, cattle are a major reservoir of *E. coli* O157:H7, with undercooked ground beef being among the most frequently implicated vehicles of transmission. Very few highly effective control measures for *E. coli* O157:H7 and other STEC types in live animals have been identified. The number of cases associated with fresh produce, such as lettuce, sprouts, and spinach, has increased substantially in recent years. An important feature of this pathogen is its acid tolerance. Outbreaks have been associated with consumption of contaminated high-acid foods, including apple juice and fermented dry

salami. Recreational and drinking waters also have been identified as vehicles of transmission of *E. coli* O157:H7 infections. GenomeTrakr and other web tools will help us identify other uncommon causes and vehicles and track down sources of contamination in the future.

Stx-producing *E. coli* strains other than O157:H7 have been increasingly associated with cases of HUS. More than 130 non-O157 STEC serotypes have been isolated from humans, but not all of these serotypes have been shown to cause illness. Although genomic analyses reveal that virulence genes are well conserved in many non-O157 STEC, in addition to the *stx* genes and the LEE island, some STEC strains may have a low potential to cause HUS; other non-O157 STEC isolates, including many found in healthy individuals, may not be pathogens. *E. coli* O157:H7 is still by far the most important serotype of STEC in North America. Isolation of non-O157 STEC requires techniques not generally used in clinical laboratories; hence, these bacteria are less frequently sought or detected in routine practice. Recognition of non-O157 STEC in foodborne illness necessitates identification of serotypes other than O157:H7 in persons with bloody diarrhea and/or HUS and preferably in implicated food. The increased availability in clinical laboratories of techniques such as testing for Stxs or their genes and identification of other virulence markers using WGS analysis will continue to enhance the detection of disease attributable to non-O157 STEC.

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12
Shigella

Foodborne infections caused by *Shigella* species remain an important source of diarrheal illness worldwide in both economically developed and developing countries. A considerable amount of research has been conducted on *Shigella* since shigellosis (bacillary dysentery) was first differentiated from amoebic dysentery in 1887 and the etiologic agent was isolated and described by Shiga during an outbreak in Japan 10 years later (1). Historically, the primary route of transmission of *Shigella* spp. was relegated to water, but as indicated by Smith (2), contaminated foods have emerged as a more important vehicle. However, the foodborne aspects of shigellosis are often a neglected area of study across the international food safety community.

Shigella spp. are endemic worldwide because of their ease of transmission. Fecally contaminated food and water can lead to rapid onset of large-scale epidemics of shigellosis with a high mortality rate in underdeveloped countries. Compounding the extent of this pathogen's effects on global health are the current lack of a protective vaccine and the extraordinary ease with which these bacteria acquire antibiotic resistance. The globalization and importation of food supply present additional risks that are being measured through the international cooperation among the World Health Organization (WHO) and public regulatory agencies (3).

This chapter presents important features of *Shigella* spp., the disease they cause, and the impact that these pathogens have with respect to food safety. Diagnosis, epidemiology, ecology, modes of transmission, and examples of recent foodborne outbreaks are discussed, along with current understanding of the genetics of

Shigella pathogenesis, the genes involved in causing disease, and how they are regulated. No single review can be completely comprehensive, so the reader is encouraged to refer to several excellent recent reviews for additional information (4–12).

CHARACTERISTICS OF THE ORGANISM

Classification and Biochemical Characteristics

Shigella organisms are Gram-negative, nonsporulating, nonmotile, facultative anaerobic rod-shaped bacteria. Adopted as a genus in 1950, *Shigella* is subgrouped into four species based on biochemical, serological, and clinical phenotypic differences: *Shigella dysenteriae* (group A; 15 serotypes), *S. flexneri* (group B; 14 classical serotypes and subserotypes), *S. boydii* (group C; 19 serotypes), and *S. sonnei* (group D; 1 serotype). *S. boydii* 13 was removed from the genus *Shigella* in 2005 (13), but a new serotype, *S. boydii* 20 serovar nov., was added in the same year (14). Serologic typing is based only on differences in the O (lipopolysaccharide [LPS]) antigen, since *Shigella* lacks the H (flagellar) and K (capsular) antigens. Consequently, *Shigella* serotyping is affected by the bacterial transition from the smooth form to the rough form, a phenotype frequently seen with *S. sonnei*, which harbors the LPS-encoding genes on a plasmid that is relatively unstable during bacterial culture. Plasmidencoded genes are also necessary for synthesis of the LPS O side chain in *S. dysenteriae* 1 (15). *S. sonnei*, *S. flexneri* 2a, and *S. dysenteriae* type 1 are the most common species isolated from cases of shigellosis, the latter being responsible for most of the deadly cases due to the unique expression of the cytotoxic Shiga toxin (Stx) common to Shiga toxin-producing pathogenic *Escherichia coli* isolates, including the enterohemorrhagic *E. coli* O157:H7 strain. Although historically *S. dysenteriae* type 1 was the only member of the genus *Shigella* to harbor the *stx* genes, recently other *Shigella* spp. were also found to carry the *stx* genes but on a lambdoid phage (16–19).

As members of the family *Enterobacteriaceae*, *Shigella* spp. are nearly genetically identical to the genus *Escherichia* and closely related to the salmonellae (20). Nevertheless, they are biochemically distinguished from other enteric bacteria by their inability to ferment acetate, mucate, and lactose (although some strains of *S. sonnei* may ferment mucate or lactose upon prolonged incubation); lack of citric acid, inositol, salicin, or adonitol utilization as a sole carbon source; and inability to synthesize lysine decarboxylase (21). The four species require nicotinic acid for growth in a minimal synthetic medium, are oxidase negative, do not produce H_2S, and do not produce gas from glucose except for *S. flexneri* 6 and *S. boydii* 14. *S. dysenteriae* strains have the additional property of not being able to ferment mannitol. *S. dysenteriae* type 1 expresses an active β-galactosidase but does not produce catalase, an extremely rare feature among the *Enterobacteriaceae*. Although *S. sonnei* strains have only one serotype, they can be subdivided into biovars on the basis of their ability to hydrolyze *o*-nitrophenyl-β-D-galactopyranoside (ONPG), xylose, and rhamnose.

The traditional classification of shigellae has been challenged by the advent of comparative genomic techniques. Niche adaptation of *Shigella* was influenced by two major genetic events: acquisition of the large virulence plasmid pINV, encoding a type III secretion system (T3SS) and effectors, and concomitant inactivation or loss of chromosomal factors, including loci referred to as antivirulence genes. Furthermore, nucleotide sequencing of multiple conserved genes has indicated that *Shigella* emerged from *E. coli*. It has been postulated that *Shigella* spp. evolved directly from commensal *E. coli* lineages and that these evolutionary steps likely occurred multiple times, with each branch-off corresponding to the independent acquisition of a large (230-kb) pINV virulence plasmid (20, 22). Additional comparative genomic analyses indicate that most *Shigella* strains can be separated into three main clusters that originated from multiple *E. coli* ancestors, with clusters 1 and 2 containing strains which branched from *E. coli* 50,000 to 270,000

years ago and cluster 3 emerging between 35,000 and 170,000 years ago (23).

The three clusters can be further subdivided into five subclusters that contain strains from mainly one serogroup, indicating that modern genotyping and traditional serogrouping are correlated (24). Furthermore, a recent genomic analysis of 336 available *E. coli* and *Shigella* genomes found that using whole-genome-based alignments resulted in phylogenetic placement of *Shigella* within a diverse set of *E. coli* genomes and formed three separate clades. When sequenced *Shigella* isolates were assessed separately, five clearly defined monophyletic clades were formed. The comparison of *Shigella*-only genomes enabled subclade designations for improved discrimination based solely on genomic content (25). Nevertheless, pathogenic *S. flexneri* and nonpathogenic *E. coli* K-12 genomes still share a common chromosomal backbone. These genomes have undergone a number of large (>5-kb) rearrangements, including inversions and translocations (26). Most *Shigella* virulence determinants are plasmid-borne; novel chromosomal open reading frames (ORFs) (i.e., chromosomal genes unique to *Shigella* which are not present in the *E. coli* genome) are scarce. As an example, *S. flexneri* 2a strain 2457T has 175 ORFs that are unique, whereas the remaining 3,030 ORFs are shared with *E. coli* K-12 strain MG1655 (26).

S. sonnei and *S. dysenteriae* type 1 appear to share an ancestor with *E. coli* O157:H7, and *S. dysenteriae* types 8 and 10 are isolated clones within *E. coli*. Modern taxonomy also revealed that enteroinvasive *E. coli* (EIEC) strains were more closely related to *Shigella* than to commensal *E. coli* strains. EIEC causes dysentery like *Shigella* and may share identical O antigens with *Shigella*, but traditional classification places them as a subgroup of *E. coli* because many are motile, are lactose, mucate, and/or acetate fermenters, and require a higher infectious dose to cause infection. It is now commonly accepted that the EIEC phenotype arose several times independently from different *E. coli* ancestors and represents

an intermediate stage in the convergent evolution leading towards the more contagious and virulent *Shigella* (23) (Fig. 12.1).

Diagnosis

Shigella spp. are not particularly fastidious in their growth requirements, and in most cases, the organisms are routinely cultivated in the laboratory on artificial media. Cultures of *Shigella* are easily isolated and grown from analytical samples, including water and clinical samples. In the latter case, *Shigella* organisms are present in the feces of patients in concentrations ranging from 10^3 to 10^{10} viable bacteria per g of stool during the acute phase of infection. Identification is readily accomplished using culture media, biochemical analysis, and serological typing. *Shigella* spp. are shed in the feces and continue to be isolated from convalescent patients (10^2 to 10^3 per g of stool) for weeks or longer after the initial infection. They have the reputation of being more fastidious and difficult to cultivate than other enteric bacteria, particularly during later stages of the illness. However, this relates more to the lack of a selective enrichment broth for *Shigella*, leading to its being outgrown by the resident bacterial fecal population. Use of the semiselective agar media Hektoen, salmonella-shigella (inhibitory for *S. dysenteriae* type 1), and xylose-lysine-deoxycholate or the recently developed chromogenic agars can increase the chance of isolating of *Shigella* (27).

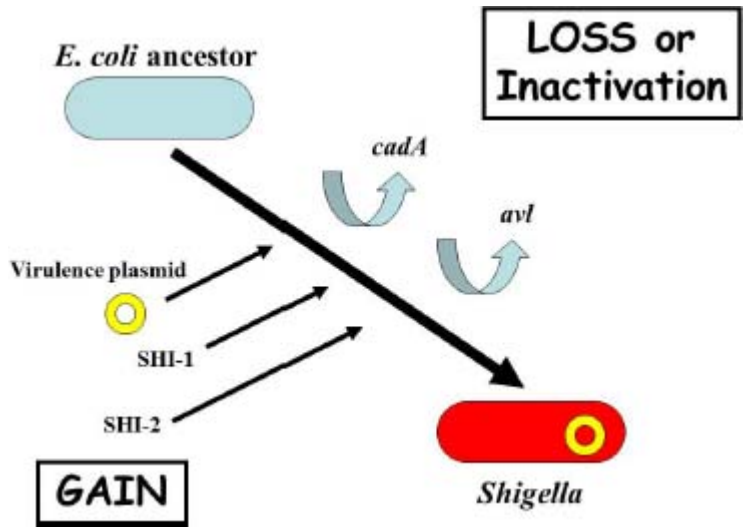


Figure 12.1 Overview of the genetic steps that contributed to the formation of pathogenic *Shigella* species from an *E. coli* ancestor. These include the acquisition of the virulence plasmid, several pathogenicity islands (SHI-1 and SHI-2 are shown), and the loss of ancestral traits (i.e., "black holes") incompatible with virulence (e.g., *cadA* and *avl*), often referred to as antivirulence genes (57).

Because the time from the initial clinical diagnosis indicating a potential outbreak to the start of an investigation to determine the cause of that outbreak can be considerable, identifying the causative agent in a timely manner can be challenging. This time lag can be exacerbated when the etiological agent has as narrow an environmental niche and survivability band as *Shigella*. Despite being saprophytic, *Shigella* spp. have a very specific host range, essentially residing only in the intestines of humans and certain other higher primates.

Shigella spp. are shed in patients with shigellosis at 10^6 to 10^8 CFU/g of stool. Although these numbers are high, the pathogens do not survive well after secretion under various environmental conditions, including temperature, exposure to sunlight, and water availability (28). Isolation of *Shigella* from foods is therefore a challenge. Many additional characteristics of the food, including its composition, physical parameters (such as pH and salt content), and the natural resident microbial flora that compete for nutrients, may

affect the successful recovery of shigellae. Considering that *Shigella* may be present in low numbers or in a poor physiological state in the suspected food samples, special enrichment procedures are often required for successful detection of foodborne *Shigella* (29).

Although molecular detection does not generally prove that an organism is alive and competent to cause disease, PCR techniques may be considered more sensitive and specific than conventional culture techniques. Several single, nested, and multiplex PCR assays have been designed to routinely amplify one or more markers present in single or multiple copies (e.g., *ipaH*) in *Shigella* and EIEC bacterial genomes (30). However, they generally do not differentiate between the four *Shigella* spp., EIEC, and/or specific serotypes. Targeting the O-serotype-specific gene(s) using PCR (31) or microarrays (32) can bring more discriminatory power to the tests. Farfán et al. (33) identified two markers that specifically differentiate between *S. flexneri*, *S. sonnei*, and diarrheagenic *E. coli* strains, while the study by Sahl et al. describing the *Shigella* monophyletic clades utilized a combination of *ipaH3* and clade-specific primers to correctly identify all *Shigella* species tested, without any false-positive results for *E. coli* strains tested (25).

Conventional analysis of isolated *Shigella* species entails the application of serological means with serotype-specific antisera. Other means to serotype isolates use PCR assays that target genes for the synthesis of O antigens. For the majority of cases, these two methods accurately determine the serotype of the *Shigella* spp. However, there have been reported situations in which discrepancies between the phenotype and genotype data have emerged. These discrepancies can arise from point mutations, cross-reactivity of the antisera used, insertions or deletions in genes responsible for O-antigen synthesis, or the emergence of novel genotypes (34). Wholegenome sequencing (WGS) has been applied to determine the serotype of *Shigella* isolates as well as to provide data to build phylogenetic relationships (35). WGS has been an extremely useful tool to identify outbreak patterns as well as the

spread of the pathogen from one geographical area to other parts of the world (global dissemination) (36).

Last, WGS is poised to become an integral part of tracking microbial pathogens that are the etiological agents of foodborne outbreaks. PulseNet, based at the Centers for Disease Control and Prevention (CDC), currently uses pulsed-field gel electrophoresis (PFGE) to match clinical and food isolates to ascertain outbreak relationships, but future surveillance tracking will be based on WGS (https://www.cdc.gov/pulsenet/pathogens/wgs.html). PulseNet International and other regional surveillance networks, e.g., PulseNet Latin America and Caribbean (37), intend to transition from PFGE to WGS analysis.

Epidemiology

Approximately 51 million *Shigella* diarrheal episodes and 15,000 deaths occur each year throughout the world (38). Although most cases of shigellosis are due to person-to-person transmission, outbreaks commonly result from food and/or water contamination. Episodes of shigellosis seem to follow seasonal variations in certain countries. In arid countries such as Egypt, transmission peaks in the hot dry season, mainly due to the consumption of contaminated water and decreased personal hygiene in times of water shortage (21). Conversely, the peak is in the rainy season in China and Thailand (39) as a result of water-washing-related transmission during heavy rains. These situations reflect the association of shigellosis with unsanitary conditions that foster fecal transmission. In developed countries, the highest incidence of shigellosis generally occurs during the warmer months of the year, when consumption of raw foods, such as fresh fruits and vegetables, and use of recreational facilities are the highest.

The distribution of *Shigella* spp. varies in different parts of the world. Fifteen years ago, the proportions of *S. flexneri*, *S. sonnei*, *S. boydii*, and *S. dysenteriae* were estimated to be, respectively, 60% (predominantly serotype 2a followed by 1b, 3a, 4a, and 6), 15%, 6%,

and 6% (30% of *S. dysenteriae* cases were type 1) in developing countries and 16% (predominantly serotype 3a followed by 1b, 1c, 2a, and Y variant [40]), 77%, 2%, and 1% in industrialized countries, with nearly half of the cases being reported among travelers (41). In some cases, specific geographical regions host one *Shigella* species, such as *S. boydii*, which is not frequently encountered outside the Indian subcontinent. Interestingly, travelers returning from India and neighboring countries and eastern, western, or northern Africa were at a higher risk for shigellosis (42).

Importantly, the distribution of *Shigella* spp. also seems to evolve with time and, of note, with the economy of a region (43). *S. dysenteriae* dominated in early parts of the 20th century but was replaced by *S. flexneri* in the 1930s and 1940s in the absence of epidemics in developing countries and in more recent times by *S. sonnei* in developed countries. With economic improvement within a developing country, a transition from *S. flexneri* to *S. sonnei* is observed, and the latter becomes responsible for the majority of shigellosis outbreaks, as seen since 2000 in Thailand (39) and Iran (44). While studying diarrheal disease caused by *Shigella* in six Asian countries, Warren et al. (11) found a link between socioeconomic factors and particular *Shigella* spp.; for example, in less resource-rich countries, the predominant species was *S. flexneri*. In addition, they observed that the variation of serotype differed temporally and with regard to geographic location, implying that the change in distribution may affect the effectiveness of any potential vaccine in areas of the world that have such shifts in serotypes.

Shigella spp. are capable of causing disease in otherwise healthy individuals. Certain populations, however, may be more predisposed to infection and disease due to the nature of transmission of the organism. Shigellae are transmitted by the fecal-oral route, direct person-to-person contact (fingers), food, water, and inanimate objects. More than two-thirds of all episodes of shigellosis are seen in children between 1 and 5 years old, who tend to explore their environment with their mouths (41, 45, 46). Sustained endemic

transmission and epidemics of bacillary dysentery are facilitated when crowding and poor sanitation conditions create an environment for direct fecal-oral contamination; examples include day care centers, custodial institutions, mental hospitals, and nursing homes, as well as refugee camps in time of war or political turmoil, where mass displacement and gathering occur. In the past 3 decades, major outbreaks have occurred in Africa, southern Asia, and Central America, causing up to 20,000 deaths in only a month (47). Oral-anal and digital-anal sexual practices favor the transmission of *Shigella* and contribute to outbreaks of shigellosis among the homosexual community (48). Additionally, human immunodeficiency virus (49)-positive individuals may present more severe and persistent forms of shigellosis.

The disease is communicable as long as an infected person excretes the organism in the stool. Secondary attack rates following exposure to the primary case can be as high as 40% among household contacts (46). The ease with which bacillary dysentery is transmitted from person to person is likely due to the very low infectious inoculum required.

Ecology

Shigella and EIEC have an extremely narrow host range. Only humans and other higher primates can develop shigellosis, but there is no evidence that the disease occurs naturally in the wild in nonhuman primates without prior contact with humans (50). While *Shigella* organisms are usually excreted for a few weeks after the illness, more than 10% of infected individuals, notably children, excrete shigellae for longer than 10 weeks (51). In areas where the organism is highly prevalent, up to 50% of all *Shigella* infections may be asymptomatic, and asymptomatic carriers of *Shigella* may exacerbate the maintenance and spread of this pathogen in developing countries. Two studies, one in Bangladesh (52) and the other in Mexico (53), showed that *Shigella* was isolated from stool samples collected from asymptomatic children under the age of 5 years.

The presence and persistence of *Shigella* in the environment is not well documented compared to those of other members of the *Enterobacteriaceae*. While the *E. coli-Shigella* ancestors gained genes that allowed them to access a new niche, i.e., the intracellular host milieu, positive selection subsequently fashioned the genome to allow virulence factors to be maintained (54) (Fig. 12.1). Accordingly, the evolution of *Shigella* was shaped by a massive loss of genes: 543 in *S. dysenteriae* Sd197, 347 to 371 in *S. flexneri*, 366 in *S. boydii* Sb227, and 255 in *S. sonnei* Ss046 (55). The majority of the lost phenotypes include surface appendages, cell motility, transport, and bacterial metabolism, reflecting the organism specialization to the intracellular pathogenic lifestyle (56–58). Because *Shigella* lacks flagella, fimbriae, and curli appendages and does not produce poly-β-1,6-*N*-acetylglucosamine or colonic acid polysaccharides, the organisms are not expected to form biofilms to protect them from damaging environmental factors (59). Nevertheless, a recent publication found that *Shigella* forms a biofilm in response to bile salts found in the small intestine (60). Current work is directed toward identifying the bacterial factors required for biofilm formation. In addition, *Shigella* incorporation into pre-established mixed-species biofilm communities has been suggested (61).

Ingestion of contaminated water is a recognized mode of transmission for shigellosis. Nevertheless, *S. dysenteriae* does not survive in water for more than 2 to 3 days. *S. flexneri* and *S. sonnei* can survive from 6 to 47 days and from 35 to 39 days, respectively (9). The community of organisms within fertile soil is biologically diverse and includes many bacterivorous organisms, including amoebae and nematodes. Over the last few years, these environmental predators have gained increasing attention as their roles as hosts for pathogenic bacteria, including *Legionella* and *Chlamydia*, are being uncovered (62). *S. sonnei* and *S. dysenteriae* can also survive phagocytosis by *Acanthamoeba* and grow in this group of ubiquitous free-living amoebae (63, 64). Considering that EIEC can paralyze and kill the nematode *Caenorhabditis elegans*

(65), agents of bacillary dysentery may well use amoebae and worms as environmental reservoirs.

SHIGELLA IN FOODS

Food Contamination

Shigella can be transmitted by consumption of raw or processed food. Generally, poor personal hygiene practices by food workers at the point of final preparation and food service are the major factor for food contamination (66). It has been recommended that infected personnel be monitored until stool samples are negative for *Shigella* (46). An extensive epidemiological investigation, for example, traced the source of a 2000 *S. sonnei* outbreak involving 406 diarrheal patients across 10 states in the United States to an employee participating in the preparation of the contaminated commercial five-layer dip (67). On at least one occasion, food (muffins and doughnuts) deliberately contaminated with *S. dysenteriae* type 2 was used as a biological weapon, leading to a dozen cases of shigellosis in Texas in 1996 (68).

Shigellae from infected individuals can spread by several routes, including food, fingers, feces, flies, and fomites. *S. sonnei* can survive for over 3 h on fingers, and *S. dysenteriae* type 1 can be recovered for up to 1 h (69, 70). *S. flexneri* can survive in feces for 12 days at 25°C (71). Flies can transmit the bacteria from fecal matter to foods. Utensils used in food preparation can also act as a vehicle for food contamination. For example, an early study by Nakamura (72) showed that *S. sonnei* could survive on metal utensils for more than 28 days at 15°C and up to 13 days at 37°C. Improper storage of contaminated foods is the second most common factor that accounted for foodborne outbreaks due to *Shigella*. Other contributing factors are inadequate cooking, contaminated equipment, and food obtained from unsafe sources. Consumption of raw vegetables harvested in fields where sewage is used as fertilizer or wastewater is used for irrigation can cause infection (73).

Survival and Growth in Foods

A number of raw or undercooked foods have been linked to shigellosis outbreaks, including lettuce, parsley, bean dip, cold sandwiches, potato salad, tofu salad, egg salad, hamburgers, tomatoes, and oysters (74). Establishments or events where these foods were served include homes, restaurants, camps, picnics, schools, airlines, sorority houses, and military mess halls. In many cases, the food source was not determined (75). Because *Shigella* spp. are not generally associated with specific foods, routine microbiological examination of foods to identify these pathogens is not usually performed. Many outbreaks are attributed to foods that require extensive manipulation during preparation and service, and investigation of food handling practices is generally more productive than microbiological examination of foods.

As discussed previously, the phylosphere (the total above-ground surfaces of plants) is not the natural habitat for *Shigella*. However, rapid growth of *S. sonnei* has been documented in shredded lettuce held at room temperature (76). Similarly, viable *S. flexneri* 2a survived for at least 72 h on damaged tomatoes (77), while *S. sonnei* did not survive desiccation on intact tomato surfaces (78). Islam et al. (79) showed that *S. flexneri* could grow on the surface of cucumbers for up to 24 h at 25°C and 37°C and survived for more than 72 h at 5°C. Cucumber skin is more textured than the waxy tomato skin and may provide sites where the bacteria are more protected from desiccation (73).

S. flexneri grew extremely well at 25°C and 37°C in sterilized milk, cooked rice, lentil soups, cooked beef, and fish and can persist for at least 3 days in all these foods except fish when stored at 5°C (79). Additionally, survival of *S. sonnei* in potato salad and raw ground beef at cold temperatures has been observed for at least 28 days and 9 days, respectively (78). *Shigella* can survive a temperature range of −20°C to room temperature; however, it survives for a longer time in foods stored frozen or at refrigeration temperatures than at room temperature. For example, two strains of *S. boydii* 18 were

inoculated into bean salad and stored at 4 or 23°C (80). No growth was observed at the lower temperatures, but both strains survived. At 23°C, a 2-order-of-magnitude increase was noted over 2 days, after which the number of viable cells declined.

Shigella tolerance to the low pH of the stomach contributes to its low infectious dose and its ability to survive in moderately acidic foods, such as fruits and vegetables. *S. flexneri* and *S. sonnei* can grow in the laboratory in rich media acidified to pH 4.75 and 4.5, respectively, and can survive in media with a pH range of 2 to 3 for a few hours. *S. sonnei* and *S. flexneri* could be recovered from tomato juice (pH 3.9 to 4.1) and apple juice (pH 3.3 to 3.4) after a few days at room temperature (81). Lowering the temperature increases the survival rate of *Shigella* in response to low pH and high salt stress. *S. flexneri* can grow at 37°C at pH 6 in the presence of 6% NaCl or at pH 5 with only up to 2% salt (82). The tolerance of *S. flexneri* to salt suggests that it may survive in salty foods, such as pickled vegetables, caviar, pickled herring, cured ham, and certain cheeses, over a long period of time. Whether *S. sonnei* survives better than *S. flexneri* in foods is still a matter of debate (83).

Foodborne Outbreaks

Foodborne outbreaks are not always easily recognizable when symptoms are mild and cases go unreported (74). As an example, a shigellosis outbreak caused by *S. sonnei* in Spain led to the infection of 60 preschool pupils and 28 family members over a 2-month period because it was undetected during the first month of occurrence (84). Even when epidemiological methods strongly imply a common food source, rapid turnover of produce may impair the ability to trace it back for analysis. Moreover, as discussed earlier, *Shigella* spp. are often not recovered and identified from foods when standard bacteriological methods are used.

The estimated number of total cases of foodborne illness in the United States each year is approximately 9.4 million annually from 31 known pathogens (85), but foodborne illness may be

significantly underreported because smaller and geographically dispersed outbreaks may not reach the attention of public health officials. Furthermore, an additional 38.4 million cases are caused by unspecified agents, bringing the total number of yearly cases to 48 million, thereby representing approximately one case for every six Americans (86). Based on the results reported by the CDC (85, 87), there are over 450,000 cases of shigellosis in the United States annually, approximately one-third of which are linked to a foodborne outbreak.

The Foodborne Diseases Active Surveillance Network (FoodNet) (88) is utilized as part of the CDC's continuing surveillance of reportable diseases caused by foodborne pathogens, including *Shigella*. From 1998 to 2004, 90 foodborne outbreaks of shigellosis were responsible for 5,324 illnesses reported to the CDC by state and local health departments (74). Compared to previous years, the incidence of foodborne shigellosis has been decreasing in the United States since 2000. Of the 17,468 laboratory-confirmed cases of foodborne infection in the United States in 2009, *Shigella* was identified in 1,849 cases, a 22% decrease compared to 2006 to 2008. A 2011 CDC publication on foodborne illnesses caused by 25 pathogens in the United States (85), which updated data from the report by Mead et al. (87), was based on laboratory-confirmed cases from five surveillance programs, some of which are indicated above. For illnesses caused by *Shigella*, most of the information cited in this new report used data from the surveillance programs covering the years 2005 to 2008. The estimated number of episodes attributed to this pathogen was given as 14,864 per year, of which nearly a third were foodborne related. This report also indicated that the numbers of hospitalizations and deaths due to illnesses acquired via foods were 1,456 and 10, respectively.

One of the striking features about foodborne outbreaks caused by shigellae is that contamination of foods usually is not at the processing plant; rather, the source is often traced to workers during final preparation of the food. As is evident from the examples below, these incidents can occur by improper food

handling in homes, small town gatherings and picnics, and largescale food service operations, such as those on cruise ships and in institutional facilities. Disease caused by the ingestion of these contaminated foods can subsequently lead to rapid person-to-person dissemination, particularly in crowded environments. *S. sonnei* has caused the majority of food-borne shigellosis in developed countries; however, *S. flexneri* and *S. dysenteriae* have also been implicated in some cases, generally via the import of contaminated produce.

Examples of Foodborne Outbreaks Caused by *Shigella* spp.

1989 and 1994—Shigellosis aboard Cruise Ships

In October 1989, 14% of the passengers and 3% of the crew members aboard a cruise ship reported having gastrointestinal symptoms (89). A multiple-antibioticresistant strain of *S. flexneri* 4a was isolated from several ill passengers and crew. The source of this outbreak was identified as German potato salad. Contamination was introduced by infected food handlers, first in the country where the food was originally prepared and second by a member of the galley crew on the cruise ship. Another outbreak of shigellosis occurred in August 1994 on the cruise ship SS *Viking Serenade* (90). A total of 586 passengers (37%) and 30 (4%) crew members reported having diarrhea, and one death occurred. In this outbreak, *S. flexneri* 2a was isolated from patients. The suspected source of contamination was spring onions.

1990—Operation Desert Shield

Diarrheal diseases during a military operation can be a major factor in reducing troop readiness. In Operation Desert Shield, enteric pathogens were isolated from 214 U.S. soldiers, and of those, 113 were diagnosed with *Shigella* infections, with *S. sonnei* being the most prevalent species isolated (91). Shigellosis accounted for more time lost from military duties and was responsible for more severe

morbidity than enterotoxigenic *E. coli*, the most common enteric pathogen isolated from U.S. troops in Saudi Arabia (91). The suspected source was contaminated fresh vegetables, notably, lettuce.

2000—Five-Layer Bean Dip

An outbreak of shigellosis caused by the ingestion of contaminated five-layer (beans, salsa, guacamole, nacho cheese, and sour cream) party dip occurred in three West Coast states (92). The causative agent was *S. sonnei*, which was isolated from at least 30 patients. The pathogen was found in just one layer (cheese) of the dip and was initially detected by a PCR assay targeting shigellae. Eventually, the pathogen was isolated by enrichment followed by plating on selective agar (67).

2001—Tomato-Linked Outbreak

Several restaurants in the New York area purchased tomatoes that were overripe and bruised from one distributor. These "special" tomatoes were identified as the source based on epidemiological data, including the fact that only restaurants that received these tomatoes were linked to the illnesses. Over 880 people reported being ill. *S. flexneri* 2a was isolated from several patrons of the restaurant as well as ill and asymptomatic employees from these restaurants. None of the workers from the restaurants reported being ill before the tomatoes arrived. PFGE of all the isolated *S. flexneri* strains linked all the restaurant patrons to this one outbreak (77).

2004—Unpasteurized Milk Curds

In October 2004, 41 persons in Vilnius, Lithuania, became sick after consuming unpasteurized milk curd produced in a local dairy. In addition to being isolated from the patients, *S. sonnei* was isolated from the dairy owner and two of her household members (93).

2004—Airline Food

In August 2004, inflight meals, likely raw carrot salad, contaminated with *S. sonnei* were served on 12 different flights departing from Honolulu International airport in Hawaii and led to 116 cases of shigellosis in two foreign countries, a U.S. territory, and nearly half of U.S. states. Between 300 and 1,500 passengers were potentially affected. The source of the outbreak was not identified with certainty (94).

2007—Raw Baby Corn

In August 2007, two shigellosis outbreaks in Denmark and Australia involving 215 and 20 cases, respectively, had a similar multidrug-resistant (MDR) *S. sonnei* isolate (95, 96). Epidemiological investigations linked the outbreaks to the consumption of raw baby corn imported from a common supply chain in Thailand. Inspection of a Thai collecting house revealed that the baby corn was placed directly on the ground and processed manually by locals without strict hygiene measures. Importantly, the water used to wash the corn contained only half of the recommended chlorine dose for disinfection (97).

2009—Sugar Peas

In April to June 2009, 37 persons became infected with *S. sonnei* in Denmark (98) and Norway (99) after consuming sugar peas imported from Kenya. *S. sonnei* was detected on the contaminated vegetable by PCR but could not be culture confirmed (99). During the same period, 35 to 47 persons became infected with *S. dysenteriae* type 2, presumably after consuming sugar peas imported from Kenya (100). However, the pathogen could not be identified from the vegetables recovered at a patient's house.

2014 and 2015—Two Outbreaks in California

Two independent foodborne outbreak clusters caused by *S. sonnei* occurred in San Jose, California, and in the San Diego, California, area. The former affected 182 people and originated in a local seafood restaurant. Notably, the *S. sonnei* isolates from patients in

this outbreak carried the lambdoid bacteriophage harboring the *stx* genes. The *S. sonnei* strain responsible for the outbreak in the San Jose area did not contain the *stx* genes but did have plasmid-borne antibiotic resistance genes, notably those encoding resistance to the fluoroquinolone class of antibiotics (101, 102).

CHARACTERISTICS OF DISEASE

Clinical Presentation

Disease caused by *Shigella* spp. is distinguished from disease caused by most of the other foodborne pathogens described in this volume in at least two important respects: the production of bloody diarrhea or dysentery and the low infectious dose required to cause clinical symptoms. Bloody diarrhea refers to diarrhea in which the stools contain visible red blood. Dysentery has the same meaning, but the passage of bloody mucoid stools is accompanied by severe abdominal and rectal pain, cramps, and fever. While abdominal pain and diarrhea are experienced by nearly all patients with shigellosis, fever occurs only in about one-third and frank blood in the stools in about 40% of the cases (103).

The clinical picture of shigellosis ranges from a mild watery diarrhea to severe dysentery. All *Shigella* spp. can cause acute bloody diarrhea. The dysentery stage of the disease caused by *Shigella* spp. may or may not be preceded by watery diarrhea. This stage reflects the transient multiplication of bacteria as they pass through the small bowel. Jejunal secretions probably are not effectively reabsorbed in the colon due to transport abnormalities caused by bacterial invasion and destruction of the colonic mucosa. The dysentery stage of disease correlates with extensive bacterial colonization of the colonic mucosa. The bacteria invade the epithelial cells of the colon and spread from cell to cell but penetrate only as far as the lamina propria. Foci of individually infected cells produce microabscesses that coalesce, forming large abscesses and mucosal ulcerations. As the infection progresses,

dead cells of the mucosal surface slough off, thus leading to the presence of blood, pus, and mucus in the stools.

The incubation period for shigellosis is 1 to 7 days, but the illness usually begins within 3 days. Strains of *S. dysenteriae* type 1 cause the most severe disease, whereas *S. sonnei* produces the mildest. *S. flexneri* and *S. boydii* infections can be either mild or severe. Despite the severity of the disease, shigellosis is generally self-limiting. If left untreated, clinical illness usually persists for 1 to 2 weeks (although it may be as long as a month), and the patient recovers. In some cases, there can be protracted asymptomatic shedding of the pathogen.

Infectious Dose

As mentioned above, an important aspect of *Shigella* pathogenesis is the extremely low 50% infective dose (ID_{50}), i.e., the oral dose required to cause disease in 50% of healthy adult volunteers challenged with a virulent strain of the pathogen. The ID_{50} for *S. flexneri*, *S. sonnei*, and *S. dysenteriae* varies, ranging from 10 CFU for virulent strains of *S. dysenteriae* to 140 CFU for *S. flexneri* and to less than 500 CFU for *S. sonnei* (see reference 104 for a review of infective doses of pathogens). Volunteers became ill when doses as low as 200 cells were given (42), and this number is generally considered an average infectious dose for *Shigella* spp. One of the host's innate defense factors is the low pH of the stomach, in which the gastric fluids can effectively kill microbial pathogens in 15 minutes (105). However, the influence of the food matrix which harbors bacterial pathogens, such as *Shigella*, may be significant with regard to surviving this harsh environment and indirectly affect the infectious dose. An example of this is when 180 CFU of wild-type *S. flexneri* 2a strain were administered with bicarbonate buffer instead of milk, which increased the shigellosis rate to 43%, indicating that resistance to gastric juice partially accounts for the high bacterial infectivity (106). The low infectious dose of *Shigella* underscores the high communicability of bacillary dysentery and

gives the disease great explosive potential for person-to-person spread as well as foodborne and waterborne outbreaks.

Complications

Shigellosis can be a very painful, incapacitating disease and is more likely to require hospitalization than many other forms of bacterial diarrhea. It is not usually life-threatening, and mortality is rare, except in malnourished children, immunocompromised individuals, and the elderly. However, complications arising from the disease include severe dehydration, intestinal perforation, toxic megacolon, septicemia, seizures, hemolytic uremic syndrome (HUS), and reactive arthritis (107). HUS is a rare but potentially fatal complication associated with infection by *S. dysenteriae* 1 (108). The syndrome is characterized by hemolytic anemia, thrombocytopenia, and acute renal failure. Epidemiologic studies suggest that Shiga toxin produced by *S. dysenteriae* 1 is the cause of HUS. This hypothesis is supported by the fact that HUS is also caused by strains of enterohemorrhagic *E. coli*, which produce high levels of Shiga toxin (see chapter 11). Shiga toxins cause HUS by entering the bloodstream and damaging vascular endothelial cells, particularly those in the kidney. The syndrome comprises three symptoms, urethritis, uveitis, and reactive arthritis, with the latter being the most dominant symptom. Reactive arthritis is a postinfection sequela to shigellosis that is strongly associated with individuals of the HLAB27 histocompatibility group (109). Infections caused by several other Gram-negative enteric pathogens also can lead to this type of sterile inflammatory polyarthropathy (110).

Treatment and Prevention

Although stool fluid losses are not as massive as with other bacterial diarrheas (e.g., cholera), the diarrhea associated with shigellosis combined with water loss from fever and the decreased water intake due to anorexia may result in severe dehydration (107). Oral intake of liquids can generally replace fluid losses, although

intravenous rehydration may be required in very young and elderly patients. Antidiarrheal drugs are not recommended because they decrease intestinal mobility and may worsen shigellosis. On the other hand, antibiotics shorten the duration of dysentery and bacterial excretion in feces. However, there is some controversy regarding the use of antibiotics in treating shigellosis. Since the infection is self-limited in normally healthy patients and full recovery occurs without the use of antibiotics, drug therapy is usually not indicated. Additionally, treatment of shigellosis has been confounded by a widespread increase in MDR *Shigella* strains, a critical health concern for treatment of infected individuals, particularly in the absence of an effective vaccine. Although the majority of outbreaks of illness caused by MDR *Shigella* in the last few years have occurred in southern Asia (India, Bangladesh, and China), there have been reports of outbreaks caused by MDR *Shigella* in South America (Chile) and the Middle East (Iran) (111–118). MDR outbreaks have been documented recently in the United States and other industrialized nations (119–122). For example, importation and domestic transmission of *S. sonnei* strains resistant to ciprofloxacin in the San Francisco area have been documented (119), as well as travel-acquired and domestically acquired MDR *S. sonnei* infections in Pennsylvania (123).

Although clonal expansion of particular resistant strains may participate in the increase of treatment failure, horizontal transfer has been proposed as the main mechanism of dissemination of antibiotic resistance determinants among *Shigella* spp. via mobile genetic elements, such as plasmids and transposons. Additionally, these genetic elements may carry integrons than can assimilate and exchange exogenous DNA cassettes, usually antibiotic resistance genes, by site-specific recombination and therefore can easily accumulate antibiotic resistance traits. Class 1 integrons have been detected in *S. flexneri* and *S. dysenteriae* (124, 125) but are less frequent in other *Shigella* spp. than class 2 integrons, which have been isolated from developed countries (e.g., Japan, South Korea,

and Ireland) (126–128) and developing countries (e.g., Brazil), especially among *S. sonnei* isolates.

The underlying explanation for the rapid worldwide distribution of MDR *Shigella* strains could be a substantial flux in the epidemiological landscape of this pathogen over the last century. International travelers are an underappreciated and significant factor for dissemination of *Shigella* spp. around the world (129, 130). These travelers, upon return to their homeland, may introduce novel MDR strains to naïve populations. These strains may then become endemic to a region, particularly if they are more resistant to elimination by antibiotics than native circulating strains. Another possible route of emergence of MDR strains is asymptomatic carriage of *Shigella* isolates, which may also account for persistence of these MDR strains, since an environmental reservoir for the bacteria has not yet been identified (131). Asymptomatic carriers of *Shigella* strains, therefore, may be exposed to broad-spectrum antibiotic drug treatment meant to target other infections. The inadvertent consequence would be ample selective pressure on *Shigella* MDR strains to perhaps circulate in the population. It is interesting that *Shigella* and other enteric pathogens utilize common drug resistance mechanisms to resist bile exposure, particularly the AcrAB efflux pump (60, 132). Therefore, exposure to bile during host infection may contribute to MDR development by inducing drug resistance mechanisms prior to antibiotic exposure. Finally, in developing nations, which account for the largest burden of *Shigella* infections, antibiotic use is often unregulated. This can easily lead to circumstances that lend themselves to the misuse and overuse of antibiotics (133).

The choice of antimicrobial drugs has changed over the years as resistance to antibiotics has occurred, with different patterns of resistance being reported around the world. Ampicillin and the combination trimethoprim-sulfamethoxazole (SXT), once the treatments of choice against shigellosis, long ago lost their efficacy in most of the world due to the development of drug resistance (134) and are no longer recommended for therapy due to this

widespread resistance pattern. In Belgium, resistance to SXT increased from 38% to 67% of *S. sonnei* isolates between 1990 and 2007 (128). In the United States, among the 552 *Shigella* isolates received by the National Antimicrobial Resistance Monitoring System at the CDC in 2008 (89.9% *S. sonnei* and 8.7% *S. flexneri*), 62.5% were resistant to ampicillin, 41.1% to SXT, and 22.8% to both treatments (135). In sub-Saharan Africa, among the 109 *Shigella* isolates isolated in Mozambique from 2001 to 2003 (86% *S. flexneri* and 14% *S. sonnei*), 56% were resistant to ampicillin, 84% to SXT, and 25% to both treatments (136).

Subsequently, first-generation quinolones have been used as a solution for the last 2 decades, but resistance to nalidixic acid is now common in Asian countries (137) and frequently found in Africa. For example, the majority of the *S. dysenteriae* type I isolates that were responsible for the devastating epidemics between 1993 and 1995 in refugee camps in Rwanda, Tanzania, and Democratic Republic of the Congo were resistant to all the commonly used antibiotics, including nalidixic acid (138). Nalidixic acid has therefore been replaced by fluoroquinolones, but ciprofloxacin resistance is now increasingly common in Asian countries (134), in particular in India, where a 48% increase has been reported between 2002 and 2007 (139). Quinolone resistance remains rare in the United States compared to Asian countries, but fluoroquinolone resistance has been reported in New York City, with half of the cases occurring in travelers from Asia or people that may have come into contact with travelers from the Indian subcontinent (140).

Ciprofloxacin has become the antibiotic of choice for MDR strains, but the emergence of ciprofloxacin resistance in *Shigella* has shifted the attention to third-generation cephalosporins, such as ceftriaxone; however, their cost is often prohibitive for patients in developing countries. The discovery of extended-spectrum β-lactamases that confer resistance to third-generation cephalosporins in *S. flexneri* and *S. sonnei* isolates in the United States (141), Vietnam (51), France (142), Lebanon (143), and India (51) compromises treatment options for shigellosis and is extremely

concerning, particularly for isolates displaying additional resistance to nalidixic acid (137). The macrolide azithromycin is recommended by the American Academy of Pediatrics for treatment of shigellosis in children and by the WHO as a second-line treatment in adults. A macrolide-resistant clinical variant of *S. sonnei* was isolated in France in 2007 (144).

Extensive use of antibiotics selects for drug-resistant organisms, and therefore, many believe that antimicrobial therapy for shigellosis should be reserved only for the most severely ill patients. On the other hand, there are persuasive public health arguments for the antibiotic management of shigellosis. Antibiotic treatment limits the duration of disease and shortens the period of fecal excretion of this pathogen (145). Since an infected person or asymptomatic carrier can be an index case for person-to-person, foodborne, and waterborne spread, antibiotic treatment of these individuals can be a significant public health tool to contain the spread of shigellosis. However, antibiotics are not a substitute for improved hygienic conditions to contain secondary spread of shigellosis. The most effective means of preventing secondary transmission are hand-washing with soap and chlorination of water. This point is highlighted by the recent *Shigella* outbreak in Flint, Michigan. Due to the lead contamination issues in the water system, residents were afraid to use and further expose their children to the water, thereby triggering an outbreak of shigellosis. It has been documented through news reports that residents were using baby wipes as alternatives to handwashing, not realizing that antimicrobial compounds are absent in the wipes (146). Food handling and preparation are important processes that also deserve attention, and persons with diarrhea should be excluded from handling food. Bardhan et al. (147) proposed that the 80% reduction in shigellosis-associated deaths observed in Asia in the past 10 years was due to measles vaccination, vitamin A supplementation, and improved nutrition in that part of the world. Breastfeeding has also been shown to provide protection during the first 3 years of life, especially in malnourished children.

Despite many years of intensive effort, an effective vaccine against shigellosis has not been developed yet. Natural infections result in about 72% protection against a second episode of shigellosis due to the homologous serotype but provide less than 30% protection against a heterogeneous serotype, suggesting that the *Shigella* O antigen is the key antigen for protection (47). A vaccine active against *S. sonnei*, *S. dysenteriae* 1, *S. flexneri* 2a, *S. flexneri* 3, and *S. flexneri* 6 would cover more than 80% of the strains currently causing morbidity and mortality in both developed and developing countries. Two main approaches were undertaken initially: (i) attenuated strains of *Shigella* were used as live oral vaccines, and (ii) O polysaccharides of *Shigella* covalently linked to carrier proteins were used as a parenteral conjugate vaccine (5). Whereas the first approach generated some degree of protection, administration of the live attenuated *Shigella* variants, for example, *S. flexneri* 2a SC602, *S. sonnei* WRSS1, or *S. dysenteriae* 1 strain WRSd1, was followed by fever and mild diarrhea. The second approach was relatively successful and safe when tested in adults and young children, but the *S. sonnei* conjugate had no efficacy in children less than 2 years old (148) and did not achieve protection in a population where the pathogen is endemic (149). New vaccine candidates include the use of new-generation conjugate vaccines that use synthetic oligosaccharides or a combination of invasion proteins and LPS named Invaplex (5).

VIRULENCE FACTORS

Hallmarks of Virulence

Shigella spp. and EIEC are the principal agents of bacillary dysentery and as such belong to the group of enteric pathogens that cause disease by overt invasion of epithelial cells in the large intestine. The clinical symptoms of shigellosis can be directly attributed to the hallmarks of *Shigella* virulence that modulate the host innate and adaptive immune systems. This modulation

provides the pathogen the ability to invade intestinal epithelial cells, escape from phagocytic vesicles, multiply intracellularly, spread from cell to cell, induce an inflammatory response, and ultimately produce typical symptoms of shigellosis such as diarrhea.

The overall picture of *Shigella* pathogenesis parallels that of other enteric bacterial pathogens. After ingestion, the organism transits the stomach and small intestine and eventually enters M cells that overlie lymphoid nodules in the colon, specifically the follicle-associated epithelium (150, 151). Exploiting the arsenal provided by the T3SS, the bacteria induce their uptake by colonic M cells and, posttranscytosis, are released into the intraepithelial pocket. Macrophages engulf the pathogens, which survive phagocytosis and use effector proteins to kill the macrophages (152, 153). After macrophage cell death, proinflammatory cytokines are released, thereby causing an acute inflammatory response. Current thought is that the host response provides the pathogen with the ability to evade the host innate and adaptive immune response and that controlling the host response may limit the severity of the disease. Bacterial cells released after the destruction of macrophages enter the basolateral membranes of adjacent epithelial cells by a macropinocytic-type process of actin polymerization and depolymerization to form filopodial and lamellipodial extensions of the epithelial membrane that result in bacterial engulfment (12, 154, 155). Lysis of the macropinocytic vacuole enables *Shigella* to enter the host cell cytoplasm, where the pathogen multiplies. Through actin polymerization, the bacteria are propelled throughout the cytoplasm and into adjacent cells through the formation of "actin rockets," similar to those observed with *Listeria monocytogenes* (see chapter 17) (156). This mechanism of intercellular spread enables the bacteria to temporarily evade host immune responses.

Shigella colonizes the small intestine only transiently and causes little tissue damage (157). Recent data demonstrate that *Shigella* forms a biofilm in response to the bile salts present in the small intestine (60). Based on their results, the authors of that study

present a model in which biofilm formation enables the bacteria to transit the small intestine, with subsequent biofilm dispersion in the terminal ileum as bile is reabsorbed into circulation. The dispersion event permits *Shigella* invasion of the colon. It is hypothesized that this biofilm formation enables the bacteria to successfully survive and transit the small intestine under otherwise bactericidal conditions. The study also identified important gene transcription changes in response to bile that included induction of virulence genes as well as genes important for anaerobic respiration. Furthermore, separate analyses have demonstrated an altered gene expression profile in response to available oxygen, indicating the genetic alterations that occur as the bacteria transition to a more anaerobic environment (158, 159). These studies highlight the alteration of *Shigella* gene expression, particularly of virulence genes, as the bacteria transit the intestines prior to infection in the colon. Finally, production of enterotoxins by the bacteria while in the small bowel likely results in the watery diarrhea that often precedes onset of dysentery (160–163), the first hallmark of *Shigella* virulence. The jejunal secretions elicited by these toxins may facilitate passage of the bacteria through the small intestine and into the colon, where they colonize and invade the epithelium.

Labrec and colleagues established the essential role of epithelial cell invasion in *Shigella* pathogenesis in a landmark study that employed both *in vitro* tissue culture assays for invasion and animal models (164). They found that spontaneous colonial variants of *S. flexneri* 2a that are unable to invade epithelial cells in tissue culture do not cause disease in monkeys. Gene transfer studies using *E. coli* K-12 donors and *S. flexneri* 2a recipients established the third hallmark, intracellular multiplication, of *Shigella* virulence. An *S. flexneri* 2a recipient that inherits the *xyl-rha* region of the *E. coli* K-12 chromosome retains the ability to invade epithelial cells but has a reduced ability to multiply within these cells. This hybrid strain fails to cause a fatal infection in the opium-

treated guinea pig model and is unable to cause disease when fed to rhesus monkeys (165).

It is necessary but not sufficient for *Shigella* to be able to multiply within the host epithelial cell after invasion. The bacterium must also be able to spread through the epithelial layer of the colon by cell-to-cell spread that does not require the bacterium to leave the intracellular environment and be re-exposed to the intestinal lumen or the host's immune response. Mutants of *Shigella* that are competent for invasion and multiplication but unable to spread between cells in this fashion have been isolated. These mutants established intracellular spread as the fourth hallmark of *Shigella* virulence and are discussed further below.

Along with the ability to colonize and cause disease, an intrinsic part of a bacterium's pathogenicity is its mechanism for regulating expression of the genes involved in virulence. Virulence in *Shigella* spp. is regulated by growth temperature. After growth at 37°C, virulent strains of *Shigella* are able to invade mammalian cells, but when cultivated at 30°C, they are noninvasive. This noninvasive phenotype is reversible by shifting the growth temperature to 37°C. The temperature change enables the bacteria to re-express their virulence properties (166). Temperature regulation of virulence gene expression is a characteristic that *Shigella* shares with other human pathogens, such as *E. coli*, *Salmonella enterica* serovar Typhimurium, *Bordetella pertussis*, *Yersinia* spp., and *L. monocytogenes* (see reference 6 for a review). Regulation of gene expression in response to environmental temperature is a useful bacterial strategy. By sensing The ambient temperature of the mammalian host (e.g., 37°C for humans) to trigger gene expression, this strategy permits *Shigella* to economize energy that would be expended on the synthesis of virulence products when the bacteria are outside the host. The system also permits the bacteria to coordinately regulate expression of multiple unlinked genes that are required for the full virulence phenotype. Temperature regulation in *S. flexneri* 2a operates at the level of gene transcription and is mediated by both positive and negative transcription factors. A

chromosomal gene, *virR* (*hns*), encodes a repressor of virulence gene expression (167), while two plasmid-borne genes, *virF* and *virB*, encode positive activators (168). These genes are discussed below. A more thorough assessment of virulence gene regulation in *Shigella* can be found in several review articles (12, 169, 170).

Genetics

Virulence-Associated Plasmid Genes

Given the complexity of the interactions between host and pathogen, it is not surprising that *Shigella* virulence is multigenic, involving both chromosomal and plasmidborne genes (Table 12.1). Another landmark study on the pathogenicity of *Shigella* demonstrated the indispensable role of a large plasmid in invasion, pINV. A 180-kb plasmid in *S. sonnei* and a 220-kb plasmid in *S. flexneri* are essential for invasion (171). Other *Shigella* spp., as well as strains of EIEC, possess homologous plasmids which are functionally interchangeable and share significant degrees of DNA homology. The DNA sequence of the virulence plasmid of *S. flexneri* was the first to be published (110, 172, 173), and the virulence plasmids of *S. sonnei*, *S. dysenteriae* 1, and *S. boydii* have also been sequenced (174, 175). The virulence plasmid from EIEC has been sequenced and can be accessed from GenBank (accession number CP001064).

A 37-kb region of the invasion plasmid of *S. flexneri* 2a contains all of the genes necessary to permit the bacteria to penetrate tissue culture cells. This DNA segment was identified as the minimal region of virulence plasmid necessary to allow a plasmid-cured derivative of *S. flexneri* (and *E. coli* K-12) to invade tissue culture cells (171, 176). The region contains about 33 genes in two groups transcribed in opposite orientation (Fig. 12.2). These genes encode proteins that are components of the T3SS apparatus, effectors, translocators, transcription activators, and chaperones. Although a precise transcription map of these genes has not been defined,

available evidence and the DNA sequence of the region suggest a multiple-operon organization.

Table 12.1 Virulence-associated loci of *Shigella*

Site and locus	Product	Role in virulence
Chromosome		
cpxR	Response regulator of CpxACpxR two-component system	Activator of *virF*
iucA to *-D; iutA*	Synthesis of aerobactin and receptor	Acquisition of iron in the host
pic	Autotransporter protein	Putative hemagglutinin, mucinase, enterotoxin, and immune-modulating functions
rfa; *rfb*	Enzymes for core and O antigen	Correct polar localization of IcsA biosynthesis
set1AB[a]	ShET1	Enterotoxin
sigA	Autotransporter protein	Enterotoxin activity
slyA	Transcriptional regulator	Activation of virulence genes; role in acid resistance
sodB	Superoxide dismutase	Inactivation of superoxide radicals; defense against oxygen-dependent killing in host
stx[b]	Shiga toxin	Destruction of vascular tissue
vacB (39)	Exoribonuclease RNase R	Posttranscriptional regulation of virulence gene expression
virR (*hns*)	Histone-like protein	Repressor of virulence gene expression
Plasmid		

icsA (virG)	Cell-bound and secreted protein	Actin polymerization for intracellular motility and intercellular spread
icsB	Secreted effector	Shields IcsA from binding autophagy protein Atg5; protects against autophagy
icsP	Outer membrane protease	Role in IcsA unipolar localization
ipaA	Secreted effector	Efficient invasion; binds to vinculin and promotes F-actin
ipaB	Secreted effector	Invasion; lysis of vacuole; induction of macrophage cell death
ipaC	Secreted effector	Invasion; induces cytoskeletal reorganization
ipaD	Secreted effector	Invasion; antisecretion plug (with IpaB) depolymerization; induction of macrophage cell death
ipaH	See below[c]	
ipaJ	Secreted effector	Disrupts entry vacuole (with VirA)
ipgB1 and -2	Secreted effector	Promotes membrane ruffling
ipgC	17-kDa protein	Cytoplasmic chaperone for IpaB and IpaC; coactivator of MxiE; activation of Rac1 and Cdc42
ipgD	Secreted effector	Promotes bacterial entry; enhances epithelial cell survival by targeting Akt
mxi-spa	20 proteins	Type III system for secretion of Ipa and other virulence proteins
mxiE	Transcriptional activator	AraC-family postinvasion activator

ospB	Secreted effector	Regulation of host cell proliferation
ospC1	Secreted effector	Regulation of neutrophil migration
ospC2	Secreted effector	Function unknown
ospC3	Secreted effector	Inhibition of host cell death
ospC4	Secreted effector	Function unknown
ospD1	Secreted effector	Antiactivator of MxiE
ospD2	Secreted effector	Function unknown
ospE1 and -2	Secreted effectors	Maintain bacterial cell integrity postinvasion; required for bile salt-induced adherence
ospF	Secreted effector	Phosphothreonine lyase
ospG	Secreted effector	Targets ubiquitin-conjugating enzymes; downregulates host innate immune response
ospI	Secreted effector	Suppresses TRAF6-mediated signaling
ospZ	Secreted effector	Blocks NF-κB activation
sen (*ospD3*)	ShET2	Enterotoxin; regulation of IL-8 secretion from infected epithelial cells
sepA	Autotransporter	Protease activity; enterotoxin activity; enables *Shigella* to gain access to the basolateral pole of the epithelium
spa15	Chaperone and secreted effector	Inhibition of epithelial-cell apoptosis
virA	Secreted effector	Disruption of entry vacuole (with IpaJ); autophagy (with IcsB)
virB	Transcriptional	Temperature regulation of

	activator	virulence genes
virF	Transcriptional activator	Temperature regulation of virulence genes

[a] The *set* locus and production of Pic and ShET1 are found almost exclusively in *S. flexneri*.

[b] The *stx* locus and production of Shiga toxin are observed only in *S. dysenteriae* 1.

[c] There are four loci located on the chromosome and five located on the virulence plasmid. The role of the *ipaH* genes appears to be to encode E3 ubiquitin ligases (141). Since there are nine genetic loci, the *ipaH* genes have been a common target in many PCR-based assays.

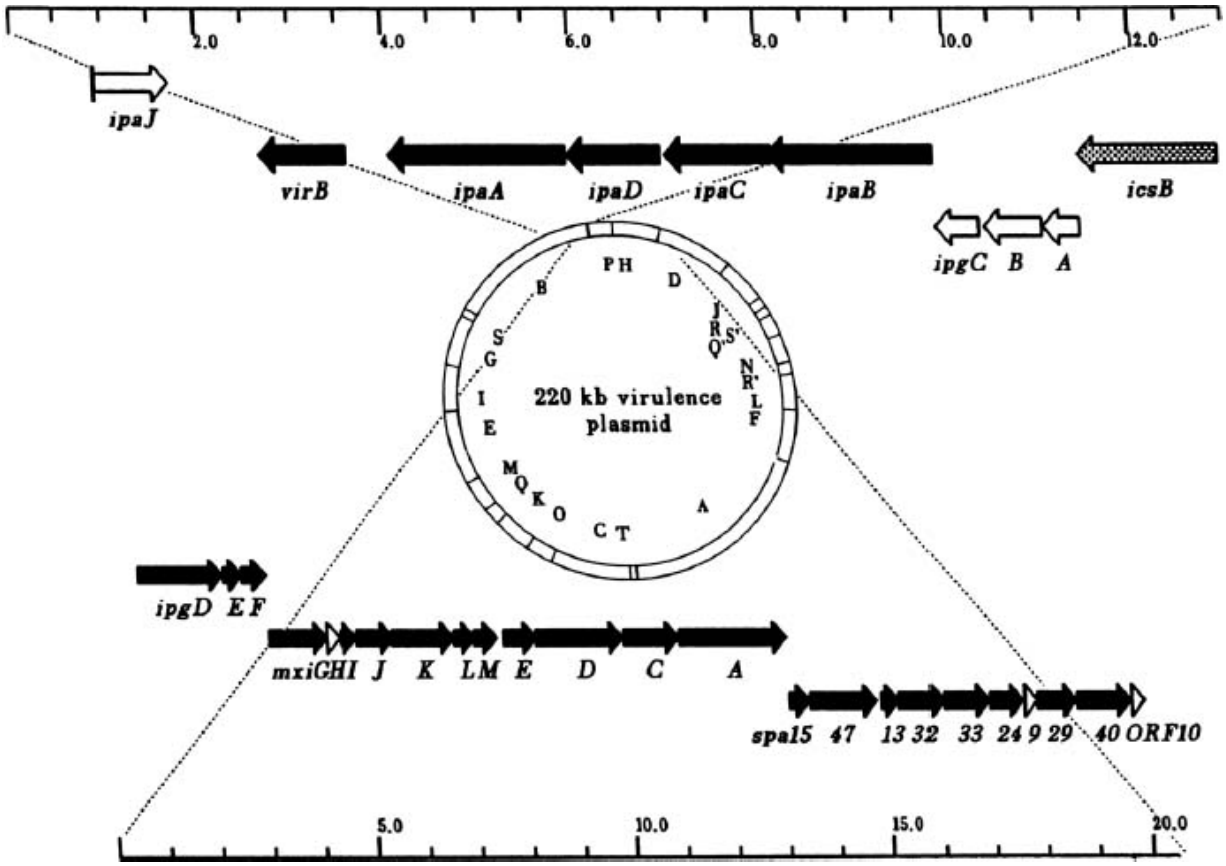


Figure 12.2 *S. flexneri* 2a virulence plasmid map. A SalI restriction map of the 220-kb plasmid is shown in the center. Sections of SalI fragments B and P (upper map) and fragments P, H, and D (lower map) are expanded to illustrate the virulence loci encoded in these regions. The expanded regions are contiguous and cover 32 kb. The ORF for *icsB* is separated from that of *ipgD* by 314 bp.

The genes composing the *ipaBCDA* (invasion plasmid antigens) cluster encode the immunodominant antigens, which were detected with sera from convalescent patients and experimentally challenged monkeys (177). *ipaBCD* have been experimentally demonstrated to be required for invasion of mammalian cells (178), while experiments have shown that *ipaA* facilitates entry (179, 180). The Ipa products act as translocators by interacting with the host cell membrane subsequent to being shunted through the bacterial membrane through the T3SS. IpaB and IpaC form a complex on the bacterial cell surface and are responsible for transducing the signal that leads to entry of *Shigella* into host cells via bacterium-directed phagocytosis (181). IpaD is also required for insertion of IpaBC into the membrane (182). However, when IpaB and IpaC are used to coat latex beads, they form a complex that promotes uptake of the beads by HeLa cells (183). This complex of Ipa proteins also binds to cell surface receptors such as $\alpha_5\beta_1$ integrins (184). Purified IpaC induces cytoskeletal reorganization via actin polymerization and depolymerization, including formation of filopodia and lamellipodial extensions on permeabilized cells (185). IpaA binds to vinculin and promotes F-actin depolymerization (186). This step is thought to facilitate reorganization of the host cell surface structures induced by contact with *Shigella* and modulate bacterial entry (179).

Although the Ipa proteins have no typical signal sequence for recognition by the usual Gram-negative bacterial transport system, these proteins are secreted into the extracellular medium via a T3SS (187, 188). The T3SS pathway requires a dedicated apparatus composed of gene products from the *mxi-spa* (membrane expression of invasion plasmid antigens and surface presentation of Ipa antigens) loci (Fig. 12.2). Secretion through T3SS is extremely orchestrated, as recently reviewed (189). Contact of the bacterium with epithelial cells induces secretion of the cytoplasmic pool of Ipa products (190). Fibronectin, laminin, collagen type IV, Congo red, bile salts, and fetal bovine serum have also been reported to induce Ipa secretion in the laboratory (190–192). IpaD forms an

antisecretion complex, or plug, with IpaB. Consequently, both *ipaD* and *ipaB* mutants are hypersecretors of the Ipa products (193).

MxiC plays a similar role in controlling the secretion of effectors, acting as a gatekeeper for secretion (194, 195). *ipgC* (invasion plasmid gene) is required for invasion and acts as a cytoplasmic chaperone which prevents IpaB and IpaC from forming complexes while in the bacterial cytoplasm (181). In the absence of IpgC, IpaB and IpaC are rapidly degraded. IpgC also plays a role as a coactivator in virulence gene regulation (see below). The product of *ipaB* has also been postulated to be the "contact hemolysin" which is responsible for lysis of the phagocytic vacuole minutes after entry of the bacterium into the host cell (196). The ability of *S. flexneri* to induce pyroptosis (197) in infected macrophages is an additional property assigned to IpaB (198), while IpaD was also recently shown to induce apoptosis in macrophages (153).

The secretion apparatus consists of the inner membrane proteins MxiG and MxiJ and an outer membrane protein, MxiD, which, along with MxiM, is proposed to form a bridge across the periplasmic space to contact MxiJ and MxiG (199). The secretion "needle" is composed of MxiH and possibly MxiI (200, 201). Spa47 plays a critical role in facilitating the transfer of proteins through the needle complex (202), while Spa32 is involved in controlling needle length (203, 204). Spa33 associates with MxiG and MxiJ in the putative C ring of the secretion apparatus and controls secretion of IpaB and IpaC (205). Interestingly, expression of both *spa32* and *spa33*, but not that of other T3SS genes, is reduced under anaerobic conditions prevalent in the lumen of the large intestine, suggesting that effective secretion of TTSS effectors would occur only when the bacteria reach the surface of intestinal epithelial cells, where oxygen is more abundant (158, 206). Nonpolar null mutations in all of the *mxi-spa* genes tested to date result in loss of the ability to secrete the Ipa proteins and loss of invasive capacity for cultured cells. Hence, T3SS is an essential component of *Shigella* virulence. The exception to this statement, i.e., loss of invasive capability, is *mxiE*, which encodes a transcriptional regulator of genes expressed

postinvasion (207). MxiE is addressed below in the section on gene regulation.

The *spa* genes encode proteins that share significant homologies with proteins involved in flagellar synthesis in *E. coli*, *Salmonella* Typhimurium, *Bacillus subtilis*, and *Caulobacter crescentus* (12). Included among these genes is *spa47*, which encodes a protein that probably functions as the energy-generating component of the secretion apparatus, since it shows sequence similarities with ATPases of the flagellar assembly machinery of other bacteria. In addition, there is an even more striking similarity in both gene organization and predicted protein sequence between the *mxi-spa* region of the *Shigella* virulence plasmid and a virulence-associated chromosomal region of *Salmonella* Typhimurium (208). The *Salmonella spa* region contains homologues of the *Shigella spa* genes in the same gene order. Sequence identities between the protein homologues are as high as 86% (Spa9 versus SpaQ). The relatedness of the *spa* regions strongly suggests that these human pathogens evolved similar mechanisms for secretion of the virulence proteins required for signal transduction with the mammalian host. Plant pathogens such as *Pectobacterium carotovorum*, *Xanthomonas campestris*, and *Ralstonia solanacearum* also contain genes that encode homologues of the *mxi-spa*-encoded proteins (209). It is now recognized that the T3SS for transport of virulence proteins is a critical element of both plant and animal bacterial pathogenesis.

The virulence plasmid encodes two distinct families of effector proteins that are secreted by the T3SS. The first, designated Osp (outer *Shigella* proteins), has several functions to regulate intracellular survival once *Shigella* is inside epithelial cells (210). Functions include modulation of host cell signaling pathways, such as the NF-κB and the ERK1/2 and p38 MAPK pathways (OspB [211, 212], OspC1 [213], OspF [212–214], OspG [215], OspI [216], and OspZ [217, 218]), maintenance of epithelial cell integrity (OspE1 and OspE2 [219]), regulation of host cell proliferation (OspB [220]), and inhibition of host cell death (OspC3 [221]). Most of the

consequences of altering host cell signaling appear to modulate the immune response, mostly by affecting interleukin 8 (IL-8) secretion from the epithelial cells and subsequent neutrophil (PMN) migration, a complex interplay during *Shigella* infection (222). Some effectors have been shown to induce IL-8 secretion and PMN migration during early infection to enhance invasion by increasing access to the basolateral pole (213, 223), while other effectors have been shown to decrease this response during later stages of infection to mitigate the immune response (212, 214, 224). OspD3 also alters IL-8 secretion (222), while the induction of PMN migration through hepoxilin A (3) has also been observed during *Shigella* infection (225). Clearly, continued research is needed to fully understand how and why *Shigella* modulates PMN migration during the various stages of infection

Additional functions described for the Osp proteins include negative regulation of virulence gene expression (the OspD1 antiactivator [226]; see below), enterotoxin activity to induce secretory diarrhea (OspD3 [160, 161]; also referred to as *Shigella* enterotoxin 2 [ShET2]), and adherence to the apical surface of epithelial cells that is mediated by bile salts exposure (OspE1 and OspE2 [227]).

The second family of T3SS effectors encoded on the virulence plasmid is the IpaH family, encoded by five separate genes (228). Most of the proteins in this family have E3 ubiquitin ligase activity, which is important for modulating NF-κB signaling and immune response signaling in epithelial cells (224, 229, 230) and enhancing pyroptosis in macrophages (231). There are an additional four *ipaH* genes (plus an additional three annotated pseudogenes or gene fragments) present on the *Shigella* chromosome whose protein products have been shown to be secreted through the T3SS (232). Thus far, function has been described only for IpaH0722, which ubiquitinates TRAF2 to further control NF-κB signaling (233).

A plasmid-borne virulence gene which is unlinked to the 37-kb region shown in Fig. 12.1 is not required for invasion but is crucial for intra-and intercellular motility. This gene, known as *virG* or *icsA*

(intracellular spread), encodes a protein that catalyzes the polymerization of actin in the cytoplasm of the infected host cell (234). The IcsA protein belongs to a family of autotransporter proteins that are large and are secreted through type V secretion (235, 236). IcsA is unusual in that it is expressed asymmetrically on the bacterial surface, being found only at one pole. The polymerization of actin monomers by IcsA forms a tail leading from the pole and provides the force that propels the bacterium through the cytoplasm. Hence, unipolar expression of IcsA imparts directionality of movement to the bacterium. A plasmid-encoded protease, SopA/IcsP, cleaves IcsA and is proposed to play a role in unipolar localization of IcsA (237). Synthesis of a complete LPS is also crucial for correct unipolar localization of IcsA (238), while the periplasmic chaperones DegP, Skp, and SurA further facilitate IcsA presentation to the outer membrane and subsequent unipolar localization (239). The use of translational fusions between IcsA and green fluorescent protein defined the amino acids that are sufficient for polar localization and confirmed correct polar localization of IcsA when it is expressed in *E. coli* as well as *Salmonella* Typhimurium, *Yersinia pseudotuberculosis*, and *Vibrio cholerae* (240). Interestingly, surface-expressed IcsA is a trigger for autophagy once the bacteria are inside the cytoplasm of epithelial cells. To protect the bacteria against this host intracellular degradation system, IcsB is secreted via the *Shigella* T3SS, which acts to shield IcsA from binding the autophagy protein Atg5 and thus protects the bacterium from being trapped and degraded within the infected host cell (241).

Characterization work has been performed on additional genes located on the virulence plasmid whose protein products encompass a variety of functions. Most of these proteins are secreted through the T3SS. First, additional proteins that facilitate bacterial entry have been identified. IpgD activates the small GTPase ADPribosylation factor 6 to promote bacterial invasion of epithelial cells (242). Invasion is further facilitated by IpgB1 and IpgB2, which activate Rac1 and RhoA, respectively, to promote bacterial entry

(243–245). Second, T3SS effectors have been shown to enhance IpaB-and IpaC-mediated bacterial escape from the macropinocytic vacuole (181) and enable *Shigella* entry into the host cell cytoplasm. IpaJ and VirA fragment the Golgi apparatus to disrupt the entry vacuole and allow the bacteria to gain access to the host cell cytoplasm (246, 247). VirA may also facilitate IcsB to disrupt autophagy (248). Third, a few T3SS effectors enhance epithelial cell survival during infection. The chaperone Spa15 (249) has also been shown to be secreted during later stages of infection to inhibit epithelial cell apoptosis upstream of caspase-3 activation (250), but a mechanism remains to be determined. IpgD further enhances epithelial survival by activating the Akt–phosphatidylinositol 3-kinase signaling pathway by phosphorylating Akt (251). Overall, *Shigella* induces several mechanisms of apoptosis inhibition during epithelial cell infection (221, 252) that require further exploration.

Regarding non-T3SS effectors, the genes in the *shfwabB-virK-msbB2* operon have been implicated in cell wall biosynthesis (253–255). MsbB2 is a second copy of the myristoyl transferase involved in lipid A synthesis. Interestingly, *msbB2* is regulated by the PhoPQ two-component regulatory system under low-magnesium conditions, which was hypothesized to be important for LPS modifications to enhance virulence (255). Shf (or SfPgdA) has been characterized as a peptidoglycan deacetylase also controlled by PhoPQ and involved in bacterial persistence in the presence of neutrophils (254). Finally, SepA is another autotransporter protein that does not require the T3SS apparatus for secretion. Originally, SepA was demonstrated to be important for the overall infection process through the rabbit ileal loop model, while subsequent studies identified proteolytic activity (256, 257). Separate research identified SepA as an overall contributor to *Shigella* enterotoxin activity (160), while recent work has demonstrated that SepA disrupts the epithelial cell barrier to enable *Shigella* invasion at the basolateral pole of the epithelium (258). A mechanism was presented in which SepA degraded the activated form of LIM kinase 1, which resulted in increased activation of cofilin and subsequent

opening of tight junctions. As epithelial barrier disruption and enterotoxin activity are related (259), this result is interesting in that it helps to explain previous research findings for SepA and provides us with a better understanding of how *Shigella* accesses and efficiently invades the basolateral pole of the epithelium.

Chromosomal Virulence Loci

Although *Shigella* and *E. coli* are very closely related at the genetic level, there are significant differences beyond the presence of the virulence plasmid in *Shigella*. Pathogenicity islands, clusters of genes that have a role in virulence, have been identified in several virulent bacteria, including *Shigella*. These regions are typically large (20 to 200 kb) and, in addition to virulence genes, may contain transposable elements, insertion sequences, and remnants of mobility plasmid or bacteriophage genes, all indicating possible horizontal transfer of genetic information.

Pathogenicity islands have been identified in the chromosome of *S. flexneri* (Table 12.1). SHI-1 (*Shigella* pathogenicity island 1) contains the *set1A* and *set1B* genes, which encode *Shigella* enterotoxin 1 (ShET1) (163). The genes are contained in the opposite orientation within the ORF of another gene, *pic*, which encodes an autotransporter protein with putative hemagglutinin, mucinase, and immune-modulating activities (260–262). The genes for ShET1 are chromosomal and are present in *S. flexneri* but have not been detected in other *Shigella* species. Pic has also been shown to result in enterotoxic activity, since a construct of *pic* with four point mutations to disrupt the *set1A* and *set1B* genes while maintaining the appropriate codons for *pic* was able to complement a mutant with a *pic-set1AB* deletion (160). A second autotransporter encoded on SHI-1, SigA, also displays enterotoxin activity (263). Therefore, data suggest that *Shigella* expresses five enterotoxins encoded on both the chromosome and the virulence plasmid (ShET1, ShET2/OspD3, Pic, SepA, and SigA).

The *iuc* locus and *iutA*, which respectively contain the genes for aerobactin synthesis and transport as well as the aerobactin-siderophore receptor, are present in SHI-2 (264, 265). A similar pathogenicity island was identified in *S. boydii* in which the *iuc* and *iut* genes were conserved, but it was located in a distinct region of the chromosome relative to *S. flexneri* and thus named SHI-3 (266). Aerobactin is a hydroxamate siderophore that *Shigella* uses to scavenge iron. Work with *S. flexneri* demonstrated that when the *iuc* locus is inactivated, the aerobactin-deficient mutants retain their capacity to invade host cells but are altered in virulence, as measured in animal models. These results suggest that aerobactin synthesis is important for bacterial growth within the mammalian host (267, 268). Since *iuc* and *iutA* are important for transport of ferric iron, the predominant form of iron under aerobic conditions, additional analysis of iron acquisition and regulation was performed under anaerobic conditions (269). Under anaerobiosis, the *feoABC* genes were activated while the *sit* and *iuciut* genes were repressed. This regulation was mediated through two transcription factors, AcrA and Fnr, in which ArcA was involved in both the activation and repression of the respective gene clusters while Fnr was also utilized for *feoABC* activation. Furthermore, the *fur* gene that encodes the iron-responsive repressor for bacterial iron acquisition was also repressed under anaerobic conditions by ArcA. These studies highlight the ability of *Shigella* to regulate iron acquisition under both aerobic (more environmental and early *in vivo* settings) and anaerobic (the human colon) conditions.

Finally, the SHI-O pathogenicity island of *S. flexneri* includes the *gtrA*, *gtrB*, and *gtr* genes, which are important for O-antigen modifications and serotype conversion (270). It was demonstrated that glucosylation of *Shigella* LPS results in shortening of the LPS to enable better contact of epithelial cells by the T3SS needle without compromising LPS protection of the bacteria (271).

S. dysenteriae type 1, like *E. coli* O157:H7, produces Shiga toxin and is responsible for some of the symptoms of HUS. Acquisition of the Shiga toxin genes *stxA* and *stxB* by *S. dysenteriae* has been

postulated to be a consequence of a lysogenic bacteriophage. Over time, the phage became stably integrated due to loss of genetic function of essential phage genes, most likely as a result of rearrangement and transposition events. Shiga toxin inhibits protein synthesis by inactivating 28S rRNA in the 60S subunit of mammalian ribosomes and preventing elongation factor 2 from interacting with the ribosome (272). The Shiga toxin produced by *S. dysenteriae* and the Shiga toxin elaborated by *E. coli* O157:H7 are nearly identical, differing from each other by just one amino acid. A mutation in the *stx* locus does not alter the ability of the bacterium to invade epithelial cells or cause keratoconjunctivitis in the Sereny test. However, when tested in macaque monkeys, the mutant strain causes less vascular damage in the colonic tissue than the toxin-producing parent (273). Hence, production of Shiga toxin may account for the fact that *S. dysenteriae* 1 infections are generally more severe than those caused by the other species of *Shigella*. Recent studies have documented the emergence of *S. flexneri* and *S. sonnei* strains harboring Shiga toxins (16, 101, 274–277), leading to the possibility of HUS and significant renal complications from infections with other species of *Shigella* (278).

Chromosomal Avirulence Loci

The early genetic studies by Formal et al. (165), and reports that select genetic loci are not present in the *Shigella* chromosome but are present in the chromosome of nonpathogenic *E. coli* (279), suggest that the absence of particular genes in *Shigella* has had a direct consequence on its pathogenesis. The absence of these genetic determinants in *Shigella* spp. occurred by either deletions or mutations and is referred to as a "black hole" (Fig. 12.1) (57). When a gene complementary to a genetic locus that is absent in *Shigella* is introduced into the pathogen, the presence of that newly acquired gene product in *Shigella* may inhibit the action of a particular virulence factor. For instance, although lysine decarboxylase activity is present in >85% of *E. coli* strains, it is missing in all strains of *Shigella* spp. and EIEC. Lysine decarboxylase (encoded by the *cadA*

gene) produces cadaverine from lysine. Cadaverine inhibits the action of the *Shigella* enterotoxins that are believed to be responsible for the diarrheal symptoms associated with shigellosis. Therefore, *cadA* is considered an antivirulence gene for *Shigella* (57). Thus, as part of the evolution of *Shigella* to a human pathogen from a nonpathogenic *E. coli* lineage, the acquisition of the large virulence plasmid may have also involved the loss of specific incompatible genetic loci. Recently, Yang et al. reviewed the role of IS elements in the evolution of *Shigella* genomes from an ancestral *E. coli* strain (24).

Virulence Gene Regulation

An important feature of *Shigella* pathogenesis is the ability of the bacterium to modulate expression of its virulence genes in response to growth temperature (12). Several activators and a repressor control the virulence regulon of *Shigella* such that genes are turned on at 37°C and turned off at 30°C. The product of the chromosomal *virR* (*hns*) locus is a histone-like protein, H-NS, which behaves as a repressor of *Shigella* virulence gene expression in response to growth temperature (167). Mutations in *virR* (*hns*) cause deregulation of temperature control and result in expression of genes in the virulence regulon even at the nonpermissive temperature of 30°C. The *virR* (*hns*) locus is allelic with regulatory loci in other enteric bacteria, and like *virR* (*hns*), these alleles act as repressors of their respective regulons (references cited in reference 169). Several different models to explain how VirR/H-NS acts as a transcriptional repressor have been proposed (169). However, because VirR/H-NS is involved in gene regulation in response to diverse environmental stimuli such as osmolarity, pH, and temperature, a comprehensive model to explain its activity has been elusive.

H-NS binds to the promoter regions of two transcriptional activators, *virF* and *virB*, and blocks their transcription at 30°C (280, 281). At 37°C, the H-NS binding sites undergo a conformational transition and no longer bind H-NS, thus leading to

increased transcription of the activator genes (12). Expression of genes in the *ipa* and *mxi-spa* clusters is dependent on VirB, and mutations in *virB* abolish the bacterium's ability to invade tissue culture cells. Transcription of *virB* is dependent on growth temperature and VirF (282). VirB is a DNA-binding protein and shares homology with the plasmid-partitioning proteins ParB of bacteriophage P1 and SopB of plasmid F. Purified VirB shows preferential binding to the intergenic *icsB-ipgD* region (Fig. 12.2) and displays a similar preference for binding to the *spa* and *virA* gene promoter regions (283). Recently, analysis of the transcriptional regulator SlyA demonstrated that the protein can rescue virulence gene expression in the absence of VirB (253). Through various reporter assays, exogenous expression of *slyA* resulted in induction of *mxiC* and *icsP* as well as restoration of the Congo red-binding phenotype. The authors hypothesize that SlyA may recognize VirB binding sites and/or affect H-NS protein levels. Future work will be required to understand the full role of SlyA, especially since the authors also demonstrated that the SlyA regulator was required for acid resistance in *S. flexneri* and that *slyA* expression is regulated by the PhoPQ regulon.

The product of the *virF* locus is a key element in temperature regulation of the *Shigella* virulence regulon. A helix-turn-helix motif in the carboxyl terminal portion of VirF is characteristic of members of the AraC family of transcriptional activators. Consistent with its predicted role as a DNA-binding protein, VirF binds to sequences upstream of *virB* (284). The binding of VirF may act as an antagonist to binding by VirR/H-NS and thereby provide a mechanism for responding to temperature. Expression of *virF* is subject to temperature regulation by H-NS. Repression of *virF* occurs at the critical temperature of <32°C and takes place through the binding of H-NS at two sites within the *virF* promoter (280). Interestingly, a new study demonstrated that VirF can also bind the small noncoding RnaG and *icsA* mRNAs via a 13-bp consensus sequence to disrupt the senseantisense binding of the mRNAs and alleviate RnaGmediated transcriptional repression of *icsA* (285).

This study identifies a new function of VirF that could have important implications in our understanding of *Shigella* virulence gene activation and the role of bacterial transcriptional regulators.

Activation of the T3SS leads to increased expression of a subset of virulence genes encoding secreted effectors. Expression of these genes is regulated by MxiE, a transcriptional activator of the AraC family (207). MxiE-regulated promoters are preceded by a 17-bp MxiE box 33 to 49 bp upstream of the transcription start site (286). Activity of MxiE requires a coactivator, IpgC, the chaperone of IpaB and IpaC (287, 288). Under nonsecretion conditions, IpgC is associated with IpaB and IpaC, whereas MxiE is associated with the T3SS substrate OspD1, which acts as an antiactivator. Hence, both activator (MxiE) and coactivator (IpgC) are bound and unavailable to interact with each other. When the secretion apparatus is activated, IpaB and IpaC are secreted and release IpgC. After OspD1 is secreted, MxiE is released and is free to bind IpgC and activate MxiE-controlled promoters (226). Expression profile analysis defined three classes of T3SS-secreted substrates: (i) those that are controlled by VirB (expressed independently of secretion activity); (ii) those that are controlled by MxiE (expressed only in conditions of secretion); and (iii) those that are controlled by both VirB and MxiE (expressed under conditions of nonsecretion and induced under conditions of secretion) (289).

In all, the virulence genes and related regulation highlight the human-adapted evolution that *Shigella* underwent to target specific pathways and ensure successful infection once inside the human host. Many of the virulence proteins have multiple functions, and as new technologies become available, additional functions may be discovered. However, despite extensive research and characterization work on virulence genes, there are still numerous annotated genes of unknown function on both the chromosome and virulence plasmid. Furthermore, functions have not been described for the T3SS effectors OspC2, OspC4, OspD2, IpaH1.4, IpaH2.5, and most of the chromosomal IpaH proteins. Continued research will certainly enhance our understanding of the *Shigella* pathogenesis

and will have important implications for successful vaccine development.

CONCLUSIONS

Foodborne infections due to *Shigella* spp., in spite of national and international efforts to reduce their incidences, remain a serious cause of diarrheal diseases. These pathogens have the potential for explosive spread due to the low infectious dose that can cause overt clinical disease. In addition, cases of bacillary dysentery frequently require medical attention (even hospitalization) and cause lost time from work, as the severity and duration of symptoms can be incapacitating. As yet, there is no effective vaccine against any *Shigella* spp. These features, coupled with the wide geographical distribution of the strains, the potential rapid spread, and the stark increase in MDR strains, make *Shigella* a formidable public health threat.

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13
Vibrio Species

The genus *Vibrio* contains 130 confirmed species (http://www.bacterio.net/vibrio.html), of which a dozen have been demonstrated to cause infections in humans. As vibrios are natural inhabitants of aquatic environments, infections are usually associated with wound exposure to seawater or consumption of raw seafood. As estimated by the Centers for Disease Control and Prevention (CDC), vibriosis causes approximately 80,000 illnesses and 100 deaths in the United States every year, mostly during the summer months, when water temperatures are warmer. In contrast to illnesses caused by other major foodborne pathogens, the number of *Vibrio* infections is steadily increasing (https://www.cdc.gov/vibrio/index.html). Several reports have recently indicated that human *Vibrio* illnesses are increasing worldwide, as well as the species responsible for these infections. Besides "the big four" (*Vibrio cholerae*, *Vibrio vulnificus*, *Vibrio parahaemolyticus*, and *Vibrio alginolyticus*) (1), additional *Vibrio* species [*Vibrio fluvialis*, *Vibrio mimicus*, *Grimontia* (*Vibrio) hollisae*, *Vibrio metschnikovii*, *Vibrio metoecus*, and *Vibrio furnissii*] have recently been associated with food consumption (2). These 10 *Vibrio* species are the subject of this chapter.

NATURAL HABITATS

Vibrios are natural constituents of estuarine and marine environments. Vibrios spend much of their life cycle outside the human host and have adapted to fluctuating conditions, including temperature shifts, nutrient limitation, osmotic stress, and predation (3). Vibrios tend to be more common in warmer waters, especially above 17°C, and depending on the species, they tolerate a range of salinities (5 to 25 ppt). Because temperature largely defines the environment where vibrios can proliferate, climate change in marine systems is greatly influencing the spread of this genus (1, 4). Reported vibriosis cases are on the rise, especially in northern countries experiencing warming sea temperatures (5). In the aquatic environments, vibrios show a wide range of niche specialization, from free-living forms to those attached to biotic (e.g., copepods, crustacean, fish, and algae) and abiotic surfaces, from symbionts to pathogens (6), contributing to their growth and persistence in the natural environment. *Vibrio* species are commonly isolated from filter-feeding shellfish, such as oysters, where they can be present at concentrations 100-fold higher than those in the surrounding water. For this reason, most foodborne *Vibrio* infections are caused by consumption of oysters and other bivalves that are consumed frequently without processing or cooking.

EPIDEMIOLOGY AND SURVEILLANCE

The cell numbers of most *Vibrio* spp. in both surface waters and shellfish exhibit a seasonal variation, generally being greatest during the warm months. Infection seasonality is most notable for *V. vulnificus* and *V. parahaemolyticus* from environmental sources, whereas some other vibrioses, such as *V. fluvialis* infections, occur throughout the year.

At the time of writing (January 2018), more than 2,000 scientific papers addressing *Vibrio* contamination of seafood and the food chain were available online. However, while cholera cases caused by *V. cholerae* O1 and O139 are notifiable for the World Health Organization, other vibrioses do not entail this requirement. Therefore, epidemiology of these infections is partial, as most cases go unreported worldwide.

In the United States, the Cholera and Other Vibrio Illness Surveillance System (COVIS) (https://www.cdc.gov/vibrio/surveillance.html) has been in use since the 1970s for reporting human infections caused by all pathogenic species of the family *Vibrionaceae*, but only in 2007 did vibriosis become a nationally notifiable disease to the CDC. The CDC also monitors vibriosis through the 10-state Foodborne Diseases Active Surveillance Network (FoodNet). Together, these systems conduct passive and active surveillance for laboratory-confirmed *Vibrio* infections. *Vibrio* infections were reported as the most common cause of seafood-associated outbreaks between 1973 and 2006, with substantial year-to-year variability observed in the number of outbreaks of *Vibrio* infections (7). This trend was confirmed by more recent COVIS data, which shows that the incidence of vibriosis in the U.S. population has been steadily increasing from 1996 to 2010, mostly for *V. parahaemolyticus*, *V. vulnificus*, and *V. alginolyticus* infections (8).

The most recent data from COVIS reported 1,252 *Vibrio* infections (excluding toxigenic *V. cholerae* O1 and O139) in 2014 (2). The most frequently reported species was *V. parahaemolyticus* (48%), followed by *V. alginolyticus* (19%) and *V. vulnificus* (10%). The majority of infections (56%) were confirmed as foodborne, were associated with mollusk (particularly oyster) and crustacean consumption, and were reported in Pacific (34%), Atlantic (26%), and Gulf (26%) Coast states. The only seven cases of cholera reported in 2014 were travel associated and caused by *V. cholerae* O1 (2).

ISOLATION

In marine and estuarine environments, vibrios are commonly isolated from sediment, the water column, and vertebrate and invertebrate animals. A complete description of the U.S. Food and Drug Administration (FDA) protocol for *Vibrio* isolation and identification can be found elsewhere (9), whereas ISO methods and guidelines are available at https://www.iso.org/obp/ui/#iso:std:iso:21872:-1:ed-1:v1:en. A schematic representation is provided in Fig 13.1. Briefly, for food and environmental samples, it is highly recommended to use a pre-enrichment step in alkaline peptone water with plating after 6 to 8 hours (to prevent overgrowth by other species) or overnight incubation (9), followed by streaking for isolation onto one or a combination of media to improve detection or isolation.

Specific solid media include thiosulfate-citrate-bile salts-sucrose (TCBS) agar, taurocholate-tellurite-gelatin agar, and CHROMagar *Vibrio*. Metabolic differences among species are the differentiating traits when these media are used. For example, TCBS agar inhibits the growth of *Enterobacteriaceae* and Gram-positive species and allows *Vibrio* differentiation between sucrose-positive (*V. cholerae*, *V. metschnikovii*, *V. fluvialis*, *V. furnissii*, and *V. alginolyticus*) and sucrose-negative (*V. mimicus*, *V. parahaemolyticus*, and *V. vulnificus*) species. Selective media have been developed for single species, such as modified cellobiose-polymyxin B-colistin and *V. vulnificus* medium (10), specific for *V. vulnificus*. New chromogenic technologies have been applied to differentiate species that show the same metabolic profile, e.g., *V. parahaemolyticus* and *V. vulnificus*, which are both sucrose negative on TCBS agar. Therefore, the use of more than one medium is suggested for completeness and convergence on the best results. A comprehensive review of media, their modes of action, their use, and typical results is presented elsewhere (11).

IDENTIFICATION

Presumptive *Vibrio* colonies isolated from these media can be confirmed by a standard series of biochemical media used for identification of *Enterobacteriaceae* and *Vibrionaceae*. Accurate phenotypic identification of *Vibrio* species is problematic, largely because of the great variability in biochemical characteristics that they exhibit. Both conventional tube tests and commercially available enteric identification systems are suitable for identifying this species, although the accuracy of commercial identification kits can range from 50 to 97% (12, 13). An updated description of the differentiating features of the 10 human-pathogenic vibrios associated with food can be found in the Bacteriological Analytical Manual

(https://www.fdagov/food/foodscienceresearch/laboratorymethods/ucm070830.htm). The main differentiating features of the 10 human-pathogenic vibrios associated with food are shown in Table 13.1.. Specific biochemical tests help discriminate ambiguous species, such as *V. fluvialis* versus *V. cholerae* or *Aeromonas* spp., thanks to their reactions to lysine, ornithine decarboxylases, arginine dihydrolase, and l-arabinose (14). The simplest trait for differentiation from *Aeromonas* spp. (especially *Aeromonas hydrophila*) is the inability of *V. fluvialis*, being halophilic, to grow in media lacking NaCl. Most members of the genus are halophilic, and the addition of NaCl is often required for isolation; however, the concentration of NaCl can affect the biochemical profile and lead to erroneous identification.

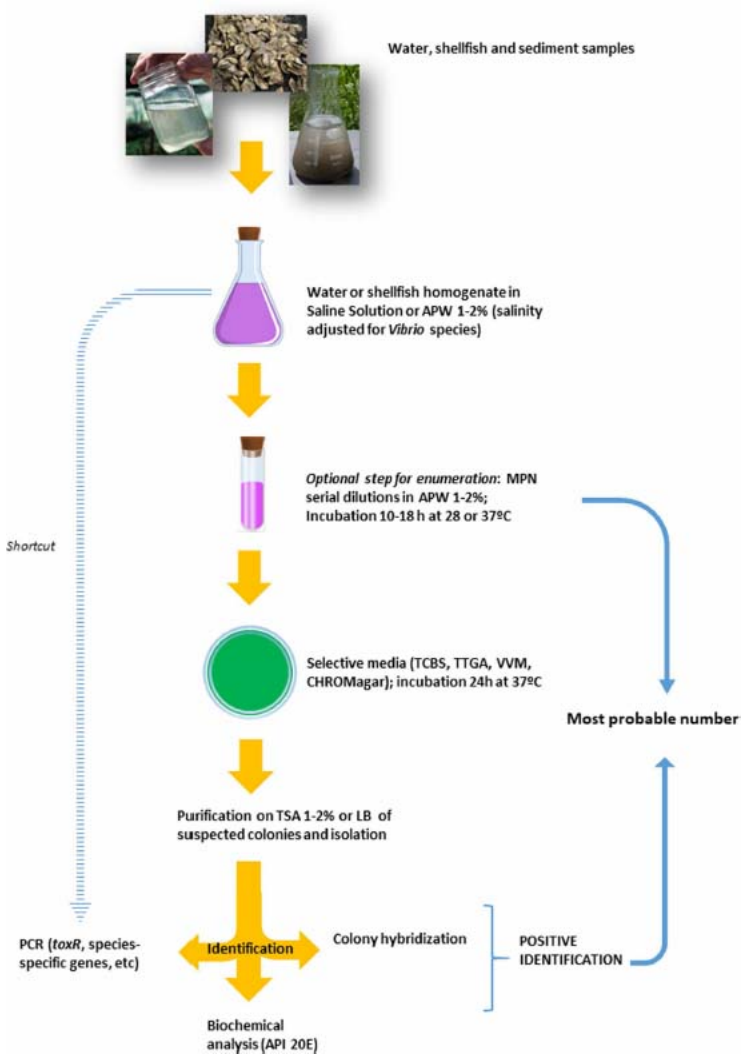


Figure 13.1 Scheme for the isolation and identification of *Vibrio* spp. from seafood, water, and sediment samples; adapted from FDA protocols (9). Once the bacterium has been isolated and identified, the pure culture should be maintained at −80°C in LB or tryptic soy broth supplemented with 15 to 20% glycerol. MPN, most probable number; APW 1-2%, alkaline peptone water containing 1 to 2% NaCl; TTGA, taurocholate-tellurite-gelatin agar; VVM, *V. vulnificus* medium. For more details and species-specific PCR, see "Isolation" and sections on individual *Vibrio* species in the text.

Molecular methods that utilize PCR and nucleotide sequence determination overcome many of the limitations of phenotypic methods and can lead to identification of an isolate within hours, including those that are not viable or are otherwise unculturable. This field of research is in continuous evolution, and new molecular techniques are available every day.

Among them, one of the latest tools to discriminate among closely related pathogenic *Vibrio* species is based on *groEL* PCR-restriction fragment length polymorphism (RFLP) analysis, where the highly conserved gene *groEL* is a better phylogeny marker than 16S rRNA gene (15). This technique can be applied for accurate identification and differentiation of *V. mimicus*, *V. cholerae*, *V. parahaemolyticus*, *V. alginolyticus*, *V. vulnificus*, *V. fluvialis*, *and V. anguillarum*. Other techniques were developed to discriminate among different *Vibrio* species in food samples and for food safety assessment. This is the case for a tetraplex PCR for simultaneous detection of *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, and *V. mimicus* in cockles (16) and for a DNA microarray for the direct identification and pathogenic characterization of harmful strains of *Vibrio* in shellfish (17). To establish the relatedness of *Vibrio* isolates from disease epidemics, discriminate among strains with more or less potential to cause disease or epidemics, and explore the population biology of these bacteria, several DNA-based techniques are employed. For species-specific methods, see the sections on individual *Vibrio* species below.

ENUMERATION

Due to the public health risk that *Vibrio* causes, it is important to have a rapid and reliable method for isolation and enumeration of *Vibrio* densities in seafood products. Depending on the pathogen, different standard methods are recommended. Most-probable-number titration of replicate samples in enrichment broth cultures and direct plating onto selective agar plates followed by incubation and membrane filtration are the most commonly used (9). Implementation of enumeration techniques is ongoing, and new protocols have been developed to address isolation and enumeration of *Vibrio* species in specific food products (18). Among others, a new selective and differential medium, *V. vulnificus* X-Gal, was developed for direct enumeration of *V. vulnificus* organisms from oyster samples (19), and a colony hybridization method was developed for the enumeration of total and potentially enteropathogenic *V. parahaemolyticus* organisms in shellfish (9, 20).

Table 13.1 Key differential traits of pathogenic food-associated *Vibrio* species[a]

					% of strains positive[b]				
Test	*V. cholerae*	*V. parahae-molyticus*	*V. vulnificus*	*V. algino-lyticus*	*V. fluvialis*[c]	*V. furnissii*[c]	*G. hollisae*	*V. mimicus*	*V. metschnikovii*
Growth on TCBS agar	Y	G	G	Y	Y	Y	NG	G	Y
Voges-Proskauer (1% NaCl)	75	0	0	95	0	0	0	9	96
Motility	99	99	99	99	70-89	100	0	98	74
Acid production from:									
Sucrose	100	1	15	99	100	100	0	0	100
D-Mannitol	99	100	45	100	97	100	0	99	96
Cellobiose	8	4	99	3	30	14	0	0	9
Salicin	1	1	95	4	0	0	0	0	9

[a] Adapted from https://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm070830.htm and references 249 and 329. Y, yellow; G, green; NG, no growth; ND, not determined.

[b] After 48 h of incubation at 36°C. Most positive reactions occur during the first 24 h.

[c] *V. fluvialis* and *V. furnissii* can be differentiated by the production of gas from D-glucose.

[d] *V. metoecus* characteristics are expressed as positive (+) or negative (-), as in reference 326. Diversity between *V. metoecus* strains exists for utilization of cellobiose (±).

CONTROL MEASURES TO MINIMIZE *VIBRIO* CONTAMINATION

Control strategies to prevent seafood-associated illnesses include consumer education, monitoring of harvest waters, and postharvest *Vibrio* reduction treatments. Consumers should be aware of the potential health risks associated with eating raw or undercooked shellfish, particularly persons with medical conditions, such as liver disease, that predispose them to severe illness. Thoroughly cooking shellfish and preventing raw seafood from cross-contaminating other food are effective measures consumers can take to reduce risk. Like other microbes present in water, vibrios can be concentrated in the tissues of filter-feeding bivalve mollusks.

Depuration is a common practice used to eliminate fecal bacteria from seawater in depuration plants, usually via treatment with either ozone or UV light (21). Depuration of naturally occurring bacteria such as vibrios presents additional challenges, since bacteria and oysters coexist in the environment and are adapted to each other. Recent data on high-salinity depuration proved this method to be promising for *V. parahaemolyticus* and *V. vulnificus* reduction in oysters (22–24). Postharvest processing methods are available to make oysters safer and greatly reduce the risk to consumers (25). The National Shellfish Sanitation Program of the FDA is constantly updating the guidelines for optimal shellfish handling and processing (26). The main postharvest treatments to reduce microbiological occurrence in seafood are summarized here.

Cold

Several options for temperature control to limit postharvest *Vibrio* growth include ice, mechanical refrigeration, and other approved means of lowering the temperature and maintaining it at 10°C or less. While reports generally indicate that vibrios are sensitive to cold temperatures, this adverse condition may also enhance virulence and resistance of the stressed bacteria (27–29), potentially increasing risks with regard to food safety. Recent proteomic studies demonstrated that *V. metschnikovii* is cold adapted (30), an advantageous feature for surviving temperature downshifts during food processing and storage. Psychrotrophic strains of *V. mimicus*, *V. fluvialis*, and *V. parahaemolyticus* from frozen seafood were found to survive well at 10, 4, and −30°C (31). The response of *V. vulnificus* to cold temperatures has been extensively investigated, and its persistence in oysters following freezing and storage at −20°C has been shown to be dependent on the length of frozen storage time and the application of vacuum-packaged samples.

Heat

The most common thermal process used to reduce microbiological occurrence and growth of vibrios in food is pasteurization. Heat shock at or above 60°C for several minutes right after harvesting, as well as boiling or steaming during cooking, is the most common method. All of the vibrios are sensitive to heat, although a wide range of thermal inactivation rates have been reported. The major pathogens targeted are *V. parahaemolyticus* and *V. vulnificus*, which are known to grow between 5 and 45°C. The FDA recommends steaming oysters, clams, and mussels for 4 to 9 min, frying shucked oysters for 10 min at 190°C, or baking oysters for 10 min at 232°C.

The heat-cool pasteurization method was developed in the mid-1990s for application to postharvest raw Gulf of Mexico oysters to reduce *V. vulnificus*. It consists of minimal heat application in shelled oysters to destroy *V. vulnificus* immediately followed by cooling. This process requires three consecutive steps: washing with potable water, immersion in water at 53°C, and immersion in water at 4°C.

Irradiation

The FDA has approved the irradiation of oysters and seafood (32). The use of gamma irradiation and, more recently, that of X rays are becoming efficient alternatives to thermal treatments.

A 6-log reduction in the number of CFU of *V. parahaemolyticus* has been observed both via X-irradiation (3.0 kGy) and gamma irradiation (3.0 kGy) treatments in ready-to-eat shrimp (33) and oysters (34), respectively, with no oyster mortalities registered. Similar results were obtained for *V. vulnificus* in oysters when X-ray irradiation was applied (1.0 and 3.0 kGy for half-and wholeshell oysters, respectively) (35).

High Pressure

High-pressure processing (HPP) is a commercial nonthermal process capable of inactivating and eliminating pathogenic and food spoilage microorganisms inside an oyster. It is applied to lower the overall bacterial load at a range between 200 and 600 MPa for 1 to 2 minutes (36). By destroying biological cells, HPP causes oyster meat and fish to become whiter, two factors that limit consumers' acceptance. Nevertheless, HPP was proved to reduce *V. vulnificus* (37) and *V. parahaemolyticus* CFU counts at low temperatures (38) in oysters.

Other Treatments

The use of phages (viruses of bacteria) to control *Vibrio* populations in the environment and within animal hosts has also been investigated. When applied to contaminated oyster meat, the *Siphoviridae* phages pVp-1 and VPp1a can significantly reduce *V. parahaemolyticus* concentrations, from 1.4×10^6 to 1.9 CFU/g within 12 h (39). However, the large and uneven surface area of the in-shell oyster represents a challenge for phage treatment on a large scale.

While the various postharvest treatments are effective at killing seafood-associated bacteria, most of these treatments also kill the mollusks. Because consumer preferences for raw, live shellfish persist, biological approaches for reducing the load of shellfish-associated *Vibrio* spp. and promoting microbiological safety of live product, such as the use of probiotics or inhibitors of quorum sensing and attachment, are also under study (40).

V. CHOLERAE

V. cholerae is a non-spore-forming curved rod naturally occurring in brackish and estuarine environments around the planet, and it is classified according to its major surface antigen, the O antigen, into ~206 serogroups (41). Historically, seven distinct cholera pandemics caused by *V. cholerae* O1 have occurred since the onset of the first report in 1817, with the seventh ongoing pandemic starting in Egypt in 1961 (42). Important distinctions within this species are based on the production of cholera enterotoxin (Ctx), serogroup, and the potential for epidemic spread. Serogroups O1 and O139 have been associated with epidemic cholera disease (42, 43). Conversely, there are occasional strains of serogroups other than O1 or O139 that are clearly pathogenic, because of the production of Ctx or other virulence factors; however, none of these other serogroups has caused large epidemics or pandemics (see "Virulence Mechanisms" below). The subject of cholera has been reviewed elsewhere, and readers are referred to these reviews for primary references, particularly in the older literature (42).

Classification

V. cholerae O1

V. cholerae organisms of the O1 serogroup that produce Ctx have long been associated with epidemic and pandemic cholera (42). Ctx-negative *V. cholerae* O1 strains are generally considered nonpathogenic, although they have been isolated from occasional cases of diarrhea or extraintestinal infections. The O1 serogroup is further classified according to the presence of somatic antigens into two major serotypes (Inaba and Ogawa) and an unstable intermediate type (Hikojima) (1). *V. cholerae*

O1 can also be divided into two biotypes, classical and El Tor, that differ in several phenotypic and genotypic characteristics, including pathogenic potential, survivability, and infection patterns in humans (43). Phenotypic tests (sheep erythrocyte hemolysis, chicken cell agglutination, Voges-Proskauer, sensitivity to polymyxin B and specific phages), *ctxB* epityping, and genotypic analyses of specific genes (*tcpA*, *rstR*, and *ctxB*) are most commonly used to differentiate the two biotypes (43–45). However, both genetic and phenotypic diversity have arisen among strains of *V. cholerae* El Tor circulating in Asia and Africa, generating new pathogenic variants known as atypical El Tor variants (43, 45). Such variants include *V. cholerae* O1 hybrids, which cannot be biotyped based on phenotypic tests and can produce cholera toxin of either biotype, and altered El Tor variants, which produce cholera toxin of the classical biotype but can be biotyped as El Tor by conventional phenotypic assays (46–48). These new variants have subsequently replaced the prototype seventh-pandemic *V. cholerae* O1 El Tor strains with respect to frequency of isolation from cholera clinical cases.

V. cholerae O139 Bengal

In early 1993, the first reports of a new epidemic of severe, cholera-like disease emerging from eastern India and Bangladesh appeared (49). Further investigations revealed that the bacterium causing the disease did not belong to the O1 serogroup but to a new serogroup, which was given the designation O139 and the synonym "Bengal," in recognition of the origin of this strain. *V. cholerae* O139 is indistinguishable from typical El Tor *V. cholerae* O1 strains in terms of clinical manifestations and Ctx sequences (49). However, this bacterium does not produce the O1 lipopolysaccharide (LPS) due to the insertion of a 35-kb region encoding the O139 antigen (50). Furthermore, it produces a polysaccharide capsule. When it initially appeared, the O139 serogroup replaced the O1 serogroup in some parts of Southeast Asia and was feared to represent a new pandemic (the eighth pandemic) of cholera. However, few cases of O139 disease were reported beyond Southeast Asia, and O1 cases rapidly became dominant again in this part of the world (51).

V. cholerae Non-O1/Non-O139

In recent years, all *V. cholerae* isolates that were identified as negative for the O1 serogroup were referred to as *V. cholerae* non-O1, or nonagglutinating vibrios. Most nonagglutinating strains do not produce Ctx and are not associated with epidemic diarrhea (50). Since the emergence of the O139 serogroup, these strains are collectively referred to as *V. cholerae* non-O1/non-O139. While most of these strains do not produce Ctx, some strains may produce other toxins and are generally associated with self-limited gastroenteritis or mild extraintestinal symptoms (although fatal cases of necrotizing fasciitis and septicemia associated with *V. cholerae* non-O1/non-O139 have also been reported [52, 53]). Strains of the O141 serogroup have been isolated from sporadic cases of severe diarrhea and have been found to produce Ctx and the toxin-coregulated pilus (Tcp) colonization factor typical of O1 and O139 strains (54). Toxigenic and nontoxigenic non-O1/non-O139 strains have caused several disease outbreaks in Asia and other countries in recent years (55), which appears to be related to ongoing climate change and ocean warming (5, 56).

Identification

Together with standard culturing techniques (see "Isolation" and "Identification" above), the key confirmation for identification of *V. cholerae* O1 is agglutination in polyvalent antisera raised against the O1 antigen. Polyvalent antiserum for *V. cholerae* O1 and O139 is commercially available and can be used in slide agglutination or coagglutination tests. Monoclonal antibody-based coagglutination tests suitable for testing isolated colonies or diarrheal stool samples for O1 or O139 are also available commercially. Oxidase-positive bacteria (determined using colonies grown on nonselective media) that agglutinate in O1 or O139 antisera can be reported presumptively as *V. cholerae* O1 or O139 and then forwarded to a public health reference laboratory for confirmation. Antisera for serogroups other than O1 or O139 are not commercially available.

Nucleic acid probes are not routinely employed for the identification of *V. cholerae* due to the ease of identifying this species by conventional methods. However, DNA probes and PCR techniques have

been extremely useful for direct detection of the bacterium in environmental (57) and complex (58) samples and in distinguishing cholera toxin-encoding strains of *V. cholerae* (59). This distinction is particularly important in examining environmental isolates of *V. cholerae*, since most of these strains lack Ctx. Subtyping of *V. cholerae* strains using a variety of techniques, such as RFLP analysis by pulsed-field gel electrophoresis (RFLP-PFGE), ribotyping, and multilocus sequence typing (MLST), has yielded significant insights into the molecular epidemiology of *V. cholerae*. MLST using only three housekeeping genes (*gyrB*, *pgm*, and *recA*) has discriminating ability superior to that of other methods, including PFGE (60). Another approach is to use a single multiplex PCR assay to simultaneously amplify 95 "diagnostic" regions from *V. cholerae* and other *Vibrio* species (e.g., species- and serogroup-specific genes, toxin genes, etc.) that are hybridized to a microarray containing these genes (61). This approach allows rapid and definitive inter-and intraspecies discrimination that can be helpful in epidemiological, environmental, and health risk assessment surveillance. Strain heterogeneity is most comprehensively captured by genomic DNA sequencing. In particular, third-generation single-molecule real-time sequencing is much faster and more productive than previously developed methods (62). Next-generation sequencing techniques combined with whole-genome enrichment protocols have also proven useful for the direct genotyping and metagenomic analysis of low-abundance (<50 genome units/liter) *V. cholerae* DNA from environmental samples (63).

Foodborne Outbreaks

The critical role of water in transmission of cholera has been recognized for more than a century (42). In developing countries, ingestion of contaminated water and food is probably the major vehicle for transmission of cholera, whereas in developed countries, foodborne transmission is more important (64). The role of food, especially shellfish, in transmitting *V. cholerae* O1 has been reviewed elsewhere (65). The spectrum of food items implicated in transmission of cholera includes crabs, shrimp, raw fish, mussels, cockles, squid, oysters, clams, rice, raw pork, millet gruel, cooked rice, street vendor food, frozen coconut milk, and raw vegetables and fruit. One shared characteristic of the implicated foods is their neutral or nearly neutral pH (66). Survival and growth of *V. cholerae* O1 in food are also enhanced by low temperatures, high organic content, high moisture content, and absence of competing microbiota. Survival is increased when food is cooked before contamination; cooking eliminates competing microbes and has also been suggested to destroy some heat-labile growth inhibitors and produce denatured proteins that the bacterium uses for growth.

In addition, food buffers *V. cholerae* O1 against killing by gastric acid. While many different food items can provide this buffering capacity, the protection provided by chitin is noteworthy because crustaceans are frequent vehicles of disease (67). In the United States, both domestically acquired and imported cases of cholera have occurred (7, 68). For domestic cases, crabs, shrimp, and oysters have been the most frequently implicated vehicles, although the largest single outbreak (16 cases) was due to ingestion of contaminated rice (66). The risk of imported cholera has greatly increased since the establishment of endemic cholera in South America in 1991. The largest such outbreak (75 cases) involved crab salad served on an airplane flying from Peru to California. Importation of cholera from Asia can also occur, even in commercially imported food. Most cases of gastroenteritis caused by *V. cholerae* non-O1/non-O139 have been linked to the consumption of raw oysters (69), and infection is enhanced in food with alkaline pH. Outside the United States, outbreaks have also been linked to consumption of contaminated potatoes, chopped eggs, pre-prepared gelatin, vegetables, and meat samples (69).

Environmental Reservoir

In the 1970s, it was widely accepted that asymptomatic and convalescent human carriers were the primary reservoir for cholera. With the recognition that *V. cholerae* is part of the normal, free-living (autochthonous) microbiota in estuarine and coastal marine areas, greater attention has been given to identification and characterization of environmental reservoirs (70). *V. cholerae* non-O1/non-O139 strains are much more commonly isolated from the environment than O1 strains, and outside areas where epidemics occur, O1 environmental isolates are almost always Ctx negative. However, it is clear

that Ctx-producing *V. cholerae* O1 can persist in the environment in the absence of known human disease, and this is most likely explained by the existence of environmental reservoirs, defined as locations outside the human body favoring bacterial persistence and replication in the environment and pathogen transmission to susceptible hosts (71). Persistence of *V. cholerae* within the environment can be facilitated by several factors, such as its ability to assume survival forms, including a viable-but-nonculturable (VBNC) state, biofilms, and a rugose survival form. In the dormant VBNC state, *V. cholerae* is still viable but not culturable in conventional laboratory media (72), and cells are reduced in size and maintain virulence properties (60). *V. cholerae* produces a chitinase and is able to bind to chitin, which is the principal component of crustacean shells (67), enabling *V. cholerae* strains to colonize zooplankton such as copepods, with 104 to 105 attached *V. cholerae* cells per copepod (73). Other aquatic biota, such as water hyacinths, filamentous green algae, and insects, as well as abiotic substrates (e.g., sediments) are also colonized by *V. cholerae* (70). *V. cholerae* can also form biofilms that can enhance survival in the environment (74) and allow toxigenic strains to persist alongside nontoxigenic strains in the aquatic environment (75). A phenomenon that is closely related to biofilms is the formation of rugose, or "wrinkled," colonial morphology due to the production of an exopolysaccharide (42). In this state, the cells are protected against adverse environmental conditions and have an enhanced capacity to form biofilms.

According to the "cholera paradigm," the establishment of cholera endemicity requires the presence of aquatic environmental reservoirs, with seasonal and weather-related increases in toxigenic *V. cholerae* O1 in these reservoirs serving as the primary trigger for human epidemics (71). A dynamic relationship between human and environmental sources of the bacterium is apparent, with carriage and amplification by human populations playing a critical role in the epidemic spread of Ctx-producing *V. cholerae*. Environmental bacteriophages capable of lysing *V. cholerae* O1 or O139 are also involved in this relationship, and a 3-year study in Bangladesh revealed that the presence of cholera phages was inversely correlated with the occurrence of viable *V. cholerae* in the environment and the number of cholera cases in the local community (76). Susceptible hosts become infectious after consuming *V. cholerae* from an environmental source, sometimes accompanied by lytic bacteriophages. Infected individuals recover through the actions of their immune systems and possibly those of lytic bacteriophages or succumb to the infection. Lytic phages and *V. cholerae* in a transient hyperinfectious state are shed in varying concentrations and can rapidly passage to the next host, can persist in the environment as culturable cells with unknown infectivity, or can transform into a VBNC state.

Characteristics of Disease

Long-term carriage of *V. cholerae* in humans is extremely rare and is not considered significant in transmission of disease. However, short-term carriage of *V. cholerae* by humans is quite important in transmission of disease. Even after cessation of symptoms, patients who have not been treated with antibiotics may continue to excrete vibrios for 1 to 2 weeks. Furthermore, a high percentage of persons infected with *V. cholerae* in areas where the organism is endemic have inapparent illness (asymptomatic carriers) and can still excrete the bacteria, although excretion generally lasts for less than a week. There have also been studies indicating that *V. cholerae* O1 can be sporadically carried by household animals, including cows, dogs, and chickens, but no animal species consistently carries the bacterium.

Cholera

The explosive, potentially fatal dehydrating diarrhea that is characteristic of cholera is actually seen in only a minority of persons infected with Ctx-producing *V. cholerae* O1 or O139. Most infections with *V. cholerae* O1 are mild or even asymptomatic. It has been estimated that 11% of patients with O1 classical infections develop severe disease, compared with 2% of those with O1 El Tor infections. An additional 5% of El Tor and 15% of classical infections result in moderate illness (42).

The incubation period of cholera can range from several hours to 5 days and is dependent in part on inoculum size. In healthy North American volunteers (77), doses of 10^{11} CFU of *V. cholerae* were required to consistently cause diarrhea when the inoculum was given in buffered saline (pH 7.2).

When stomach acidity was neutralized with 2 g of sodium bicarbonate immediately prior to administration of the inoculum, attack rates of 90% were seen with an inoculum of 10^6 CFU. Under natural field circumstances, the inoculum size needed to cause cholera may be even lower, because attack rates are lower than in volunteer studies, and many patients have low gastric acid production. Further studies revealed that most volunteers who received as few as 10^3 to 10^4 vibrios developed diarrhea, although lower inocula correlated with a longer incubation period and diminished severity.

After the *V. cholerae* inoculum is ingested, the onset of illness may be sudden, with profuse, watery diarrhea, or there can be premonitory symptoms, such as anorexia, abdominal discomfort, and simple diarrhea. Mucus in the stool imparts the characteristic "rice water" appearance. In its most severe form, termed cholera gravis, the rate of diarrhea may quickly reach 500 to 1,000 ml/h (excreting up to 10^{11} to 10^{13} CFU of *V. cholerae* per gram of stool), leading rapidly to tachycardia, hypotension, and vascular collapse due to dehydration. Such severe dehydration can lead to death within hours of the onset of symptoms unless fluids and electrolytes are rapidly replaced.

Gastroenteritis and Extraintestinal Infections

While cholera gravis is a striking clinical entity, milder illnesses are not readily differentiated from gastroenteritis due to other causes in areas of cholera endemicity. Gastroenteritis associated with *V. cholerae* non-O1/non-O139 is generally of mild to moderate severity, although severe, cholera-like illness has also been reported occasionally. Strains of *V. cholerae* non-O1/non-O139 have also been studied in volunteers (69). Of three non-Ctxproducing strains fed to volunteers, only one strain caused diarrhea. This strain produced a heat-stable enterotoxin-like toxin and caused diarrhea in six of eight volunteers at doses of 10^6 to 10^9 vibrios after neutralization of stomach acid with sodium bicarbonate. The severity of disease was generally mild, but in one volunteer, diarrheal stool volume exceeded 5 liters.

V. cholerae non-O1/non-O139 is also frequently isolated from extraintestinal infections such as septicemia and wound and ear infections; these infections usually involve exposure to fresh or brackish water (5). The case fatality rate of extraintestinal infections can exceed 50%, and individuals with preexisting liver disease are particularly at risk.

Virulence Mechanisms

In order to cause a diarrheal disease, *V. cholerae* must successfully colonize the intestine. After passing the acid barrier of the stomach, *V. cholerae* enters the mucus layer covering the epithelium of the small intestine and proliferates on its surface (Fig 13.2). Recent developments on the detailed mechanisms facilitating this colonization are thoroughly summarized elsewhere (78). We describe here the major virulence factors that take part in the development of cholera and other vibrioses and their association with different *V. cholerae* serotypes.

Cholera Toxin

Cholera is caused by the action of the cholera toxin (Ctx), which is encoded by *V. cholerae* O1 and O139 and is rarely found in other nonpandemic serotypes. The *ctxAB* genes, encoding Ctx, reside on a filamentous bacteriophage (CTXφ) that is capable of transducing *ctx* genes into nontoxigenic strains (79) and has played a critical role in the evolution of pathogenic *V. cholerae* (43). Ctx is among the best-characterized bacterial toxins and has been extensively reviewed (Fig 13.2) (80, 81). It consists of two subunits, A and B, organized in a pentamer of identical B subunits and a single A subunit, formed by an active A1 subunit and a bridging A2 portion. The B pentamer binds the GM1 ganglioside eukaryotic receptor, whose conversion triggers toxin A internalization via endocytic vesicles. Binding of Ctx to epithelial cells is enhanced by the neuraminidase NanH, which catalyzes the conversion of higher-order gangliosides to GM1, thus enhancing Ctxspecific receptors. Once translocated into the cell cytosol, the A1 subunit possesses specific enzymatic activities (ADP-ribosyltransferase and NAD-glycohydrolase) that irreversibly ADP-ribosylate the G proteins. G proteins regulate an extensive set of metabolic pathways, including the adenylate cyclase (AC) complex, which mediates the transformation

of ATP to cyclic AMP (cAMP), a crucial intracellular messenger for a variety of cellular pathways. Activation of adenylate cyclase and the subsequent increase in intracellular level of cAMP lead to protein phosphorylation of, among others, the major Cl– channel in epithelial cells. Increased Cl^- secretion by intestinal crypt cells and decreased NaCl-coupled absorption by villus cells result in a transepithelial osmotic gradient that causes water flow into the lumen of the intestine. The massive volume of water overwhelms the absorptive capacity of the intestine, resulting in diarrhea.

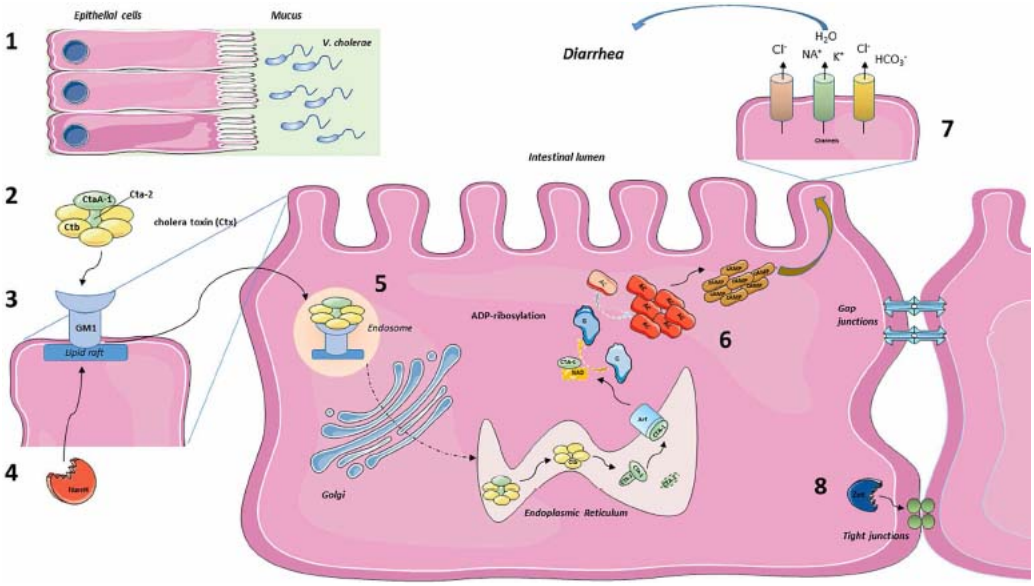


Figure 13.2 Cholera toxin mechanism. After ingestion, *V. cholerae* cells colonize the intestinal lumen (step 1), where they start secreting cholera toxin (Ctx), which is composed of two toxic subunits (A1 and A2) and five binding (B) units (step 2). The complete toxin binds to the GM1 ganglioside receptors on the cell membrane (step 3) via the binding subunits. NanH facilitates Ctx binding to host cells by converting cell surface gangliosides to GM1 gangliosides (step 4). This interaction triggers the internalization of the toxin via an endosome in the cell (step 5). Once in the cell, the reduction of the A and B subunits occurs in the endoplasmic reticulum, leaving the B subunits in the endoplasmic reticulum. The active A1 subunit is then released into the cytosol, where it catalyzes the ADP-ribosylation of the G regulatory protein, which regulates the activation of the adenylate cyclase (AC) system (step 6). The constant active state of G provokes a persistent activation of AC, which results in accumulation of cyclic AMP (cAMP) along the cell membrane. The cAMP causes the active secretion (step 7) of sodium (Na^+), chloride (Cl^-), potassium (K^+), bicarbonate (HCO^{3-}), and water (H_2O) into the intestinal lumen, which is clinically associated with profuse diarrhea and dehydration. The accessory toxin Zot (step 8) induces modifications of cytoskeletal organization that lead to the opening of tight junctions, increasing the permeability of the small intestine. Data are from reference 328.

Other Toxins Produced by *V. cholera*

A variety of studies on the first recombinant *V. cholerae* vaccine strains showed that volunteers administered *V. cholerae* strains not producing Ctx still experienced mild to moderate diarrhea, abdominal cramps, and lowgrade fever. These results triggered the search for and eventually underlined the role of additional toxins produced by *V. cholerae*.

The zonula occludens toxin (Zot) induces modifications in cell cytoskeletal organization that lead to the opening of intercellular tight junction, or zonula occludens, increasing the permeability of the small intestine (82). The accessory cholera enterotoxin (Ace) causes fluid accumulation in rabbit ligated ileal loops and increases potential difference in intestinal tissue, although its role in the infection is not completely clear yet (83). The *ace* and *zot* genes are located immediately upstream of the *ctx* genes and are also believed to be components of the CTXφ filamentous phage, which encodes

cholera toxin (79). *V. cholerae* also produces the small soluble cytotoxin hemagglutinin/protease (HA/P). This zinc-dependent metalloprotease can perturb the barrier function of epithelial cells by digesting the host protein occludin and rearranging the distribution of ZO-1 in tight junctions, suggesting an important role in microbial pathogenesis (84).

The El Tor hemolysin (also called cytolysin or hemolysin) consists of two major toxin groups, the *V. cholerae* O1 group (VCC1) and the *V. cholerae* non-O1/non-O139 group (VCC2). The hemolysin triggers an outward transcellular flux of chloride that, in association with the movement of Na^+ and water, contributes to diarrhea caused by certain nontoxigenic strains of *V. cholerae* (85).

Finally, the RtxA toxin possesses cytotoxic activity for HEp-2 cells *in vitro*, and its gene is physically linked to the cholera toxin phage in the *V. cholerae* genome (86). RtxA is the representative member of the multifunctional autoprocessing repeats in toxin (MARTX) family produced by various Gram-negative bacteria (see "*V. vulnificus*" below) (87).

Although *V. cholerae* non-O1/non-O139 strains generally do not produce cholera toxin, other factors contribute to their pathogenicity, including the El Tor hemolysin, RtxA, and HA/P. The heat-stable enterotoxin (also called nonagglutinable *Vibrio* heat-stable enterotoxin) was identified as a secondary factor in volunteers who received *V. cholerae* non-O1/non-O139 and showed diarrhea and abdominal cramps (88).

Overall, the role of toxins other than Ctx in the pathogenesis of disease due to *V. cholerae* is mostly unknown, although they may contribute in a secondary secretogenic mechanism when conditions for producing cholera toxin are not optimal.

Colonization Factors

Toxin-Coregulated Pilus

Besides Ctx, a type IV pilus, the toxin-coregulated pilus (Tcp) is the most important virulence factor of *V. cholerae* and is essential for colonizing the intestine of the host. The name of the pilus derives from the fact that its expression is correlated with Ctx expression and that Tcp is also the receptor for CTXφ on the *V. cholerae* cell surface (89). The synthesis of the Tcp pilus is complex, and there are up to 15 open reading frames in the *tcp* gene cluster (90). The *tcp* gene cluster along with *toxT* (see "Virulence Regulation" below) is present on a 40-kb pathogenicity island (called the *Vibrio* pathogenicity island) that is present in all O1 and O139 clinical isolates and absent from nearly all *V. cholerae* non-O1/non-O139 strains (91). The role of Tcp in *V. cholerae* colonization has been proven in volunteers ingesting *V. cholerae* strains with specific mutations in the *tcpA* gene; these volunteers did not experience diarrhea, and vibrios were not recovered from their stools. Direct binding of Tcp to epithelial cells has not been observed, and the role of this factor may be to mediate interbacterial aggregation and thereby facilitate intestinal colonization.

Other Potential Colonization Factors

A number of potential colonization factors have been described for *V. cholerae*. The sialidase NanH has been shown to provide *V. cholerae* with the capacity to utilize sialic acid as a carbon and energy source, an advantage in the mucus-rich environment of the gut. Mutants for the gene *nanH* are defective in early intestinal colonization, suggesting a role in bacterial pathogenesis (92). A type 3 secretion system (T3SS) enables *V. cholerae* to translocate effector proteins directly into the host cytosol without passing through the extracellular environment; these proteins can elicit diarrhea and can induce pathological changes in the intestine (93). A type 6 secretion system (T6SS) confers cytotoxic effects against both prokaryotic and eukaryotic cells, suggesting its employment by *V. cholerae* to compete with commensal bacteria in the human intestine and/or environmental reservoirs (94). Finally, a mannose-sensitive hemagglutinin type IV pilus is crucial for surface selection and attachment (95), and although it has been proven not to be necessary for human colonization, it appears to be involved in biofilm formation and adherence to zooplankton (96). The LPS of *V. cholerae* O139 has also been

implicated in intestinal colonization, probably by multiple mechanisms involving direct mucosal adherence and increased intestinal survival in the presence of bile and other factors (97).

Flagella and Chemotaxis

V. cholerae cells rapidly enter the mucus gel overlying the intestinal epithelium by means of a single, polar, sheathed flagellum, and organisms can be found in intervillus spaces within minutes to a few hours after ingestion of the organisms (98). The role of chemotaxis in *V. cholerae* infection is complex and involves modulating the direction or speed of flagellar rotation in response to the surrounding environment. Even though chemotaxis surprisingly inhibits the ability of *V. cholerae* to colonize the small intestine, this outcompeting phenotype correlates with a reduction in the number of bacteria that are required to cause infection (99). The increased infectivity of nonchemotactic *V. cholerae* might contribute to the transient "rice-water stool" state of *V. cholerae* to improve its chances of infecting new human hosts.

Virulence Regulation

Regulation of virulence gene expression in *V. cholerae* is highly complex, and this is understandable in light of the different environments—the human intestine and the aquatic environment—that are involved in the *V. cholerae* life cycle (3). Two important systems that allow the pathogen to respond to its environment and regulate virulence gene expression are briefly described.

The ToxR regulon is a virulence regulatory cascade that controls the expression of several critical virulence factors, including Ctx and Tcp, and is the most extensively characterized regulatory system in *V. cholerae* (100). The key activator of Ctx and Tcp expression is ToxT, which binds upstream of *tcpA* and *ctxA* genes. Expression of ToxT itself is modulated by ToxR in concert with ToxS and Tcp proteins, which are encoded upstream of *toxT*. These protein complexes permit the transmission of environmental signals to modulate expression of *toxT*. This intricate signaling pathway is essential to ensure maximal virulence factor induction within the human intestine.

Quorum sensing (QS) is a microbial signaling process for monitoring population density and complexity. Small molecules termed autoinducers act as signaling molecules among bacterial cells to coordinate gene expression, allowing unicellular microbes to act as multicellular organisms. Contrarily to other bacteria, high densities of *V. cholerae* cells lead to repression of virulence factors (101) and biofilm formation (102). Thanks to four parallel signaling receptors (CqsS, LuxPQ, VpsS, and CqsR), a common response regulator, LuxO, is regulated by phosphorylation. Early in infection, at low cell densities, Tcp and Ctx are expressed along with biofilm, which helps intestinal colonization. Later in the infection, at high cell densities, phosphorylation of LuxO ultimately leads to repression of toxin genes and to activation of HA/P, allowing *V. cholerae* to exit the host and adapt to an environmental reservoir, where toxin expression is not necessary.

V. PARAHAEMOLYTICUS

V. parahaemolyticus represents an important cause of foodborne illness in Asia, South America, and the United States, and it is recognized as the leading cause of human gastroenteritis associated with seafood consumption, particularly raw fish and shellfish. Since its first description in 1950 as the etiological agent of a severe foodborne outbreak in Osaka (Japan) following consumption of a small, semidried fish ("*shirasu*"), *V. parahaemolyticus* has been implicated in several outbreaks of diarrheal disease throughout the world. Presently, CDC FoodNet data on laboratory-confirmed cases indicate a *V. parahaemolyticus* incidence rate of 0.24 per 100,000 persons in 2015, confirming this microorganism as the *Vibrio* species most frequently isolated from clinical specimens in the United States (59.5% of the infections attributed to members of the *Vibrionaceae*). Overall, considering underreporting and underdiagnoses, an estimated 35,000 cases of foodborne *V. parahaemolyticus* infection occur each year in the United States (103).

Identification

Beside standard phenotypic identification (see “Isolation” and “Identification” above), a peculiar biochemical feature of *V. parahaemolyticus* is its variable expression of urease, encoded by the *ure* gene, which is associated with relevant virulence factors, such as those encoded by the *trh* genes (see “Virulence Factors” below), and is therefore used for phenotypical screening of potentially pathogenic strains. It should be noted that the reliability of commercial identification systems for the identification of food and environmental isolates of *V. parahaemolyticus* may vary greatly (12), particularly in relation to the requirement for sodium ion of this microorganism, and that media used for testing the biochemical reactions of *V. parahaemolyticus* should contain 2% or 3% NaCl to improve accuracy of identification (9).

V. parahaemolyticus possesses three antigenic components: H (flagellar), O (somatic), and K (capsular). The H antigen is common to all strains and is not used for serotyping, which is instead based on O (13 lipopolysaccharide antigens, named O1 to O13) and K (65 acidic polysaccharide antigens, named K1 to K71, following exclusion of some proposed antigens). Notably, many environmental and some clinical isolates are untypeable by the K antigen (KUT), but most of the strains can be classified by their O type. Until the mid-1990s, there appeared to be no correlation between serotype and virulence, but the emergence of the pandemic strain O3:K6 in 1996 (see “The O3:K6 Pandemic Clone” below) provided a new insight into the value of serotyping for outbreak investigation and for epidemiological studies.

Transmission and Disease

V. parahaemolyticus displays the features common to the genus *Vibrio*, including the natural occurrence in marine and estuarine environments, and is therefore commonly present in seafood products. Additionally, *V. parahaemolyticus* is characterized by remarkably rapid growth, with a replication time as short as 8 to 9 minutes under optimal conditions and in the range of 12 to 18 minutes in seafood, which allows the organism to rapidly reach the estimated infectious dose (10^5 to 10^7 CFU for pathogenic strains) in raw, undercooked, or cross-contaminated seafood products that are incorrectly stored. Foodborne transmission accounts for an estimated 86% of *V. parahaemolyticus* cases (103), the remainder being mainly associated with wound infections following marine recreational activities and, less commonly, handling of sea-food.

Clinical manifestations of *V. parahaemolyticus* infection include watery diarrhea (and in the cases that are most severe, bloody diarrhea), abdominal cramps, nausea, vomiting, fever, and headache. Symptoms usually occur within 24 hours of microorganism ingestion (the incubation period is 4 to 90 hours), and the illness is self-limiting, lasting 1 to 12 days (median duration, 2.4 days), though occasionally hospitalization may be necessary. In individuals with underlying clinical conditions, infection with *V. parahaemolyticus* may also lead to life-threatening septicemia, requiring aggressive antimicrobial therapy.

Foodborne Outbreaks

Many factors (geographical origin, environmental parameters [temperature, salinity, and trophic status] of the fishing/farming areas, animal species, storage and transport conditions, and structure of the supply chain, etc.) may influence the presence and levels of *V. parahaemolyticus* in seafood products; further to this, the wide variation in the analytical methods used may significantly influence the results of studies of prevalence in food matrices (104). To date, most of the studies on *V. parahaemolyticus* prevalence in seafood involve bivalve molluscs and crustaceans, with species detection reported at a frequency of 77 to 79% in oysters collected in the United States, ~10 to 78% in studies on seafood products in European countries, and up to 100% in samples from other countries. An extended review of *V. parahaemolyticus* prevalence data can be found elsewhere (105). Significantly, most of these studies reported a much lower prevalence of pathogenic *V. parahaemolyticus*, ranging from null to ~23%. Similarly to prevalence, quantitative levels of *V. parahaemolyticus* vary widely depending on the studies, though average contamination in products at harvesting mostly ranges between 10^1 and 10^3 CFU/g.

In Japan, several large outbreaks (>500 cases) were reported between 1996 and 2007 following consumption of prepared meals containing boiled crab, sushi, salted squid, or other seafood dishes (106). In China, *V. parahaemolyticus* represented the most relevant etiological agent for bacterial foodborne outbreaks in 1994 to 2005, with 211 disease events and almost 11,000 cases (107). A number of outbreaks have also been reported in several other Asian countries, particularly in relation to the emergence of the pandemic strain O3:K6 (see "The O3:K6 Pandemic Clone" below).

In the United States, outbreaks of *V. parahaemolyticus* gastroenteritis have been reported at least since 1973 (reviewed in reference 108). More recently, several oyster-related multistate outbreaks have claimed attention. In 1997, approximately 1,200 cases were reported in seven states following consumption of raw oysters from the Pacific coast that harbored *V. parahaemolyticus* serotypes O4:K12 and O1:K56 (109). A similar episode, also associated with the O4:K12 serotype, took place in 2006, when 177 cases were reported in three different states after consumption of shellfish from the Northern Pacific region (110). In 1998, 416 gastroenteritis cases (*V. parahaemolyticus* serotype O3:K6) were reported from 13 states in association with eating oysters from Galveston Bay, Texas (111), and in July to September of the same year, a smaller outbreak (23 cases, mostly characterized as being caused by O3:K6) were reported in three states on the northeastern coast after consumption of oysters and clams harvested from Long Island Sound (112). The latter outbreaks represented the first occurrence of the O3:K6 pandemic strain in the United States. In 2004, an outbreak (caused predominantly by serotype O6:K18) was surprisingly recorded in Alaska among participants in boat cruises who had consumed locally produced oysters (113), an event that highlighted the presence of pathogenic strains of *V. parahaemolyticus* in waters previously thought to be too cold to support the growth of the microorganism. The Alaskan *V. parahaemolyticus* isolates and the O4:K12 serotype responsible for the 1997 and 2006 outbreaks have historically been linked to the aquatic environment of the Pacific Northwest coast of the United States (and are therefore sometimes referred to as Pacific Northwest [or PNW] strains). In the summer of 2012, however, the O4:K12 serotype was reported in gastroenteritis cases associated with the consumption of oysters harvested in Oyster Bay Harbor, New York. In 2013, the same strain was traced to shellfish harvested along the eastern coast of the United States and was responsible for 104 gastroenteritis cases in 13 states (114), highlighting the spread of the PNW *V. parahaemolyticus* strain to the Atlantic coast.

Similar to what has been observed in the United States, *V. parahaemolyticus* outbreaks associated with O3:K6 and O4:K12 strains were repeatedly reported in Latin America in the period between 1989 and 2009 (115), with outbreaks as large as 3,700 cases (Chile, 2005). In Europe, the pandemic strain O3:K6 appeared in 2004 in Spain and in samples from cases in France and Italy (116–118), while the serotype O4:K12 was reported in 2012 in Galicia (northwestern Spain), simultaneously with the spread of the PNW complex to the Atlantic coasts. This outbreak, affecting 100 passengers travelling on a cruise ship and associated with consumption of cross-contaminated shrimps, revealed the presence of genetically undistinguishable O4:K12 strains in both the U.S. East Coast and Western Europe, suggesting that these strains may be becoming endemic in an expanding area of the Atlantic Ocean (119).

Virulence Factors

Toxins

Although *V. parahaemolyticus* occurs naturally in the marine environment, only a minority of environmental isolates have been proved to be pathogenic. A key feature of *V. parahaemolyticus* pathogenicity is the ability of certain strains to produce hemolysins (120). The production of the thermostable direct hemolysin (TDH) was first detected in the late 1970s in clinical strains showing beta-hemolytic activity on a special blood agar called Wagatsuma agar, a process designated the Kanagawa phenomenon (KP). KP-positive strains were therefore associated with pathogenicity. Early studies by Sakazaki and colleagues (121) on the production of TDH hemolysin showed that the vast majority of clinical isolates (96.5%) were KP positive, whereas the prevalence of the KP in environmental isolates was around 1%. This significant difference has been confirmed in subsequent studies, both through assessment of the KP and by detection of genes responsible for TDH production,

with the prevalence of KP-positive isolates among environmental strains being on the order of 1 to 2% (122). TDH is an amyloid, pore-forming toxin with a variety of biological activities that include hemolytic activity, cytotoxicity, cardiotoxicity, and enterotoxicity (123). The fairly large (~2-nm) pore formed in erythrocyte membrane allows both water and ions to flow through the membrane. These alterations in ion flux in the intestine are the basis of the mechanism causing the diarrhea observed during *V. parahaemolyticus* infection. To date, five alleles of the *tdh* gene with 96 to 98% nucleotide homology have been described, and with the exception of *tdh3*, located on a plasmid, all the variants are chromosome borne, with TDH-producing strains usually harboring both *tdh1* and *tdh2* (124, 125). TDH production in such strains is mainly contributed by *tdh2* (>90%) (126), while other *tdh* genotypes, probably in relation to different transcriptional control or nucleotide changes within the promoter region, were shown to have very low levels of gene expression (127). In addition to being unevenly distributed among environmental *V. parahaemolyticus* isolates, *tdh* genes are sporadically present in other *Vibrio* species, including *V. mimicus*, *V. cholerae* non-O1/non-O139, and *G. hollisae* (122).

Investigation of an outbreak in the Maldives in 1985 highlighted the fact that *V. parahaemolyticus* can express pathogenicity even in the absence of *tdh*, and this finding led to the discovery of a second hemolysin, the TDH-related hemolysin (TRH), which, although it is immunologically similar to TDH, is heat labile, being inactivated following treatment at 60°C for 10 min (128). Later studies on the *V. parahaemolyticus* type strain isolated by Fujino and colleagues in 1950 (strain EB101, WDCM 00037) proved that it is also KP negative and expresses TRH (129). TRH is believed to act similarly to TDH by the activation of Cl^- channels, resulting in altered ion flux in intestinal epithelial cells. The *trh* genes show a higher strain-to-strain sequence variation than the *tdh* genes and can be clustered into two main subgroups (*trh1* and *trh2*), which share 84% identity with each other (130) and approximately 68% identity with *tdh* sequences (131). The two variants usually do not coexist in the same strain (132), and their distribution among environmental isolates, where *trh* prevalence varies between <1 and 17% (133, 134), seems to indicate a preponderance of *trh2* (135). As for *tdh*, *trh* genes are occasionally detected in *Vibrio* species other than *V. parahaemolyticus*, such as *V. alginolyticus* (136), and have been recently identified also in non-*Vibrio* species (*Aeromonas veronii* isolates from subjects with acute diarrhea [137]). Strains carrying both *tdh* and *trh* genes are also detected in a nonnegligible number of cases and were found to produce TDH at far lower levels than isolates harboring only the *tdh* genes (138).

A third, unrelated hemolysin, called thermolabile hemolysin (encoded by the gene *tlh*), is often reported in *V. parahaemolyticus*, being present in all strains of clinical and environmental origin (139), irrespective of their virulence. Given its distribution, *tlh* is often used as a species-specific marker for detection or identification of *V. parahaemolyticus*.

Secretion Systems

The pathogenicity of *V. parahaemolyticus* and the presence of *tdh* and *trh* have been clearly correlated by molecular epidemiological analysis (140). An increasing number of studies, however, have reported the absence of both *tdh* and *trh* in about 10% of strains isolated from human clinical cases (120), indicating that other virulence factors exist and may play a significant role in the pathogenesis mechanism of *V. parahaemolyticus*. A first step to elucidating such a contribution was the genome analysis of *V. parahaemolyticus* strain RIMD 2210633, belonging to the pandemic clone O3:K6, which emerged in 1996 (see "The O3:K6 Pandemic Clone" below). Whole-genome sequencing of this strain identified the presence in the genome of genes for two novel T3SSs, named T3SS1 and T3SS2, respectively, located on chromosome 1 and chromosome 2 (141). Such apparatuses usually consist of 20 to 30 proteins, assembled in a basal body (stretching through the inner and outer bacterial membranes), a needle structure (extending through extracellular space), and a translocon pore (inserting into the eukaryotic cell membrane). The most common targets of the effectors translocated via T3SSs are actin cytoskeleton, innate immune signaling, and autophagy (142).

T3SS1 is conserved in all environmental and clinical *V. parahaemolyticus* strains, irrespective of their pathogenicity or lack thereof. The T3SS1 gene cluster is composed of 42 genes showing similarity to the

genes encoding the Ysc secretion system in *Yersinia*, and cytotoxic activity against several cell lines has been demonstrated. Depending on the pathogen's needs, the systems can be either up or down regulated. The three main effectors translocated by T3SS1 (VopQ, VPA0450, and VopS) provide destabilization of the eukaryotic cell structure by causing detachment of the plasma membrane from the actin cytoskeleton (blebbing of plasma membrane) and collapse of the actin cytoskeleton (cell rounding and shrinkage) and by inducing autophagy, which, by sequestering of membrane resources, antagonizes phagocytosis of the infecting bacteria.

The T3SS2 genes are present on a *V. parahaemolyticus* pathogenicity island (VPaI; see "Pathogenicity Islands" below) present on chromosome 2 and carrying several virulence-related genes. T3SS2 is found in clinical isolates, being responsible for both cytotoxicity and enterotoxicity. Two distinct lineages (T3SS2α and T3SS2β) have been reported, correlating with the presence of *tdh* and *trh* genes, respectively (143). T3SS2α was first identified in strain RIMD 2210633 in close association with a pathogenicity island (VPaI-7) and flanked by two *tdh* genes, which appear to be coregulated with T3SS2α (144). The six effectors associated with T3SS2α (VopC, VopT, VopA/P, VopL, VopV, and VopZ) allow *V. parahaemolyticus* to invade, survive in, and replicate in nonphagocytic host cells (144) through the induction of several responses, including changes in the actin cytoskeleton and activation of the mitogen-activated protein kinase signaling pathway. T3SS2β was first identified in strain TH3996 by sequencing of the region surrounding the *trh* gene (143). T3SS2β was also confirmed to be part of a pathogenicity island (which also includes the *ure* gene cluster, previously reported to be in association with *trh*), and the effectors VopC, VopA/P, and VopL have been described. Interestingly, different studies on clinical and environmental strains have shown that while T3SS2α and T3SS2β are reliably found in *tdh*-positive, *trh*-negative strains and in *tdh*-negative, *trh*-positive strains, T3SS2-related genes may also be found in *tdh*-negative, *trh*-negative strains (145). Additional details on T3SSs are provided in references 142 and 146.

To further enhance the complexity of *V. parahaemolyticus* pathogenic mechanisms, a second set of secretion systems was recently described, namely, the T6SSs. T6SSs, which usually contain 13 to 20 proteins, have been reported in different *Vibrio* species (i.e., *V. cholerae*, *V. alginolyticus*, and *V. harveyi*) as well as in several non-*Vibrio* genera (e.g., *Pseudomonas*, *Aeromonas*, *Burkholderia*, etc.) (147) and are present in *V. parahaemolyticus* in two sets, T6SS1 and T6SS2, encoded by genes located on chromosome I and chromosome II, respectively (148, 149). Although the exact functionality of T6SS1 has not yet been demonstrated, preliminary data suggest its involvement in competition against other bacterial species and against other strains of the same species, as environmental signals (temperature, salinity, cell density, etc.) and the master QS regulator (OpaR) regulate T6SS1 activity and expression (150). As for T6SS2, available data suggest that this secretion system is functional to the adhesion to host cells in the first step of infection (151). Furthermore, since T6SS2 and T3SS2 coexist, it has been suggested that the two systems might cooperate during an infection, with T6SS2 acting primarily on adhesion and T3SS2 expressing virulence by inducing enterocytotoxicity.

Pathogenicity Islands

Acquisition of genetic material by horizontal transfer plays a key role in the evolution and the ecological fitness of *V. parahaemolyticus*, and recent comparative studies on available genomes have highlighted the presence of several genomic islands in this species. Genomic islands have been classified based on the different functions they encode, and the different types include metabolic, degradation, resistance, symbiosis, and pathogenicity islands (VPaI in *V. parahaemolyticus*). Seven VPaIs (VPaI-1 to VPaI-7) have been identified in the pandemic *V. parahaemolyticus* RIMD2210633 genome sequence (148, 152), while two further VPaIs (VPaI-8 and VPaI-9) were described in the *tdh*-positive prepandemic strain AQ3810 (92) (for further definitions of pandemic and prepandemic strains, see "The O3:K6 Pandemic Clone" below). Since their first description, VPaIs have been reported in several studies on clinical and environmental *V. parahaemolyticus*, with variable genomic distribution among different isolates. VPaIs are characterized by a G+C content lower than that of the overall genome (~45%); they possess a phage-like integrase gene and are flanked by repeated sequences, although there are exceptions (e.g., VPaI-7, where the integrase gene is replaced by several

transposase genes) (152). Comparative studies showed that VPaI-1 and VPaI-5 are harbored only by pandemic strains, whereas VPaI-7 and T3SS2 are closely associated with *tdh*-positive strains of *V. parahaemolyticus*. In particular, VPaI-1 (~23 kb and 24 open reading frames) encodes a DNA methyltransferase (VP0394) that is regarded as an additional colonization factor (152), and VPaI-7 (formerly known as Vp-PAI or *tdh*VPA; ~8 kb and 87 open reading frames) was shown to be consistently present in KP-positive strains and undetectable in KP-negative strains, suggesting that not only *tdh* but the entire region is required for pathogenicity of KP-positive clinical *V. parahaemolyticus* (149). Compared to VPaIs of *tdh*-positive strains, islands containing *trh*, T3SS2β genes, and the *ure* cluster (described as Vp-PAI$_{TH3966}$ or *trh*VPA) have scarcely been investigated. Interestingly, recent studies on emerging pathogenic lineages in the northeastern United States highlighted the reassortment of genes within VPaIs, demonstrating the mosaic nature of these genomic islands (153).

Adhesion Systems

The first stage in bacterial infection is adhesion to the host cell, a step that is a prerequisite for the efficient translocation of effectors and triggering of signaling pathways that facilitate subsequent pathogen invasion. To date, several adhesive organelles, including T6SSs, capsular polysaccharide, and mannose-sensitive hemagglutinin pilus, have been reported to contribute to attachment of *V. parahaemolyticus* to host cells, and other adhesin genes are periodically discovered. The major adhesion factor, crucial for the expression of pathogenesis, is MAM7 (multivalent adhesion molecule 7), a constitutively expressed transmembrane protein conserved in many Gram-negative bacteria that consists of a hydrophobic stretch of 44 amino acids at its N terminus (transmembrane domain) and seven mammalian cell entry domains that mediate its adherence to different types of cells by binding fibronectin and phosphatidic acid (154).

Iron Uptake Systems

V. parahaemolyticus, like other bacteria of the genus *Vibrio*, requires iron (Fe) for growth and has developed systems to acquire Fe for sustaining metabolism and replication. The Fe concentration in coastal waters ranges from ~1 to almost 600 nM, while the level of free Fe in mammalian bodily fluids is variable (~10^{-18} M) but always far below the concentrations required for bacterial growth. In the human host, *V. parahaemolyticus* can utilize host sources, such as the iron proteins hemoglobin, heme, hemin, and transferrin, for its metabolic needs. One of the strategies of *V. parahaemolyticus* to obtain the iron from the host iron proteins is to secrete siderophores (vibrioferrin) or to use siderophores produced by other bacteria (e.g., aerobactin and enterobactin). Other strategies may include expression of transferrin receptors, for which putative genes have been found in the *V. parahaemolyticus* genome. A review of the strategies adopted by *Vibrio* species for iron uptake is provided in reference 155.

The O3:K6 Pandemic Clone

In 1996, in Kolkata, India, a sudden increase of gastroenteritis attributed to *V. parahaemolyticus* serotype O3:K6 was recorded (156). Analysis of these O3:K6 isolates highlighted a specific pattern that included a *tdh*-positive, *trh*-negative and urease-positive profile. Within a few months, strains with the same characteristics were isolated in several Southeast Asian countries as well as in Japan and Korea (157). Further analysis demonstrated other distinctive molecular properties, including an associated filamentous phage (*f*237) with a unique open reading frame (*orf8*) and different sequences of the *toxR* and *toxS* genes (158), subsequently used for the development of specific PCR for the detection of the pandemic strain (group-specific PCR). The screening for such a molecular profile, together with the evidence obtained through RFLP-PFGE, rapidly led to the detection of genetic patterns indistinguishable from that of the epidemic O3:K6 clone in isolates belonging to different serotypes, such as O4:K68, O1:K25, O1:K41, O1:KUT, and O6:K18 (159). All these clonal serotypes showing different antigenic formulas (to date, 27 different serotypes have been reported [160]) are collectively referred to as O3:K6 serovariants, the O3:K6 pandemic clone, or the pandemic clonal complex. By the end of 2006, the O3:K6 *V. parahaemolyticus* serovariants had been isolated in Europe, Africa, the

United States, and several South and Central American countries (157), marking the first pandemic of *V. parahaemolyticus*. An extended review of the emergence, spread, and evolution of the O3:K6 pandemic strain is provided in reference 161.

The emergence and rapid diffusion of the O3:K6 pandemic clone have provided significant insight into the molecular epidemiology of *V. parahaemolyticus*, leading to the discovery of the above-mentioned virulence factors, including T3SSs and VPaIs (see "Virulence Factors" above). Furthermore, the need for a reliable assessment of the clonality of pathogenic strains, which is challenged by the serological heterogeneity and high genome plasticity shown by *V. parahaemolyticus*, has led to the progressive implementation of an MLST scheme for *V. parahaemolyticus* based on seven loci representing housekeeping genes (*recA*, *dnaE*, and *gyrB* on chromosome I and *dtdS*, *pntA*, *pyrC*, and *tnaA* on chromosome II) (162, 163). Based on MLST analysis, a sequence type (ST) can be attributed to clinical or environmental isolates and, based on phylogenetic relatedness, STs (to date, over 1,800) can be assigned to defined clonal complexes (CCs). Based on this typing scheme, the O3:K6 pandemic strains are described as ST3, CC3, with CC3 including the O3:K6 serovariants, while another relevant *V. parahaemolyticus* strain, the O4:K12 strain, which was responsible for repeated and diffused outbreaks on the Pacific (1997 and 2006) and Atlantic (2012) coasts of the United States as well as in Spain (2012), is reported as ST36, CC36.

V. VULNIFICUS

V. vulnificus is responsible for the majority of seafood-associated deaths worldwide (164). This pathogen is classified as accidental (i.e., "accidentally" infecting humans) as well as opportunistic or secondary (i.e., causing severe disease only when the host's immune system is impaired).

Ecology and Animal Reservoirs

V. vulnificus is an autochthonous inhabitant of coastal brackish water ecosystems located in tropical, subtropical, and warm temperate areas (164); it can survive in cold water (below 10°C) by entering the metabolically inactive VBNC state (165). Ecological data on the isolation of *V. vulnificus* from the environment suggest that water salinity and temperature determine its geographical distribution (166). The ideal salinity for *V. vulnificus* survival in nature ranges between 2 and 25 g of salt per liter (‰), with an optimum around 10 to 18‰, and a temperature between 10 and 33°C, with an optimum around 25 to 28°C (165). However, because of the combined effect of both parameters, the true temperature limits for *V. vulnificus* survival depend on salinity, being narrower (22 to 33°C) at high salinities (>10‰) and wider (10 to 33°C) at low salinities (5 to 10‰) (167). According to this, *V. vulnificus* would be an inhabitant of water bodies of higher salinity in the tropics and of lower salinity in warm temperate areas. Lately, global climate change has extended the geographical distribution of *V. vulnificus* to colder areas; it has been isolated from coastal habitats located in Northern Europe, mainly in association with heat waves (1, 168).

V. vulnificus, like the other *Vibrio* species, switches between free-swimming and sessile lifestyles during its life cycle in water (165). Sessile *V. vulnificus* is mainly associated with abiotic (i.e., sediment) and biotic (i.e., the chitinous exoskeleton of crustaceans and the internal and external mucosa of fish) surfaces by forming biofilms. In addition, free-living and microparticle-associated *V. vulnificus* can be concentrated to more than 100 times the concentration found in the surrounding waters by filter feeders, such as small crustaceans and bivalves (clams, oysters, etc.) (169). Oysters, shrimp, and fish are considered the main environmental reservoirs for *V. vulnificus*, whose presence in these hosts is mainly controlled by the same parameters that control its survival in water (169, 170). Figure 13.3 shows a model for the life cycle of *V. vulnificus* in nature.

Identification

V. vulnificus can be isolated from water and seafood samples by using the FDA protocol (Fig 13.1). Experiments with seawater microcosms revealed that the *V. vulnificus* recovery rate increases significantly when the selective and differential medium modified cellobiosepolymyxin B-colistin is

replaced with *V. vulnificus* medium (10) or TCBS agar (170). Then, identification can be performed by Southern blot hybridization or PCR by targeting the species-specific cytolysin gene *vvhA* (9). In addition, multiple systems have been reported for identification and differentiation of *V. vulnificus* from the main pathogenic *Vibrio* species, including multiplex PCR and real-time PCR, mostly targeting *toxR*, or even analyzing new DNA targets by comparative genomics (171, 172). Among them is a multiplex assay for the simultaneous identification of 10 bacterial pathogens (including vibrios and other common pathogens) in a single reaction by using a multiplex ligation reaction based on probe melting curve analysis (173). This protocol was used with 100% accuracy in the case of environmental and clinical *V. vulnificus* strains. According to the authors, the assay can be used directly on blood and skin tissue samples or even tissues from the suspected prime source of the infection and could be implemented in public health and food laboratories.

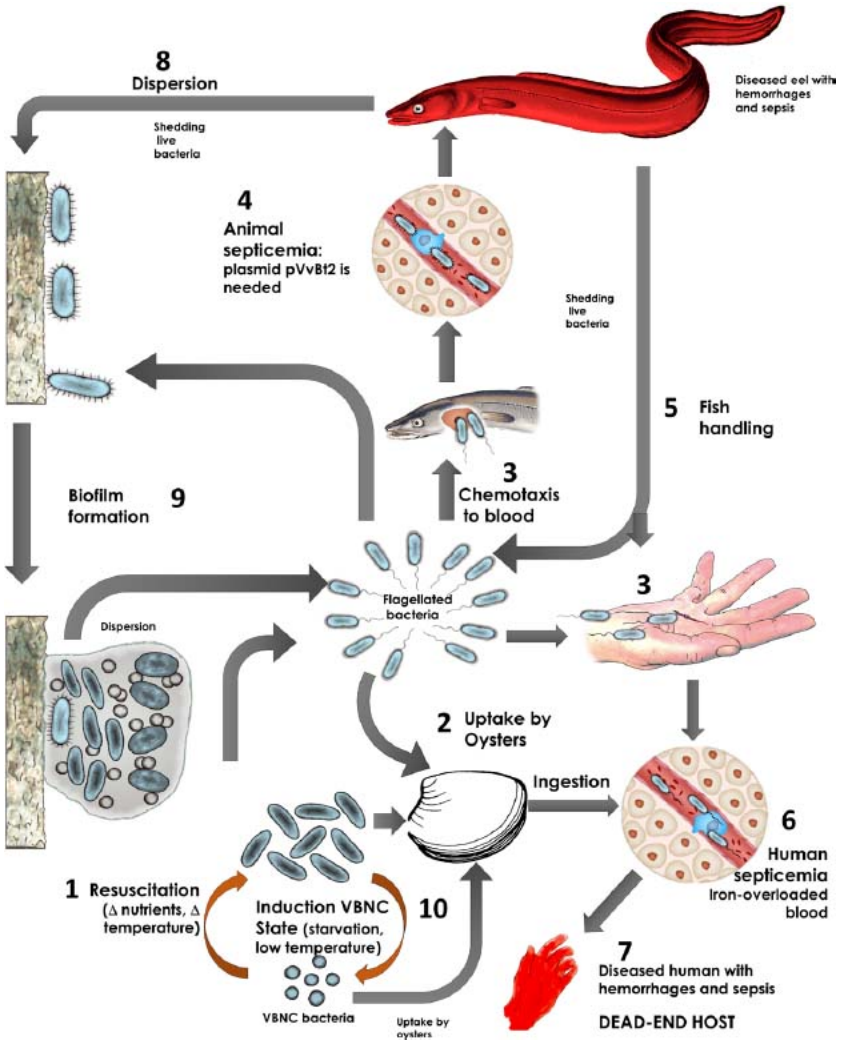


Figure 13.3 Life cycle of *V. vulnificus*. (Step 1) Resuscitation. VBNC bacteria resuscitate when temperature increases to more than 10°C. (Step 2) Uptake by filter feeders, ingestion by humans, and infection. Bacteria with or without flagella can be taken up by filter feeders, such as oysters, which, in turn, can be ingested by humans. (Step 3) Chemotaxis through blood. Flagellated bacteria can be attracted by blood and colonize wounds on animal and human surfaces. (Step 4) Animal septicemia: fish vibriosis. In the case of animals, only bacteria possessing the virulence plasmid pVvBt2 colonize and invade blood, causing fish vibriosis. (Step 5) Human infection by fish and seafood handling. (Step 6) Human septicemia. In the case of humans, regardless of the route of infection (ingestion or contact), bacteria have success in invasion and in causing septicemia, mainly in iron-overloaded patients. (Step 7) Humans are the end hosts for *V. vulnificus* infection. (Step 8) Dispersion of *V. vulnificus* cells from infected aquatic animals into the water. (Step 9) Bacteria can form biofilms on mucosal and inert surfaces, from which they can be dispersed again into the water. (Step 10) Induction of the VBNC state. When the temperature drops below 10°C, bacteria enter the VBNC state, closing the cycle. Adapted from reference 207.

Intraspecific Variability and Virulence

V. vulnificus was defined as a bacterial species by Farmer in 1979 from a series of lactose-and indole-positive vibrios isolated from the environment and clinical cases reported in the United States (174). The isolation of vibrios that had similar characteristics but were indole negative from diseased farmed eels was simultaneously reported in Japan. Subsequently, the indole-positive and -negative strains

were assigned to two different biotypes (175). Later, a third biotype was established in Israel to group atypical isolates (cellobiose, salicin, and lactose negative) from human infection outbreaks associated with handling of farmed tilapia (176). The three biotypes can cause human diseases, but only biotype 2 (Bt2) is also able to cause fish vibriosis, an ability that relies on a plasmid (pVvBt2) encoding a resistance system to the fish innate immunity (177). Moreover, among Bt2 strains, only those belonging to the clonal complex Bt2-SerE are recognized as truly zoonotic (178, 179). Although strains of the three biotypes could originally be phenotypically and genetically discriminated, diversity as well as the number of isolates has increased, and at present, there is no test or set of tests that clearly differentiate among them, with the exception of Bt2, which can be targeted by PCR for unique plasmid genes (180). A recent phylogenomic analysis of the core genomes of 80 *V. vulnificus* strains belonging to the three biotypes demonstrated that this species is subdivided into five well-supported phylogenetic groups or lineages that do not correspond to biotypes (181). Lineage 1 comprises a mixture of clinical and environmental Bt1 strains, most of them involved in human clinical cases related to raw seafood ingestion. Lineage 2 accounts for a mixture of Bt1 and Bt2 strains from various sources related to the aquaculture industry. Lineage 3 comprises exclusively all Bt3 strains, and lineages 4 and 5 comprise a few Bt1 strains associated with specific geographical areas. This study also found strong evidence that pVvBt2 has been acquired independently by different clones, probably in fish farms, and proposes a new updated classification of the species based on phylogenetic lineages rather than on biotypes, as well as the inclusion of all Bt2 strains in a unique pathovar with the particular ability to cause fish vibriosis (*V. vulnificus* pv. piscis).

Regardless of lineage and biotype, a single strain or clone can be virulent or avirulent. There have been several unsuccessful attempts to find a genetic virulence marker by comparing environmental and clinical strains. However, it seems that there are differences in several polymorphisms and in repetitive extragenic palindromic sequences that could be used to predict the virulence potential of a strain. The most used genetic markers for virulence-related polymorphisms are *pilF* (pilus type IV assembly gene; clinical [C] strains are positive and environmental [E] strains are negative) (182), *vcg* (open reading frame VV0401; virulence-correlated gene of strain YJ016; C or E) (183), capsular polysaccharide (type 1 [C] or 2 [E]) (184), and 16S RNA (type A [E] or B [C]) (185). A multiplex PCR that combines some of these genetic markers would probably be more discriminant than a PCR that uses only one, since none of them is infallible when used in a single PCR. Another approach is to use MLST analysis based on housekeeping gene sequencing (https://pubmlst.org/vvulnificus/info/primers.shtml) (179, 186). However, this analysis is also not completely reliable, since there are multiple exceptions. At present, our conclusion is that all *V. vulnificus* strains should be considered potentially virulent for humans. This conclusion is supported by the phylogenomic study of Roig et al. (181), who found that most of the described virulence genes were present in the core genome of the species.

Disease

V. vulnificus is an extremely diverse pathogenic species. This pathogen can accidentally infect fish, shrimps, and even humans, causing diseases with multiple clinical manifestations. There are two main forms of human vibriosis according to the transmission route, i.e., contact or ingestion. In the first case, the pathogen causes severe tissue necrosis after wound contact with contaminated seawater or any carrier species (mainly during handling of farmed fish and seafood) that can lead to debridement and/or amputation or even secondary septicemia (164). Since people infected by contact are usually swimmers, bathers, and fishers, this pathogen is sometimes known as the “marine flesh-eating bacterium.” In the second case, *V. vulnificus* causes gastroenteritis and/or primary septicemia after ingestion of raw or undercooked seafood (mainly oysters in Western countries and shrimp in Asia) (164) (Fig 13.3), and for this reason, it is considered a foodborne pathogen.

Fish vibriosis is a hemorrhagic septicemia produced by contact with water or fish (carrier or diseased) that has a short incubation time (around 24 h on average) in common with human vibriosis (177). Thus, *V. vulnificus* can be also considered an extremely fast-acting multihost septicemic pathogen. Finally, *V. vulnificus* can be transmitted from diseased fish to humans, which suggests that it is also a zoonotic agent (177). From here, this section focuses mainly on foodborne human infections and the

particular relationship that *V. vulnificus* establishes with the most studied reservoir for this type of disease, the oyster.

Epidemiology

From its first isolation by the CDC in 1964, *V. vulnificus* has been extensively isolated in the United States, especially in the Gulf Coast states, where it is considered a human pathogen of concern. The most complete epidemiological data set on *V. vulnificus* isolation comes from COVIS, and no similar databases are available anywhere else. The most recent COVIS data differentiate between contact and foodborne infections and permit some relevant conclusions to be drawn: (i) foodborne infections occur as sporadic cases in warmer months (May to October), with a maximum in the hottest months (July and August), in the geographical areas where *V. vulnificus* is a native bacterium; (ii) although the prime vehicle for foodborne infections in the United States is oysters, there are other aquatic animals of concern, such as clams, crabs, and finfish, all of them thought to be ingested raw or undercooked; (iii) the average human target host is 60.5 years old and male; (iv) confirmed foodborne infection cases are extremely low (~125 cases per year, with ~21% caused by seafood ingestion) considering the abundance of *V. vulnificus* in edible filter feeders in the warmer months (i.e., more than 10^7 CFU/g of oyster), likely due to the majority of cases not being severe enough to require medical assistance at home or hospitalization; (v) among reported medical cases, the hospitalization rate is high (84% of total cases), and the case fatality ratio is also high (25% considering total cases and up to 31.2% considering only hospitalized cases). Although COVIS data do not distinguish mortality caused by ingestion from that caused by contact, previous studies report values of ~50% and 15%, respectively, which correlate with the reported epidemiological data. It is safe to extend COVIS conclusions globally, since they do not differ substantially from recently reviewed data from Europe (France, Denmark, and Germany), Asia (Japan, Korea, and Taiwan), and the United States (187–189), although the primary source of infection varies according to dietary habits (i.e., oysters and clams in the United States versus mud shrimp in Japan) (190). Recently, a tendency toward an increase in *V. vulnificus* infection cases in COVIS data from 1988 to 2010 was detected (187). However, our analysis reveals that the case number in the United States seems to have been stable from 2011 to 2014. Therefore, our conclusion is that the preventive measures applied in the most heavily affected states appear to have counteracted this global tendency.

Pathogenesis of Foodborne Infections

The infectious dose of *V. vulnificus* probably depends on the variant of the pathogen present in the ingested animal species (see “Intraspecific Variability and Virulence” above) as well as on the health status of the consumer. In healthy people, the disease seems to be self-limiting, but in susceptible populations, fewer than 100 total bacteria may cause primary septicemia (septic shock), with fever, chills, altered mental status, and hypotension as the main clinical signs (164, 188). More than 60% of septicemic patients develop secondary lesions on the extremities, similar to those found in wound infections (cellulitis, bullous lesions, and necrosis) (188), which makes it difficult to discriminate primary from secondary septicemia when the source of infection is unknown (Fig 13.4). Primary septicemia occurs when the pathogen crosses the intestinal barrier, arrives in the bloodstream, resists the immune defenses, and multiplies, causing sepsis. In case of *V. vulnificus*, there is a clear correlation between sepsis and an underlying disease (<5% cases occur in individuals without any identifiable risk factor) (188). The main reported risk factor is chronic liver disease (cirrhosis, hepatitis, liver transplantation, or liver cancer), followed by hemochromatosis, a hereditary blood disease (188, 189). Additional risk factors are a compromised immune system (chemotherapy, immunosuppressive medication, AIDS, etc.), diabetes, and chronic renal or intestinal diseases (188). Nutritional immunity due to iron sequestering by transferrin seems to be the key defense mechanism to keep *V. vulnificus* under control. Specifically, the two main risk factors (chronic liver diseases and hemochromatosis) can lead to an iron overload in serum because of low transferrin and hepcidin (an iron-regulatory hormone) production and hereditary hepcidin deficiency, respectively. Another innate mechanism that can fail and contribute to the rapid expansion of *V. vulnificus* is bacterial killing either

by phagocytoses or by complement, both of which are impaired in immunodeficient patients. Individuals with chronic liver disease or various immunodeficiencies have been reported to be 80 times more likely to develop primary sepsis than healthy individuals (191).



Figure 13.4 Clinical signs of human sepsis caused by *V. vulnificus*. The photo shows typical clinical signs of primary and secondary sepsis caused by *V. vulnificus*: swelling, erythema, development of vesicles or bullae, and tissue necrosis. Picture courtesy of Ching-Chuan Liu, Department of Pediatrics, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, Tainan City, Taiwan.

Virulence Factors

The animal models most often used to study host-pathogen interaction in *V. vulnificus* are normal or iron-overloaded (by preinjecting different sources of iron) mice that are intraperitoneally (model for sepsis) or intragastrically infected. Of all the *V. vulnificus* virulence factors that have been discovered by using these models, we have selected a handful with a clear role in the three main steps of the infection process: colonization, invasion, and sepsis (Fig 13.5).

Colonization

V. vulnificus can resist, although only partially, the acidic pH of the stomach due to the combined action of CadA and CadB, a lysine/cadaverine antiporter and a lysine decarboxylase that synthesize and excrete cadaverine to counteract external pH (192). Antacids or deficiencies in the natural pH of the stomach are considered another risk factor for foodborne infections caused by this pathogen. Surviving *V. vulnificus* organisms arrive at the intestine, where they can resist the bactericidal action of bile salts and/or microbicidal peptides thanks to a sialic acid-containing LPS, the outer membrane protein OmpH, and a potassium pump (TrkA), among other factors (193–195). Resistant bacteria must adhere to the intestinal epithelium or they will be eliminated by the constant turnover of the mucus layer together with the peristaltic movements of the intestine. According to recent literature, *V. vulnificus* can first attach weakly to the intestinal epithelium thanks to the interaction between mucin and the mucin-binding outer membrane protein GbpA and then thanks to the interaction between mucin on the bacterial surface and different mucin receptors on the intestinal cells (196). Upon initial attachment, bacteria can then produce the multifunctional protease VvpE, which can inhibit the expression of mucin (mucin 2), facilitating a stronger and direct attachment (193), in this case through the interaction of different adhesins (flagellin, type IV pilin, and several outer membrane proteins, such as IlpA and OmpU, among other factors) and their putative receptors on intestinal cells (194, 195). Once firmly attached, *V. vulnificus* multiplies by using as nutrients different carbon sources naturally present in the mucosa (e.g., *N*-acetylneuraminic acid) (197).

Invasion and Spreading

V. vulnificus produces a protein known as multifunctional autoprocessing repeats-in-toxin (MARTX or RtxA1) that is directly involved in invasion and spreading (198). MARTX is an early-expression protein that is expressed upon contact of the bacterium with multiple types of eukaryotic cells. MARTX toxins are modular proteins of high molecular weight with conserved external modules (in particular, N-and

C-terminal repeat regions) that form pores in the eukaryotic membrane, through which the toxin's central module is translocated (199). Then, the effector domains in the central module are liberated after activation of the cysteine protease domain, an endoprotease with autoproteolytic activity, in the cytosol (199). Several types of MARTX have been described in *V. vulnificus* according to the repertoire and organization of the effector domains. The best known is MARTX type I or RtxA11 (Fig 13.6), which induces a rapid intestinal barrier dysfunction and increases paracellular permeability prior to onset of cell lysis. In conjunction with cytolysin VvhA, MARTX causes intestinal tissue damage and inflammation contributing to *V. vulnificus* dissemination to the bloodstream and other organs (200). The metalloprotease VvpE also collaborates in this step by triggering a local inflammatory response that culminates in the disruption of tight junctions (201). Once in the blood, *V. vulnificus* uses the sialic acid-like LPS plus the capsule to resist the bactericidal action of complement and/or phagocytosis/opsonophagocytosis (202). It is possible that TrkA could also contribute by increasing serum resistance (203). Recently, it was demonstrated that capsule biosynthesis and *trkA* gene expression are up-regulated under iron excess conditions, which could partially explain the highest susceptibility to septicemia of patients with high iron levels in the blood (Fig 13.3) (207). *V. vulnificus* can also use MARTX to inhibit phagocytosis and kill phagocytic cells by diverse mechanisms (205, 206). Finally, surviving bacteria multiply in the blood by using either free iron in iron-overloaded patients or, in the case of healthy patients, two high-affinity iron uptake systems that are expressed only under iron starvation conditions (207), a siderophore called vulnibactin together with its receptor, VuuA, and a heme receptor, HupA (208). Additionally, *V. vulnificus* can use sugars naturally present in blood as nutrients. Recently, it was reported that MARTX can act on endothelial cells, thus favoring the spread to and invasion of internal organs (209). Once the bacteria are there, the combined action of these and other factors (collagenase, hyaluronidase, etc.) could contribute to cause lesions in the organs.

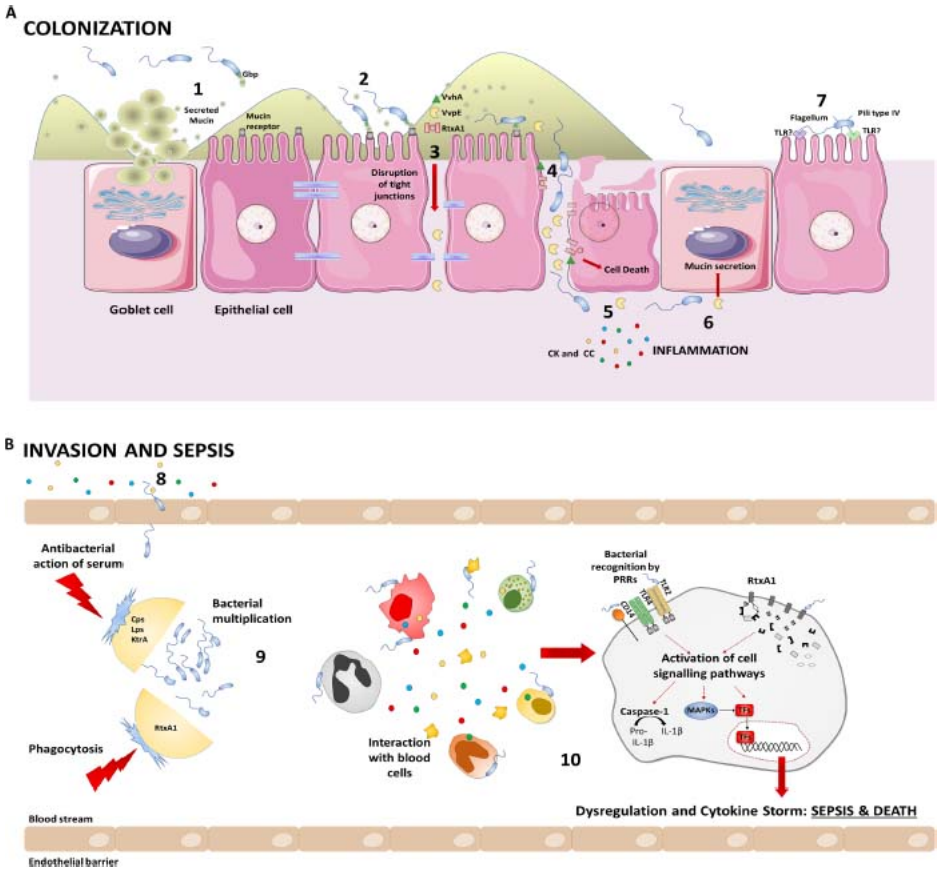


Figure 13.5 Steps in colonization, invasion, and sepsis caused by *V. vulnificus* and role of selected genes. (A) Colonization. *V. vulnificus* cells arrive at the intestine; some are eliminated, but others are attracted by mucin and bind to it by Gbp (step 1). Mucin-coated *V. vulnificus* cells bind to mucin receptors on epithelial cells (step 2). Attached cells produce VvpE, VvhA, and RtxA1, whose joint activity results in increased permeability and tight-junction disruption (step 3). Cells pass through the epithelium and continue producing Vvp, VvhA, and RtxA1, which cause cell death by different mechanisms (step 4). Attacked cells secrete cytokines (CK) and chemokines (CC), which triggers local inflammation (step 5). VvpE inhibits mucin secretion (step 6), which in turn facilitates vibrio adhesion, in this case probably by the interaction between pili/flagellin and Toll-like receptors (TLR) (step 7). Established bacteria will multiply on the epithelium. (B) Invasion and sepsis. Inflammation alters endothelial cells, enabling bacteria to cross the endothelial barrier (step 8). Bacteria multiply in blood thanks to the combination of a series of protective mechanisms in which capsule, LPS, KtrA, and RtxA1 are involved (step 9). Bacteria interact with different immune cells and, finally, cause sepsis and death, probably by inducing a cytokine storm (step 10). The figure is not to scale. PRRs, pattern recognition receptors. Adapted from reference 204.

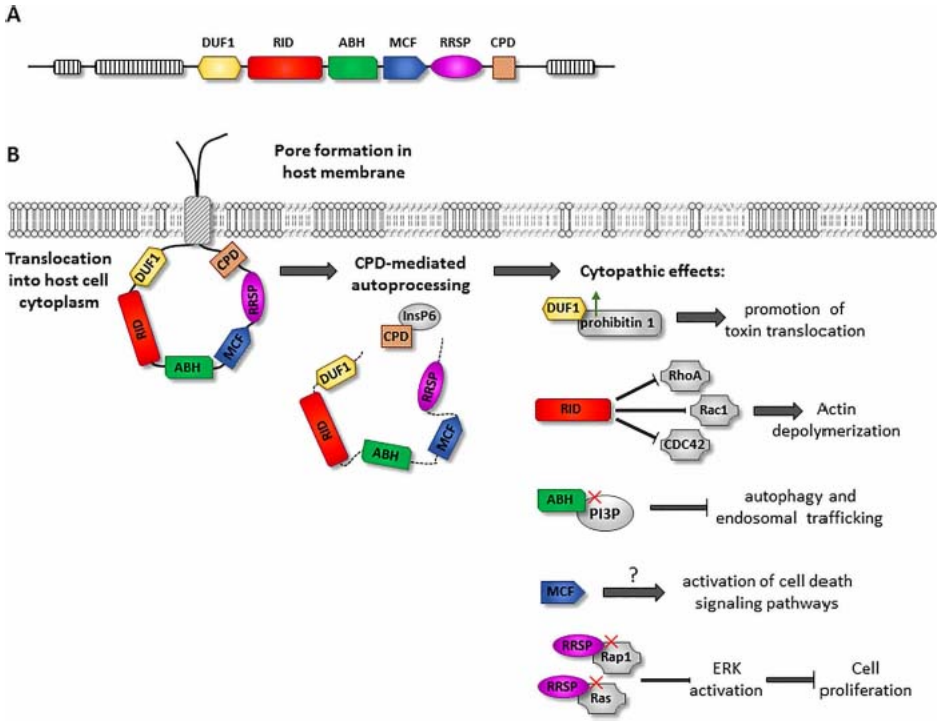


Figure 13.6 Structure (A) and mode of action at the cellular level (B) of MARTX type I of *V. vulnificus* (also called RtxA11). (A) The scheme shows the conserved external modules and the internal module, containing the five effector domains. (B) The toxin is secreted and the external module is associated with the target cell membrane by forming a pore that allows the central module to be exposed to the cytosol. The cysteine protease domain (CPD) catalyzes the release of the rest of internal domains after being activated by binding inositol hexakisphosphate. The domain with unknown function (DUF1) probably binds prohibitin-1, promoting the translocation of the toxin into the cell. The Rho GTPase inhibitor (RID) activates actin depolymerization, altering the cell cytoskeleton. The alpha/beta hydrolase domain (ABH) binds inositol 3-phosphate and inhibits autophagy and endosomal trafficking. The MCF (makes caterpillars floppy)-like domain induces depolarization of the mitochondrial membrane potential, which causes activation of cell death. The Ras/Rap1 specific endopeptidase domain (RRSP) suppresses the ERK–mitogen-activated protein kinase pathway by proteolytically processing Ras and Rap1 GTPases, preventing Ras from activating ERK, and then inhibiting cell proliferation. For more details on MARTX action at the cellular level, see reference 198. The figure is not to scale.

Sepsis

Sepsis caused by *V. vulnificus* is mainly due to the action of the immune system against the bacterium that, eventually, causes severe injury to tissues and organs, including the endothelium. Almost every cell of the body has Toll-like receptors to detect pathogen-associated molecular patterns and is able to secrete cytokines and chemokines when attacked by a microorganism. Neutrophils, macrophages, and probably endothelial cells are the master cells in articulating the early response against pathogens. Several of the cytokines secreted by these cells can mediate the systemic inflammatory response syndrome caused by *V. vulnificus*. In accordance with this, the proinflammatory cytokines interleukin 1β (IL-1β), IL-6, IL-8, and tumor necrosis factor alpha have been detected in the blood of patients with sepsis caused by *V. vulnificus* (210). However, patients with chronic liver diseases, who should be predisposed to sepsis by *V. vulnificus*, are paradoxically deficient in these cytokines (211). The key is probably not the quantity but the quality of the immune response: *V. vulnificus* likely induces a dysregulated and unbalanced immune response in which these and other cytokines and chemokines can be involved. Recently, MARTX type III produced by *V. vulnificus* zoonotic strains (all of them

related to infections by contact) was shown to be clearly associated with an early and unbalanced cytokine storm by using an animal model of sepsis (204). These strains present two copies of the gene, one on pVvBt2 and another on chromosome II, which is unusual in *V. vulnificus*, and cause sepsis without evidence of multiplication in the blood (204). Although the role of MARTX in foodborne infections is not clear and more studies are necessary, it seems that *V. vulnificus* multiplies in the bloodstream and that LPS (a potent inducer of tumor necrosis factor alpha) (212), IlpA (213), and the capsule (214) can act as strong inducers of cytokine and chemokine production, contributing to death by sepsis. However, the key factor inducing the hypothetical early and dysregulated cytokine response associated with foodborne infections, if any, remains to be determined. Figure 5 summarizes some of the mechanisms used by *V. vulnificus* to colonize, invade, and cause sepsis.

Diagnosis, Treatment, and Prevention of *V. vulnificus* Foodborne Diseases

Patients who show clinical symptoms of foodborne infection caused by *V. vulnificus*, especially signs of septicemia, and who present one or more risk factors should be treated immediately because of the high death rate associated with *V. vulnificus* disease (188, 215). Diagnosis is routinely performed in hospital laboratories from external lesions, diarrheal stools, or blood, and in addition to antibiotics, many patients require supportive therapy and intensive care (215). The mortality rate seems to be strongly dependent on the time after infection of antibiotic treatment (around 30% before 24 h, 53% between 24 and 48 h, and 100% for patients given antibiotics >72 h) (216). Recommended antibiotic therapy for *V. vulnificus* infection includes doxycycline and ceftazidime or other third-generation cephalosporins (188). *V. vulnificus* is usually susceptible to most antibiotics used in clinical practice (215). However, the extensive use of antibiotics in health care, agriculture, and especially aquaculture systems has led to an increase in resistance to antibiotics, including those prescribed for *V. vulnificus* infection treatment (217, 218). Recently, tigecycline, which is active in stopping the passage of *V. vulnificus* to the bloodstream, was successfully tested *in vitro* (219).

The best prevention measure against *V. vulnificus* is to trace the contaminated oysters (geographical area from which they were harvested and the month and/or season, among others) and to apply a series of measures from harvesting to consumption in order to reduce the presence of vibrios below risk levels (ideally no detectable levels, i.e., a most probable number of <30/g) (see “Control Measures to Minimize Vibrio Contamination” above). These measures should be complemented by instructing people to avoid raw or undercooked shellfish ingestion during hazard season(s) in areas where *V. vulnificus* is native. A successful example of these measures is represented by the State of California, which implemented a regulation restricting sales of raw oysters harvested from the Gulf of Mexico during April 1 to October 31, unless the oysters had been previously processed to reduce *V. vulnificus* to undetectable levels. The application of these measures has set to zero the number of deaths due to foodborne *V. vulnificus* infections (220).

V. ALGINOLYTICUS

V. alginolyticus was originally classified as a biotype of *V. parahaemolyticus* (221). The two species are genetically similar, but they can easily be differentiated phenotypically, most readily by the fermentation of sucrose by *V. alginolyticus*. *V. alginolyticus* has been included by Sawabe et al. (222) in the Harveyi clade of the genus *Vibrio*, which comprises several species isolated from seawater, saltmarsh mud, marine animals, and mucus of corals. Some differences in taxonomic traits between clinical and environmental isolates of *V. alginolyticus* have been suggested, but whether these are significant has not been demonstrated. Conventional, multiplex, and real-time PCR assays are now available for the detection and discrimination of *V. alginolyticus* in different sample types (223, 224). Diagnostic methods based on loop-mediated isothermal amplification, matrixassisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry, and aptamer technologies have also been developed (225, 226). An innovative method based on the use of multiwalled carbon nanotubes, which function as immunomagnetic fluorescent sensors for *V. alginolyticus* quantitative detection, was recently developed to be employed in fishery and environmental samples (227).

V. alginolyticus has been reported to inhabit seawater and seafood obtained worldwide. It is easily isolated from fish, clams, crabs, oysters, mussels, and shrimp, as well as water (228). Several studies from different countries revealed this species as one of the most common vibrios in environmental and food samples, and a correlationlation between temperature and its rate of isolation, which is greater in the warm months, has been reported (229–232).

Foodborne Outbreaks

In general, disease due to *V. alginolyticus* mainly affects persons who had direct contact with seawater or handled shellfish. *V. alginolyticus* has been commonly associated with ear infections (otitis media and otitis externa) and superficial wounds after exposure to contaminated water sources (233). In some cases, these infections can progress to bacteremia and necrotizing fasciitis, particularly in immunocompromised patients (234). The implication of this organism as a food-associated pathogen has also been documented. *V. alginolyticus* has been detected and isolated from watery diarrheal stools of patients with acute enterocolitis and also from the seafood they consumed. Chien et al. (234) described a case of *V. alginolyticus* bacteremia in an immunocompromised patient who ate a large amount of seafood.

According to COVIS, the incidence of *V. alginolyticus*, one of the three most common reported vibrios, increased between 1996 and 2010. *V. alginolyticus* accounted for 11.5% of the total cases of vibriosis and for 8.7% of foodborne cases (8). Hospitalization and death rates for infection with *V. alginolyticus* were similar to those for infection with *V. parahaemolyticus*. *V. alginolyticus* wound infections are rare in Europe, with only sporadic cases being reported in the United Kingdom, the Netherlands, and Denmark (235). This species has been isolated from 0.5% of healthy people in Japan, with no clinically associated intestinal disease evident.

Characteristics of Disease

Few reports describe the gastroenteric symptoms caused by *V. alginolyticus*, and acute diarrhea has rarely been associated with this infection. A leukemic patient with *V. alginolyticus* infection displayed confusion, shock, anemia, a temperature of 40°C, and a systolic blood pressure of 80 mm Hg. Despite antibiotic administration and treatment for shock, the patient died 12 days after admission. The exact role of *V. alginolyticus* in this case is unknown, however, as *Pseudomonas aeruginosa* was also isolated from the patient's blood and the presence of the latter pathogen was very possibly a factor in the fatal outcome.

V. alginolyticus is also pathogenic to fish and shellfish. The clinical signs observed in finfish species include septicemia, exophthalmia and corneal opaqueness, ascites, lethargy, melanosis, and ulcers (228). *V. alginolyticus* caused large-scale mortalities in cultured fish throughout the world, and some vaccine formulations for prevention of fish vibriosis by *V. alginolyticus* have been developed (236). In shellfish, *V. alginolyticus* has been associated with necrosis, shell disease and white spot in shrimp, and mass mortalities in clam larvae (237).

While extraintestinal infections are typically selflimiting and relatively mild, systemic infections are generally severe. Most such cases appear to involve patients who are immunocompromised due to severe burns, cirrhosis, or cancer. *V. alginolyticus* infections usually respond well to antibiotic therapy. However, in recent years, multiresistant strains, including extended-spectrum cephalosporins, have been isolated from fish and food products, including shrimp, chicken, pork, and beef (238, 239). The emergence of resistant strains may have important implications in public health. For this reason, phage therapy was suggested as an alternative sanitizing agent for seafood in order to prevent associated *V. alginolyticus* infections (240).

Virulence Mechanisms

The pathogenicity mechanism of *V. alginolyticus* is not yet fully understood, despite its being an important zoonotic pathogen causing disease in marine animals and in humans. Several virulence factors have been described (241). The presence of a capsule and lateral flagella, playing a role in

adhesion and swarming motility, as well as the production of hydroxamate siderophores, may facilitate the first stages of colonization and invasion of *V. alginolyticus*. Genes coding for components ofT3SS and T6SS were found in environmental isolates of *V. alginolyticus*. *toxR* and *ompK* genes, detected in some strains of *V. alginolyticus*, were found to cause bacillary necrosis and mortality in larval and juvenile mollusks. Another common virulence factor is collagenase, which may play a role in wound infections, as it is able to hasten bacterial dissemination through the protein components of the extracellular matrix and causes hemorrhagic tissue damage through destruction of the basement membrane (242). It has been reported that some *V. alginolyticus* virulence factors are derived from pathogenic strains of *V. cholerae* and *V. parahaemolyticus*, as is the case for TDH of *V. parahaemolyticus*, which is variably present among *V. alginolyticus* strains. Finally, QS seems to be an important regulatory system in virulence expression in *V. alginolyticus*, as in other *Vibrio* species (243–245). The alkaline serine protease, known to have toxic effects in oyster and shrimp, is regulated by the QS system through AphB, a LysR-type transcriptional regulator, also essential in other vibrios' pathogenesis.

V. FLUVIALIS

In 1977, Furniss et al. (246) reported the isolation from humans suffering from severe diarrhea of a new bacterium, designated group F, with phenotypic properties intermediate between those of the genera *Vibrio* and *Aeromonas*. In numerical taxonomy studies, Lee et al. (247, 248) determined that group F strains constituted a tight cluster distinct from known species of *Vibrio*, *Aeromonas*, *Plesiomonas*, and *Photobacterium*. Group F strains could be divided into biovars I and II. Biovar I was anaerogenic and isolated from aquatic environments and diarrheal cases, whereas biovar II was aerogenic and not disease associated. Subsequent studies revealed that aerogenic strains were a unique species, and these were reclassified as *V. furnissii* (249) (see "*V. furnissii*" below).

Reservoirs and Identification

V. fluvialis has frequently been isolated from brackish and marine waters and sediments in the United States as well as other countries. It has also been isolated from fish and shellfish, in some cases causing disease in these animals. Its detection rate is usually high in marine mollusks, mainly in bivalves, as they may accumulate the bacterium during the process of filter feeding. Thus, isolation from bivalves has been reported in Senegal, Brazil, Costa Rica, Malaysia, and Taiwan, among other countries. The bacterium has only rarely been isolated from freshwaters.

Alongside culturing methods (see "Isolation" and "Identification" above), molecular tools have been developed for the identification of *V. fluvialis*, including PCR, PCR coupled with denaturing gradient gel electrophoresis, quantitative PCR targeting the *toxR* gene, *groEL* PCR-RFLP analysis, and MALDI-TOF analysis (15, 250–253).

Foodborne Outbreaks

According to the World Health Organization (254), *V. fluvialis* infections are common in areas that have high levels of feces-contaminated water and food supplies and consumption of raw or contaminated seafood. The greatest outbreak occurred in Bangladesh in 1976–1977, where *V. fluvialis* was isolated from over 500 patients during a 9-month period. Since this outbreak, however, *V. fluvialis* has only occasionally been reported as an enteric pathogen. Klontz and Desenclos (255) described 12 persons with gastroenteritis in Florida between 1982 and 1988, and a subsequent survey of vibriosis in Florida during an 8-year period found that 5.6% of 125 gastroenteritis cases were caused by *V. fluvialis* (256). Consumption of raw oysters was implicated in most cases, although shrimp and fish consumption was also sporadically associated. Foodborne diarrheal outbreaks caused by *V. fluvialis* have also been reported in Brazil, Russia, and India (257–259). Incidence of the organism among travelers with diarrheal symptoms seems to be low compared to that of other pathogens.

Characteristics of Disease

V. fluvialis-related illness is characterized by gastroenteritis, nausea, loss of appetite, vomiting, and watery bloody diarrhea with abdominal cramps or significant fever (260). Moderate to severe dehydration, hypokalemia, metabolic acidosis, and occasionally hypovolemic shock can occur in 4 to 12 hours if fluid losses are not replaced. *V. fluvialis* has also been reported to cause necrotizing fasciitis and septicemia in the Gulf of Mexico and Southeast Asia, associated with minor trauma and exposure to fish, raw oysters, shellfish, crabs, or seawater, especially in the summer months (261). A case of biliary tract infection in an immunocompromised patient with end-stage renal disease and liver cirrhosis was also reported (262). The source could not be identified, although ingestion of undercooked seafood or contamination of a cutaneous lesion was highly probable. A case of acute otitis was also reported in a 40-year-old female who had been swimming in a seawater pool (263). Severe infection by *V. fluvialis* is favored by different host factors, including liver disease, an immunocompromised state such as HIV/AIDS, iron overload (e.g., hemochromatosis), and diabetes mellitus (260). In cases of wound infection and sepsis, since *V. fluvialis* causes vascular necrosis, therapeutic levels of antibiotic may not reach the organism and amputation may be necessary to prevent progression.

The infectious dose of *V. fluvialis* is not known. Infections are most common in infants, children, and young adults (264), and successful resolution of infection without antibiotic therapy has been reported; however, treatment with quinolones is generally administered along with intravenous fluids. The emergence of *V. fluvialis* strains that are resistant to quinolones and carbapenem has been a public health concern in recent years (14, 265). Some of these resistance genes are found on large conjugative plasmids that could be easily transferred to other enteric pathogens (265).

Virulence Mechanisms

V. fluvialis strains, of either clinical or environmental origin, express many putative virulence factors, including proteases, cytolysins, enterotoxin-like substance (immunologically indistinguishable from cholera toxin), Chinese hamster ovary (CHO) cell elongation factor, CHO cell killing factor, and hemolysin (14, 260, 264). In addition, endotoxin activity of *V. fluvialis* has been observed *in vitro* (266).

The hemolysin VFH, closely related to the El Tor hemolysin of *V. cholerae* and the heat-labile hemolysin of *V. mimicus* (267), has received special attention as the major *V. fluvialis* virulence determinant. Kothary et al. (268) reported that, in addition to lysing erythrocytes of different animal species, VFH is cytotoxic and elicits fluid accumulation in suckling mice. VFH also plays a key role in mediating IL-1β secretion and histopathology in the colon during *V. fluvialis* infection. In addition, VFH-mediated activation of the NLRP3 inflammasome is involved in the proinflammatory response upon infection (269).

Two QS systems were identified in *V. fluvialis*: an acyl-homoserine lactone-based system and a CAI-1/AI-2-based one (270). QS positively regulates the production of at least two potential virulence factors, an extracellular protease and VFH, and also affects cytotoxic activity against epithelial tissue cultures. Therefore, *V. fluvialis* integrates QS regulatory pathways to play an important physiological role in pathogenesis. Recently, Huang et al. (264) determined that there is at least one functional T6SS in *V. fluvialis* that may play a role in virulence as well as constituting an important factor for successful competition with other microorganisms in the marine environment.

V. FURNISSII

The taxonomy of *V. furnissii*, including phenotypical and genetic traits, was extensively described by Brenner et al. when they reclassified the aerogenic (gas-producing) biogroup of *V. fluvialis* as a new species (249). They compared *V. furnissii* not only with its closest relative, *V. fluvialis*, but also with other vibrios and with *Aeromonas* and *Alteromonas* species. Thus, *V. furnissii* can be differentiated from *V. fluvialis* primarily by its production of gas from glucose and can be differentiated from *A. hydrophila*, another phenotypically similar bacterium, by its ability to grow in 6% NaCl. Different molecular procedures were developed and compared for the detection of *V. furnissii* in different

sample types, as well as to discriminate it from closely related species. Conventional and quantitative PCR assays targeting the *toxR* gene as well as MALDI-TOF analysis proved useful for rapid and reliable identification of this bacterial species (250, 271).

V. furnissii can be considered a widespread marine species (272), and it has been isolated from brackish and estuarine waters and from marine mollusks and crustaceans around the world. Wong et al. (273) determined that approximately 7 to 12% of the oysters, clams, shrimp, and crabs examined in Taiwan are *V. furnissii* positive, whereas Matté et al. (274) observed incidences up to 17% in mussels in Brazil. In addition, an association with eel disease in Spain and with mortalities of tiger shrimp (*Penaeus monodon*) has been suggested (228).

Foodborne Outbreaks

The largest documented outbreaks were reported in 1969, when *V. furnissii* was isolated from American tourists with acute gastroenteritis after returning from Tokyo and Hong Kong. The first outbreak affected 23 out of 42 people, and *V. furnissii* was recovered from stool specimens, sporadically in association with *V. parahaemolyticus*. Epidemiological investigation implicated shrimp and/or crab salad as the possible source of infection. The second outbreak affected 24 of 59 people, and *V. furnissii* was isolated together with several other potentially enteropathogenic bacteria, hampering a reliable etiology. A food vehicle could not be identified. The CDC reported 16 cases of *V. furnissii* gastroenteritis to the end of the 1980s and 10 cases between 1997 and 2008, with isolation of the bacterium from stool, blood, or wounds, although no further epidemiological information was given. According to COVIS, since 2009, the incidence of *V. furnissii* infection accounted for less than 1% of total *Vibrio* infections. Although *V. furnissii* incidence may appear low, this bacterium can be a serious pathogen, causing septicemia by blood poisoning and skin lesions in patients with underlying comorbidities (250, 275).

Characteristics of Disease

V. furnissii gastroenteritis symptoms included diarrhea (91 to 100%), abdominal cramps (79 to 100%), nausea (65 to 89%), and vomiting (39 to 78%), with no fever reported (249). Onset of symptoms is between 5 and 20 hours, with patients recovering within 24 hours. The symptoms of a case of bacteremia and skin lesions were reported, including bilateral lower extremity swelling and erythema associated with burning, weeping lesions (275). The infectious dose of *V. furnissii* is not known, and no significant preference for age or gender can be established after the analysis of the data reported by COVIS.

Virulence Mechanisms

Potential virulence factors of *V. furnissii* include exocellular metalloproteases with elastolytic and hemagglutinating abilities (276), phosphomannomutase implicated in lipopolysaccharide biosynthesis (277), and oligopeptide permeases with multifaceted functions, including solute binding, *in vitro* hemolysis, cytotoxicity, and antibiotic resistance (278). *V. furnissii* also produces the siderophore fluvibactin and has the ability to gain iron from a xenosiderophore, desferrioxamine B, through an outer membrane receptor similar to that of *V. vulnificus* (279, 280). In addition, the siderophore fluvibactin is responsible for the antibacterial activity of *V. furnissii* against other vibrios (281). The complete genome sequence of *V. furnissii* strain NCTC 11218 was recently determined (272, 282). Although *V. furnissii* lacks several features usually associated with virulence in other pathogenic vibrios (*toxRT*, *zot*, *ctxAB*, and a T3SS), it produces a novel RTX-type toxin, which confirmed its potential as an emerging marine and human pathogen, especially in tropical coastal regions.

G. (*V.*) *HOLLISAE*

The species *V. hollisae* was first described in 1982 with the characterization of 16 strains, most of them obtained from stool samples or intestinal contents of patients with diarrhea (283). Twenty years later,

V. hollisae was reclassified into a newly formed genus as *Grimontia hollisae* on the basis of results obtained from fluorescent amplified fragment length polymorphism fingerprinting and from 16S rRNA gene sequence analysis, which demonstrated that this species was quite different from other vibrios (284, 285). *G. hollisae* is unusual among vibrios in its inability to grow on the two media routinely employed for the examination of stool samples, TCBS agar and MacConkey agar. Therefore, it is possible that infections caused by this species are not detected in clinical laboratories. However, *G. hollisae* grows well on blood agar and marine agar, and culture on xylose-lysinedesoxycholate agar recovers this species. *G. hollisae* can be properly identified with the API-20E system. In recent years, several molecular methods were developed for the detection and discrimination of *G. hollisae*, including DNA colony hybridization and PCR protocols targeting *gyrB* or *toxR* (286), loop-mediated isothermal amplification assay (287), and use of a PCR-ligase detection reaction-universal array (17).

Reservoirs

The distribution of *G. hollisae* is not well documented, although it is likely a marine species, and it appears to prefer warm waters (288). *G. hollisae* has rarely been detected in environmental samples, and it has been hypothesized that it may be present in the environment as VBNC cells (286). A possible selective retention in shellfish was suggested for this species, since it could be detected in mussels but not in surrounding waters in southern Italy (289). In addition, it has been isolated from different fish species, such as healthy sharpbeak terapon (*Rhynchopelates* [*Therapon*] *oxyrhynchus*) and diseased amberjack (*Seriola dumerili*) suffering from fish vibriosis (228, 290). *G. hollisae* displays a temperature-regulated rugose phenotype characterized by the production of copious amounts of exopolysaccharide consisting partially of *N*-acetylglucosamine and mannose (291). A role for this phenotype in the survival of *G. hollisae* in the environment has been suggested.

Foodborne Outbreaks

According to the literature, most *G. hollisae* cases correspond to episodes of moderate to severe gastroenteritis in adults, although some extraintestinal diseases and even septicemia were occasionally associated with this bacterium (288, 292). A strong correlation between *G. hollisae* infections and consumption of raw seafood has been suggested, although cases have also been reported following consumption of fried catfish and dried and salted fish, suggesting the ability of *G. hollisae* to survive these treatments. In addition, other sources could play a role as in the case of a 61-year-old male suffering from *G. hollisae*-associated diarrhea who denied recent travel or seafood consumption (288).

Until 1996, all *G. hollisae* cases were reported in the United States, mostly on the Atlantic coast and the Gulf of Mexico and less frequently on the Pacific coast and in Hawaii. Morris et al. (293) described nine cases of *G. hollisae*-associated diarrhea, with raw oysters, clams, or shrimp as the possible source. Between 1981 and 1988, 10% of vibrio illnesses occurring in Florida were due to *G. hollisae* (256). Of these, 62.5% followed ingestion of raw oysters, with development of gastroenteritis or septicemia. The most severe form of gastroenteritis caused by *G. hollisae* was reported in Vietnam in a man presenting with profound hypotension and acute renal failure secondary to hypovolemic shock (292).

Cases of septicemia associated with *G. hollisae* are scarce. The first case was in a 65-year-old male who reported consumption of fried Mississippi River catfish and no history of exposure to saltwater or other seafood consumption (294). This unusual case occurred following consumption of a freshwater fish that had been fried, suggesting that incomplete cooking or recontamination of the fish occurred. An additional case of foodborne septicemia was associated with dried and salted fish that had been obtained from a Southeast Asian food store and eaten uncooked (295). The first case of gastroenteritis and bacteremia reported in Europe was associated with consumption of cockles in France (296), whereas a severe case of gastroenteritis and hepatitis occurred in the Mediterranean area in 2009 after consumption of raw oysters (297). It is noteworthy that of the four cases of septicemia reported, three occurred in patients with underlying liver disease.

Characteristics of Disease

Symptoms of gastroenteritis caused by *G. hollisae* are similar to those caused by *V. cholerae* non-O1/non-O139 (293) and typically include severe abdominal cramping, vomiting, fever, and watery diarrhea (288).

The period of hospitalization, if needed, ranges between 2 and 9 days, and most patients recover with antibiotics like ciprofloxacin (296) or tobramycin and cefamandole for septicemic cases (294). One fatal case due to complications such as encephalopathy and sepsis by *Cryptococcus neoformans* was reported (293). There is no evidence of seasonality-associated infections, and most cases are in healthy individuals, whereas underlying disease appears to be the norm for septicemic cases (288, 293).

Virulence Mechanisms

G. hollisae produces a number of toxins and also invades host epithelial cells (298, 299), indicating that its pathogenicity may be multifactorial. These features are consistent with septicemia, which is an invasive disease, as observed in some patients (294). A hemolysin that is related to the TDH of *V. parahaemolyticus* was characterized in *G. hollisae* (299, 300). The two toxins seem to have similar lethal toxicities in mice, although different lytic activities on erythrocytes from various animals have been described (299). It has been reported that all strains of *G. hollisae* carry the *tdh* gene, which is phylogenetically distant from and dissimilar in prevalence to homologous genes in other vibrios (301). An extracellular enterotoxin which is structurally and functionally different from cholera toxin or the heat-labile toxin of *Escherichia coli* and which elongates CHO cells and causes fluid accumulation in mice has also been characterized (302). In response to iron limitation, *G. hollisae* produces aerobactin as a major siderophore, which may play a role in the ability of this species to cause infection (303). In one of the bacteremia cases, the patient had improperly dosed himself with ferrous sulfate as a supplement for chronic anemia, and such treatment may have exacerbated the disease process.

V. MIMICUS

Given its peculiar biochemical characteristics (no sucrose fermentation), *V. mimicus* was initially believed to be an atypical *V. cholerae* non-O1/non-O139 strain responsible for gastroenteritis and ear infections. Only in the early 1980s was it recognized as a new pathogenic species of the genus *Vibrio* (304). The differences in biochemical metabolism between *V. mimicus* and *V. cholerae* are mainly caused by gene deletion (305). The absence of sucrose fermentation is associated with the loss of genes coding for a sucrose-6-phosphate dehydrogenase and a fructokinase; the different lipase reactions and the negative Voges-Proskauer test for *V. mimicus* are also due to the absence of a lipase and an acetolactate synthase, respectively (305).

V. mimicus relies on a few virulence factors that are either acquired by horizontal gene transfer from other vibrios (306) or species specific. Based on whole-genome sequencing, *V. mimicus* shares almost 64% of its genome with *V. cholerae*, including genes coding for hemolysins and Ctx (cholera toxin) as well as toxin-coregulated pilus pathogenicity islands, which are responsible for the similar pathogenicity observed in *V. mimicus* and *V. cholerae* (305). *V. mimicus* strains carrying the genes encoding the cholera toxin (*ctxAB*) may produce Ctxlike enterotoxins and cause severe watery diarrhea.

The most important virulence factor of *V. mimicus* is a heat-labile hemolysin designated *V. mimicus* hemolysin (VMH) (307). The gene encoding VMH is ubiquitous in clinical and environmental isolates, and this protein is responsible for fluid accumulation in the gut (similar to cholera enterotoxic activity) with consequent severe diarrhea, abdominal pain, and fever (308). The *V. mimicus* protease is another extracellular product that has no enterotoxic activity of its own but has been proven to modulate the activity of VMH and/or its precursor, pre-VMH, through limited proteolysis (309). Recently, a third virulence-associated protein was detected in a *V. mimicus* isolate from brackish water. This protease is an enzyme similar to trypsin and is able to activate pro-VMH to its mature form, VMH (310).

V. mimicus has been recognized as a cause of gastroenteritis transmitted worldwide by raw oysters, fish, prawns, squid, and crayfish (311). In 2014, the CDC reported 31 cases of vibriosis whose etiological agent was recognized as *V. mimicus*, with a 3% death rate and almost 80% of the cases being of

confirmed foodborne origin (2). *V. mimicus* outbreaks are not frequent, and the largest known foodborne outbreak to date, involving 306 people, was caused by the ingestion of contaminated freshwater fish and seafood in Thailand (312). Recurrent outbreaks took place in Taiwan between 2004 and 2006 (313). The first case of *V. mimicus* infection associated with mass mortality in freshwater catfish was recently reported in China, highlighting the risk of cross-contamination to humans, since catfish are the most widely cultured fish and an important food source in China (314).

V. METSCHNIKOVII

Taxonomically, *V. metschnikovii* was defined as a new species of the genus *Vibrio* in the late 1970s, although it is distinguished from other *Vibrio* species by its inability to produce cytochrome oxidase and to reduce nitrate (315). *V. metschnikovii* is a natural inhabitant of the aquatic environment and has been isolated from marine water, estuarine water, and wastewater, as well as from fish, crabs, oysters, and diseased birds. The largest occurrences were documented in rural communities in Peru, Brazil, South Africa, and Nigeria, with seafood, stream water, and final effluent wastewater likely being the causes of human infection.

Although *V. metschnikovii* is present in a wide range of aquatic products and is associated with human diseases, this species has not been extensively studied, and little published work is available. The first clinically significant isolate of *V. metschnikovii* was recovered in the early 1980s from the bloodstream of a patient suffering from peritonitis, and since then, it has been associated with cases of septicemia, wound infection, diarrhea, pneumonia, and, most recently, severe septic shock and cardiac arrest (316). *V. metschnikovii* can act as both a human enteric pathogen and a wound pathogen, and its prevalence accounted for <1% of vibriosis cases in the United States in 2014 (2).

Recent proteomic studies on the prolonged exposure of *V. metschnikovii* to low temperatures have demonstrated differentially expressed genes coding for outer membrane proteins, proteins involved in energy conversion and metabolism (317), and proteins involved in the modulation of fatty acid composition to control membrane fluidity (30). *V. metschnikovii* seems to be cold adapted, an advantage when temperature downshifts during food processing and storage are encountered. Similarly, *V. metschnikovii* shows a pattern of seasonal abundance different from that of other pathogenic *Vibrio* species, as it can be isolated in cold waters (e.g., the Black Sea and the Russian Far East) during winter months (30, 318).

The exact mechanism of pathogenicity is still not clear, although a few virulence factors have been described. *V. metschnikovii* is able to cause hemolysis of erythrocytes from different animal species, with higher susceptibility being observed in human cells. This phenomenon has been associated with a cytolysin responsible for vascular permeability changes and fluid accumulation in the intestines (319). Various extracellular alkaline serine proteases have been found in *V. metschnikovii* strains as early as the 1980s. For two of these enzymes, kinetic properties and substrate specificity for various human blood coagulation-associated proteins (plasminogen, fibrinogen, and fibrin polymer) have also been demonstrated (320, 321). Although the complete genome of *V. metschnikovii* was sequenced in 2009 (GenBank accession no. NZ_ ACZO01000006.1), most of its proteins are hypothetical, and their potential involvement in pathogenicity is yet to be discovered.

V. METOECUS

As of 2014, one case of confirmed foodborne vibriosis by *V. metoecus* was reported by the CDC (2). *V. metoecus* was first described as a new species, closely related to *V. cholerae*, isolated from a water sample from the Chesapeake Bay estuary (Maryland) in 1998 (322). *V. metoecus* is a ubiquitous inhabitant of coastal marine lagoons (323–325). Recently, phenotypic and biochemical analysis showed that although *V. metoecus* strains resemble *V. cholerae*, they are able to grow using *N*-acetyl-D-galactosamine and D-glucuronic acid as sole carbon sources and are negative for the Voges-Proskauer assay, in contrast to *V. cholerae* (326). While 16S rRNA gene sequencing does not differentiate between the two species, multilocus sequence analysis successfully outlined the phylogenetic relationship of *V.*

metoecus to both *V. cholerae* and *V. mimicus* (326). In an effort to further define differences and similarities among these species, the genomes of clinical and environmental isolates of *V. metoecus* from the United States were recently fully sequenced, together with *V. cholerae* strains originating from the same environmental reservoir (Oyster Pond, MA) (327). Overall, *V. metoecus* shares 84% of its open reading frames with *V. cholerae*, but the major *V. cholerae* virulence factors (cholera toxin and toxincoregulated pilus) are absent from both clinical and environmental *V. metoecus*, confirming the absence of toxigenic strain reports. However, *V. metoecus* carries both the entire *Vibrio* pathogenicity island 1 and the core of pathogenicity island 2, suggesting interspecies movement of pathogenicity islands and evolution of pathogenic variants (327). The occasional presence of pathogenicity islands that originated from pandemic *V. cholerae* might play a role in the virulence at the base of *V. metoecus* vibriosis, although the exact mechanism of infection is still unknown.

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14
Cronobacter Species

TAXONOMY AND NATURAL HISTORY OF *CRONOBACTER*

Cronobacter species (formerly known as *Enterobacter sakazakii*) represent a genus of foodborne opportunistic pathogens which are characterized as being Gramnegative, rod-shaped bacteria that are non-spore-forming, facultatively anaerobic, oxidase negative, catalase positive, and motile by means of peritrichous flagella (Fig 14.1). In 1980, Farmer et al. (1) reported results of DNA-DNA hybridization studies and phenotypic observations, which led to redefining a group of previously designated yellow-pigmented *Enterobacter cloacae* strains as a new species called *E. sakazakii*, a name which was chosen in honor of the Japanese microbiologist Riichi Sakazaki (1920–2002). These seminal studies described the *E. sakazakii* species complex as a diverse group of microorganisms that embodied 15 phenotypically distinct biogroups. Subsequently, Iversen et al. in 2006 described a 16th biogroup (2), and the novel genus *Cronobacter* was proposed in 2007 by Iversen et al. (3). The genus was further taxonomically characterized by Iversen et al. (4) using a polyphasic approach that encompassed results from full-length 16S rRNA gene-based sequencing, ribotyping, fluorescent amplified fragment length polymorphism analysis, and DNA-DNA hybridization studies, in addition to phenotypic data to help differentiate strains among the seven species. These data together led to the revision of the taxon and the proposal that *Cronobacter sakazakii* subsp. *malonaticus* be given its own species epithet, *Cronobacter malonaticus*. The genus *Cronobacter* currently consists of seven species, including *C. sakazakii*, *C. malonaticus*, *C. turicensis*, *C. muytjensii*, *C. dublinensis*, *C. universalis*, and *C. condimenti* (4, 5).

The ability to reliably and specifically detect and identify *Cronobacter* species and differentiate them from phylogenetically related members of the *Enterobacteriaceae*, which may occupy similar econiches and foods, would contribute immensely toward reducing health risks for at-risk individuals. This chapter summarizes the current taxonomy, epidemiology, and laboratory-based approaches that can be used to detect, characterize, and trace this important human pathogen.

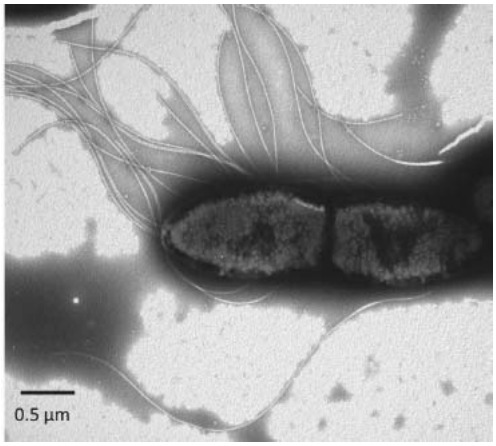


Figure 14.1 Transmission electron photomicrograph of *C. sakazakii* strain BAA-894 negatively stained with 0.5% sodium phosphotungstate (pH 6.8) showing numerous peritrichous flagella. Bar, 0.5 µm.

Epidemiology and Related Public Health Trends

The notoriety of *Cronobacter* was elevated to a global public health concern when contamination of powdered infant formula (PIF) was linked to several neonatal meningitis outbreaks (6). Though historically, PIF was thought to be the source of the infections, it is clear now that contamination of reconstituted, temperature-abused PIF can occur intrinsically and extrinsically, although the main reservoir(s) and routes(s) of contamination have yet to be determined. Except for *C. condimenti*, all species of *Cronobacter* have been isolated from clinical specimens, and currently, *C. sakazakii*, *C. malonaticus*, and *C. turicensis* are the primary human pathogens of interest.

These pathogens cause infantile necrotizing enterocolitis (NEC), septicemia, and meningitis and have mortality rates which have been reported to be as high as 80%, posing a serious threat to neonates, particularly those that present as underweight and are immunocompromised. Because PIF is not manufactured as a sterile product, it poses a significant risk if the product is reconstituted and handled inappropriately. The consensus is that the most likely cause of some of these infections may be poor personal hygiene practices of those involved in PIF preparation. However, Jason (7) recently reported that 7 of 82 (8%) infants surveyed between 1958 and 2010 became ill with invasive disease (defined here as a confirmed culture-positive case of septicemia or meningitis) after ingesting breast milk exclusively (without supplementation with PIF or powdered human milk fortifiers) prior to illness onset. To emphasize this point, Bowen et al. (8) recently reported an infantile case of septicemia and meningitis where the child consumed pasteurized donated human milk and expressed maternal milk (EMM) during the first week after birth. Thereafter, the infant received EMM mixed with a commercial liquid human milk fortifier. The infant did not receive PIF products. Maternal milk was expressed using a dedicated bedside hospital breast pump and the mother's personal breast pump throughout the infant's hospitalization and subsequent home care. *C. sakazakii* was subsequently cultured from the valves of the personal breast pump, frozen EMM samples, and the drain of the kitchen sink at the infant's home. It is thought that the breast pump kit became contaminated and was not adequately cleaned or sanitized, leading to contamination of the milk expressed with this pump kit. Since then, the CDC has developed guidance to optimize breast pump hygiene practices (https://www.cdc.gov/healthywater/hygiene/healthychildcare/infantfeeding/breastpump.html).

As mentioned above, neonates and infants are particularly vulnerable to invasive *Cronobacter* infection; and from the onset of symptoms, death can occur within hours. Recovery from these infections often progresses to lifelong chronic neurological sequelae, such as hydrocephalus, permanent neurological damage, and mental disabilities, which result in later developmental challenges or early death (9).

Until recently, *Cronobacter* was thought to be only a neonatal pathogen. A current trend that has been observed is that more full-term infants leave the hospital only to return with an invasive disease caused by a *Cronobacter* infection. Thus, of primary pediatric concern is that *Cronobacter* causes three types of disease: NEC, septicemia, and meningitis.

Necrotizing Enterocolitis

NEC is the most common inflammatory intestinal disorder observed in neonates, especially in preterm infants with very low birth weights (VLBW; defined as a weight at birth of <1,500 g). Although the exact etiology of NEC remains to be determined, multiple risk factors have been implicated in the pathogenesis of the disease. For example, prematurity (birth at less than 36 weeks of gestation), a history of formula feeding, and underdeveloped microbial colonization of the gastrointestinal tract have been identified as risk factors (10–13). The incidence rate of NEC in VLBW infants has somewhat stabilized over the years, and this disease is currently observed in approximately 5 to 7% of all patients Admitted to neonatal intensive care units (14). Even though NEC has been associated with *Cronobacter* species, to date no single pathogen has been identified as being more involved in pathogenesis than any other (14).

The sequence of events leading to infantile NEC can rapidly evolve over a short span of time (within 6 h) and is complex; damage to the intestinal epithelium is believed to involve weakening of the intestinal barrier by unknown perinatal insults, possibly secreted lipopolysaccharide (LPS), followed by colonization of the luminal epithelial surface and translocation across the weakened epithelium. An "out-of-control" inflammatory response ensues, and exacerbation of the gut barrier damage by host inflammatory factors is thought to lead to the intensive cycle of inflammation-inflicted epithelial damage (13, 15). Infantile disease, mortality, and morbidity vary with the degree of mucosal damage, and pathology can range from mild to severe, affecting both the large and small bowel. Often with newborns, the formation of gas cysts within the bowel wall (called pneumatosis intestinalis) occurs. Pneumatosis intestinalis is considered diagnostic for NEC, and the gas within the cysts is produced as a result of bacterial colonization and metabolism within the cysts. Typically, this is followed by intestinal ischemia, which leads to sepsis, peritonitis, disseminated intravascular coagulation, shock, and death in more severe cases (15). Grishin et al. (13) suggest that nitric oxide (NO), produced by inducible NO synthase (iNOS), and subsequent oxidation of its physiological intermediates play a prominent role in the intestinal barrier damage by inducing enterocyte apoptosis and inhibiting epithelial compensation processes, such as enterocyte proliferation and migration. The factors that govern iNOS upregulation in the intestine are not well understood, a feature that has also hampered efforts in developing NO/iNOS-targeted therapies. However, microbiome studies of hospital-associated outbreaks of NEC reveal an infectious nature of this disease (15, 16). A NEClike disease was observed and described by Hunter et al. (16) using a rat pup infection model. In these studies, exposure to *C. muytjensii* induced apoptosis and caused an increase in the production of interleukin 6 (IL-6) within IEC-6 tissue culture cells and in the rat pup infection animal model, which were hallmarks of the experimental infection. These data suggest that *C. muytjensii* induced NEC and triggered intestinal disease by modulating enterocyte intracellular signaling pathways. However, these animal studies also revealed that the induction of NEC may be related to unknown properties of specific strains rather than a *Cronobacter* species. Nonetheless, a greater understanding of the infectious nature and host response involved in NEC is warranted, but this will require extensive microbiological experiments, genome-wide analysis of bacterial virulence factors, and bacterial and host transcriptomic studies.

Septicemia

It is thought that after successful intestinal colonization by *Cronobacter* species (see the description of adherence factors in "Virulence Factors" below), the pathogen invades the intestinal epithelial cells in a process (either by active adherence and invasion of epithelial cells or via NEC, as described above) that progresses with the pathogen translocating across the intestinal tissue layers to enter the systemic circulatory system, resulting in an extraintestinal infection, such as sepsis or meningitis (17). Much of what is known about this process comes from extrapolating data from adherence and invasion studies using intestinal and immune tissue culture cell lines. Singamsetty et al. (17) suggested that human brain microvascular endothelial cells (HBMECs) are most susceptible to *Cronobacter* invasion, after demonstrating a higher invasion frequency in HBMECs than in endothelial and epithelial cells from different tissues. Townsend et al. (18) determined that *Cronobacter* invasion within HBMECs was strain dependent, and there was no direct correlation between attachment and invasion rates for intestinally derived Caco-2 cells. However, Kim and Loessner (19) reported that *Cronobacter* invasion of Caco-2 cells was accomplished by an active process that needed *de novo* protein synthesis and depended on exposure time and multiplicity of infection. Recently, Giri et al. (20) also determined that *Cronobacter* isolates could undergo transcytosis across tight monolayers of Caco-2 cells and HBMECs, thus mimicking the *in vivo* ability to cross the intestine and subsequently the blood-brain barrier (BBB) to cause meningitis. In addition, the rate of transcytosis of some strains was equivalent to that of the control meningitis-causing *Escherichia coli* K1 strain; however, this rate varied significantly among isolates.

Most pathogenic bacteria that enter a host cell typically manipulate the cytoskeleton of the host cell either through the secretion of effector molecules or by interfering with various phosphorylation cascades that are associated with the intracellular signal transduction pathways (18, 20–22). Adherence and invasion studies (18, 19, 22) clearly revealed that *Cronobacter* exploits host

cytoskeleton elements such as microfilaments and microtubules to invade host cells. Actin microfilaments are the best-known structures involved in this process. This was supported by the fact that treating intestinally derived INT-407 cells, Caco-2 cells, and HBMECs with the actin-depolymerizing agent cytochalasin D completely blocked invasion by *C. sakazakii* (17, 20, 23). It is also thought that *C. sakazakii* invasion of HBMECs involves participation of distinct signal transduction processes in which the phosphatidylinositol 3-kinase (PI3K)–Akt signaling pathway also plays an essential role in initiating the rearrangement of actin filaments followed by bacterial internalization (18, 20, 24). Liu et al. (24) determined that host cytosolic phospholipase A2a is a downstream effector of PI3K-Akt signaling, required for actin reorganization in HBMECs elicited by *C. sakazakii*. In contrast, it was shown that *Cronobacter* severely impaired the phosphorylation of p38, extracellular-signal-regulated kinase 1/2, and c-Jun N-terminal kinase, which are major components of the mitogen-activated protein kinase (MAPK) pathway, and prevented the activation and maturation of dendritic cells by disarming the MAPK pathway, a process that may aid the pathogen in evading the immune system (22).

Meningitis

Meningitis caused by Gram-negative bacteria was first described in the 1940s as a likely cause of mortality and morbidity throughout the world despite advances in antimicrobial chemotherapy and supportive care. *Cronobacter* species are well-known etiological agents of nosocomial meningitis, especially in the neonatal intensive care unit setting. However, its tropism for the central nervous system in neonates and infants still remains a mystery. Our general lack of knowledge about the pathogenesis of meningitis has also contributed to the high mortality and morbidity rates (as high as 80%, as mentioned above) that are seen in infants infected with *Cronobacter*. Meningitis often results in ventriculitis, brain abscess, or cyst formation, as well as late development of hydrocephalus, requiring ventricular peritoneal shunting (25, 26). This disease process has an extremely poor clinical outcome, with a current estimated mortality rate of 42% (27). It usually manifests in infants that are born at full gestational age, and it occurs in the first few days of life (28, 29). Mortality and morbidity associated with *Cronobacter* meningitis are high, and virtually all patients recovering from the central nervous system infection suffered mental and physical developmental delays. Some information describing some of the mechanisms involved in meningitis has come from *in vitro* studies. *Cronobacter* can exploit dendritic cells, and they can persist within human macrophages, enabling them to avoid the host's immune response to reach and penetrate the BBB (19, 22, 30).

In general, pathogens that cause bacterial infantile meningitis and their antimicrobial susceptibilities have changed dramatically in the era of increasing antimicrobial resistance, so use of conjugated vaccines and maternal antibiotic prophylaxis is applicable for pathogens such as group B *Streptococcus*. The outcome of *Cronobacter* meningitis has improved with the availability of the third-generation cephalosporins. However, continued surveillance is warranted, because antimicrobial resistance will continue to provide challenges for clinicians.

Friedemann (28) has summarized the epidemiology of invasive neonatal *Cronobacter* infections between 2000 and 2008. She reported that the overall lethality of 67 invasive infections was 26.9%, whereas the lethality of *Cronobacter* meningitis, bacteremia, and NEC combined was 41.9%. Her report revealed some interesting trends. For example, included as significant factors were the following: (i) a higher gestational age at birth is an important factor which lowers the risk of infection; (ii) having parentage not from Europe leads to a higher probability of reporting neonatal *Cronobacter* meningitis; and (iii) neonates with *Cronobacter* meningitis not originating from North America have a higher risk for a lethal outcome than those with other neonatal *Cronobacter* infections. Also, her study confirmed that neonatal *Cronobacter* infections are mainly associated with the contamination of infant formula and of the relevant cleaning and preparation equipment. However, she also noted 11 neonatal *Cronobacter* infections that were not associated with consumption of contaminated infant formula.

Adult Infections

Cronobacter is also known to cause infections in adults, most notably in elderly and immunocompromised individuals (27, 31–34). For example, recent surveillance data from the United States revealed that there is a higher percentage of *Cronobacter* infections in adults than are reported in infants, even though greater invasive disease or more severe disease and higher mortalities occur in the latter group (31). Adults usually present clinically with extraintestinal infections such as urinary tract (urosepsis) and wound infections, as well as cases of septicemia, osteomyelitis, and pneumonia. Most adult cases are nosocomial in origin (26).

Cronobacter Associated with Foods and Other Natural Habitats: Strategies for Survival, Persistence, and Virulence

Cronobacter species are considerably more widespread than was once thought, and as mentioned previously, besides PIFs and follow-up formulas (FUFs), they have been associated with many low-water-activity (a_w) foods and environments, such as milk protein products, cereals, cheeses (and cheese powders), licorice, candies, spices, teas, nuts, herbs, ready-to-eat foods (such as pastas), and a variety of vegetables, as well as filth, stable flies, PIF and milk powder production facilities, and household environments (35–37). *Cronobacter* can survive in conditions of extreme desiccation (high osmotic stress), such as low-a_w foods, and this property is thought to influence its environmental persistence in PIF and FUF manufacturing facilities and in other dried products and desiccated environments (38, 39). The primary response to highosmotic-stress environments (such as low-a_w foods) involves the rapid accumulation of certain electrolytes (potassium [K^+], trehalose, and glutamate) to increase the cell's internal osmotic pressure, thus counteracting the high external osmolar conditions. When a cell internalizes elevated potassium concentrations, glutamate also is involved. Together, these molecules act as temporary osmoprotectants. The principal compatible solutes accumulated by *Cronobacter* under osmotically stressful environments include glycine betaine, proline, carnitine, and trehalose. For more information about the osmotic response, see the article by Feeney and Sleator (40).

It is now thought that the widespread occurrence of *Cronobacter* and its resistance to desiccation are factors contributing to the commercial distribution of contaminated low-a_w foods (if proper surveillance is not maintained), which pose a risk to susceptible consumers and widen the scope of public health concerns (41, 42). It is likely that a detailed understanding of the *C. sakazakii* osmotolerance response will eventually lead to more effective control measures to limit survival and growth of *C. sakazakii* in low-a_w products, such as PIF.

The ability of *Cronobacter* species, as is the case for any microorganism, to withstand the harmful effects of exposure to different environmental stresses is crucial for their survival, persistence, and virulence. Besides the ability to tolerate osmotic stress, *Cronobacter* spp. have also adapted quite well to exposure to high-growth-temperature conditions (43). For example, Williams et al. (44) identified a biomarker in heat-tolerant *Cronobacter* strains that Edelson-Mammel and Buchanan (43) determined was highly heat resistant. The protein was determined to be a hypothetical protein found in the thermotolerant bacterium *Methylobacillus flagellatus* KT. Gajdosova et al. (45) described an 18-kbp region that contained a cluster of genes, including that for the hypothetical protein found by Williams et al., that had significant homology with other known bacterial gene clusters encoding proteins involved in some type of stress response, including heat, oxidation, and acid stress. However, not every strain that possesses a thermotolerant phenotype has this gene cluster (46), suggesting that another molecular mechanism involving thermotolerance exists. Many studies have revealed that *Cronobacter* species can grow in environments with low pH levels (pH 3.9 to 4.5) and are somewhat more acid tolerant than most other related enteric pathogens (47). However, more information is needed to fully understand how *Cronobacter* species can survive under these stressful growth conditions and whether multiple commercially available treatment regimens can be used to successfully prevent growth in ready-to-eat foods and PIF. A study by Jang and Rhee (48) revealed an inhibitory effect when caprylic acid and mild heat were combined to reduce *Cronobacter* growth in reconstituted PIF. The combined treatment resulted in a "synergistic effect," in which *Cronobacter* populations were reduced much

more rapidly with increased temperatures and concentrations of caprylic acid. Besides providing an understanding of the mode of action of caprylic acid (damage to the bacterial cell wall), these data also reveal that the addition of this natural antimicrobial agent to infant formula may have potential use for controlling this pathogen prior to consumption. The investigation of how *Cronobacter* persists under conditions of osmotic, thermal, and acidic stress is still in its infancy, but it holds the key to controlling this foodborne pathogen within the food manufacturing environment.

There are a few reports revealing that *Cronobacter* is a member of the human intestinal tract flora and may be carried in the intestinal tracts of healthy adults and neonates (41, 42). However, more epidemiologic information is needed to understand its role as a member of the gut microflora and to determine whether its presence represents transient colonization or other host associations. Also, more information regarding its association with the postpartum intestinal microbiota is needed. Lastly, surveillance studies using species-specific identification schemes that include at least results of species-specific PCR assays or at best species identities obtained from whole-genome sequencing (WGS) studies are needed so that a clearer epidemiologic picture can be obtained as to which *Cronobacter* species are responsible for which infections (49–52).

ISOLATION AND DETECTION METHODS

Microbiological Detection and Isolation Procedures

Early culture methods for the detection and isolation of *Cronobacter* from foods, specifically PIF, were developed by Muytjens et al. (53). Each sample was dissolved in buffered peptone water held at 45°C for 5 min until the PIF was dissolved. After overnight incubation at 36°C, a 10-ml volume of the culture was withdrawn from each flask and transferred to 90 ml of *Enterobacteriaceae* enrichment broth. After another overnight incubation, 1 ml of each broth culture was mixed with 20 ml of molten violet red-bile glucose agar (VRBGA) in duplicate and was solidified. The cultures were incubated overnight at 36°C. Suspected colonies were then isolated and identified using an API 20E biochemical test strip and other phenotypic observations, i.e., the production of yellow colonies on nutrient agar after incubation for 48 h at 25°C, the production of extracellular DNase on toluidine blue agar after 6 days at 36°C, and a positive α-glucosidase reaction after 4 h (53, 54). Noted findings from this study were the facts that 52.5% of 141 PIFs, obtained from 35 countries, were contaminated and that the contamination level was low (<1 CFU/g in any product). Since the introduction of this groundbreaking method, other methods utilizing PCR, improved selective enrichment broths and selective agars (such as *Cronobacter* screening broth and chromogenic agars), and more accurate microbial identification schemes (such as the automated Vitek 2 system) have been described.

An example of one of the newer protocols is the International Organization for Standardization (ISO) method published as a technical standard protocol, known as ISO 22964:2017, for the detection of *Cronobacter* species in food products for humans and animal feeds and in environmental samples, which has also gone through a validation study using a 15-laboratory horizontal method for the detection of *Cronobacter* spp. (https://www.iso.org/standard/64708.html). This method, which technically revises an earlier protocol, ISO/TS 22964:2006, in addition to PIF now includes all food products for humans and feeds for animals as well as environmental samples. Other changes include replacing the selective enrichment broth, modified lauryl sulfate tryptose broth, with *Cronobacter* selective broth (55) and the isolation agar, *E. sakazakii* isolation agar (which is a formulation of Druggan-Forsythe-Iversen [DFI]) agar), with chromogenic *Cronobacter* isolation agar.

The U.S. Food and Drug Administration (FDA) established two culture-based methods for the detection and isolation of *Cronobacter* species. The first method was similar to that described by Muytjens et al. (53) and has been since replaced with a revised method (Fig 14.2) utilizing only a buffered peptone water enrichment followed by a real-time PCR assay that incorporated an internal amplification control for rapid screening of products (http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm289378.htm). The revised FDA method utilizes a real-time *Cronobacter* PCR assay designed to detect the partial macromolecular

synthesis operon, consisting of the 3′ end of *rpsU* and the 5′ end of *dnaG* (56). This revised method also replaced VRBGA with two chromogenic agars, DFI agar (synonymous with Brilliance *E. sakazakii* agar) and *E. sakazakii* plating medium, developed by Restaino et al. (55, 57). Both ISO 22964:2017 and the revised FDA method are currently accepted as reference methods for the detection and identification of *Cronobacter* species from foods, feeds, and environmental samples.

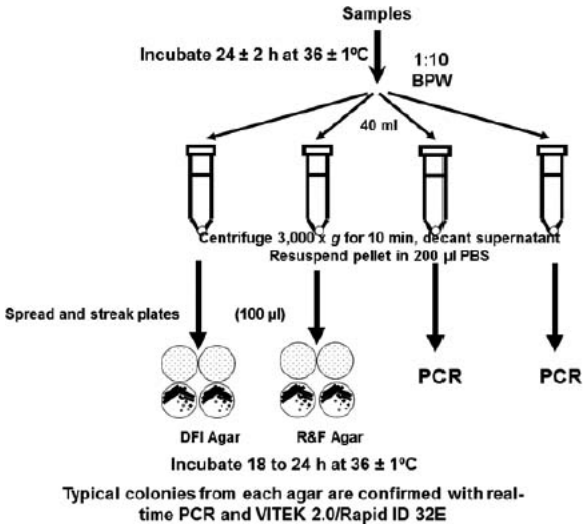


Figure 14.2 Revised FDA *Cronobacter* isolation and detection method, adapted from chapter 29 of the Bacteriological Analytical Manual (https://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm289378.htm). BPW, buffered peptone water; PBS, phosphate-buffered saline; R&F, *Enterobacter sakazakii* plating medium.

Alternative Detection and Isolation Procedures

Mullane et al. (58) described in 2006 a protocol utilizing paramagnetic beads in a novel capture system called Pathatrix, which had undergone precollaborative validation studies. The principle behind Pathatrix includes the use of positively charged magnetic beads, which electrostatically attract the negatively charged LPS on the surface of *Cronobacter* cells (and Gram-negative bacteria in general) as the enrichment broth is circulated over a magnet using a peristaltic pump. This method requires a short enrichment period (6 to 8 h, using buffered peptone water and an incubation temperature of 42°C), followed by capture of the bacteria and subsequent identification after plating onto DFI agar. All presumptive *Cronobacter* colonies were confirmed by using the qPCR procedure described by Seo and Brackett (56). The protocol could detect between 1 and 5 CFU in 500 g of PIF in less than 24 h, and a sample-pooling strategy was found to be useful.

Song et al. (59) described another alternative detection and isolation procedure that includes a fluorescence-based liposome immunoassay that was developed as a sensitive and rapid diagnostic system for detection of *C. muytjensii* in broth cultures. This research group further developed other liposome-based detection assays to be used in an indirect noncompetitive enzyme-linked immunosorbent assay (ELISA) for the detection of *Cronobacter* in PIF (60) and in combination with magnetic beads (61). Finally, an immunochromatographic strip assay which combined antibodies and liposomes with the immunochromatographic strip to detect *C. sakazakii* in enrichment cultures has also been described by this research group (62).

ELISA technology has also been applied commercially in the Vitek immunodiagnostic assay system (known as VIDAS; bioMerieux, Marcy l'Etoile, France) for the detection of enterotoxins of several foodborne pathogens, including *Salmonella* species, *E. coli* O157:H7, *Listeria* species, *Campylobacter jejuni*, and *Staphylococcus* species. Kits for *Cronobacter* species detection have been developed (63).

Trinh et al. (64) described a "lab-on-a-chip" device (or flow cell) that was designed to conduct PCR assays that detected *E. coli* O157:H7 and *C. condimenti* in broth cultures. The lab-on-a-chip PCR device

could successfully detect these pathogens by amplifying nucleotide regions of the *E. coli* O157:H7 Shiga toxin and *C. condimenti rpoB* genes (50). The levels of detection for each DNA target were 1 ng/µl and 3 ng/µl, respectively, and the test could be accomplished within 25 minutes. This highly portable system has the potential to enable PCR-based investigations of *Cronobacter* infections as well as many other diseases quickly and accurately.

MOLECULAR GENUS AND SPECIES IDENTIFICATION AND SUBTYPING CHARACTERIZATION METHODS

Iversen et al. (4) and others (49, 65, 66) observed that phenotypic assays as well as 16S rRNA gene analysis could not identify some *Cronobacter* isolates to the species level. Table 14.1. summarizes the phenotypic assays used to typically identify members of the *Cronobacter* species complex and differentiate them from phylogenetically related species. This is the case for many medically important pathogens and has led to the use of DNAbased detection methods, particularly conventional and real-time PCR assays, which now have become routine laboratory procedures to detect and identify foodborne pathogens from foods, including foods contaminated with *Cronobacter* species. Typically, these assays are designed to target unique pathogen-specific genes. Several studies describing a variety of genes used in such assays have been published. Yan et al. (67) provided a summary of common gene targets that have been useful for the detection of *Cronobacter* spp. using PCR-based methods. These gene targets include 16S rRNA genes, the 1,6-α-glucosidase gene (*gluA*), the macromolecular synthesis gene (*dnaG*), a gene encoding zinc-containing metalloprotease (*zpx*), and *ompA*. Many of these were designed prior to the reclassification of the genus, i.e., when *Cronobacter* species were one species, *E. sakazakii*.

Additionally, Ye et al. (68) compared the efficacy of several of these PCR assays, which target the 16S-23S rRNA gene internal transcribed spacer (ITS) region, *ompA*, and *gluA*, with the ISO method for detecting *Cronobacter* in 243 commercial infant formula samples. Twelve samples were positive for *Cronobacter* by all PCR assays used. Only 10 of the samples were positive by the ISO method, and all 10 were positive by all PCR assays. In contrast, four false-positive results were generated with the ITS region PCR assay and one false-positive result was found using the *ompA* PCR assay, whereas there were two false-negative results with the ISO method. The authors also reported that combining the selective enrichment step(s) with either a duplex PCR assay targeting both the ITS region and *ompA* or targeting *ompA* and *gluA* or a single PCR targeting *gluA* was useful for detecting *Cronobacter* in infant formulas. Similar findings were reported by Jaradat et al. (69). Recently, Li et al. (70) described a real-time (quantitative PCR [qPCR]) assay based on *rpoB* that also detects *Cronobacter* species and used this PCR assay to identify the bacterium in 70 food samples that included 3 dry cereal products, 5 maternal milk samples, and 1 infant food formula sample which were positive by qPCR.

Table 14.1 Typical phenotypic (biochemical) characteristics of *Cronobacter* species[a]

					Reaction for:			
Phenotype reaction	*C. sakazakii*	*C. malonaticus*	*C. dublinensis*	*C. muytjensii*	*C. turicensis*	*C. condimenti*	*C. universalis*	*S. turi*
Voges-Proskauer[b]	V	+	+	+	+	+	+	–
Methyl red[c]	V	-	-	-	-	-	-	+
Mucate	-	-	-	-	-	-	-	+
Dulcitol	V	-	-	+	+	-	+	+
D-Arabitol	-	-	-	-	-	-	-	+
5-Keto-D-gluconate	-	-	-	-	-	-	-	-
Sucrose	+	+	+	+	+	+	+	-
α-L-Rhamnose	+	+	+	+	+	+	+	+
Raffinose	+	+	+	+	+	+	+	-
Fumarate	+	+	+	+	+	ND	+	-
Quinate	+	+	+	+	+	ND	+	-
Ornithine decarboxylation[d]	V	V	+	+	+	+	V	-
Acid from[e]:								
D-Cellobiose	+	+	+	+	+	+	+	-
Palatinose	+	+	+	+	+	+	+	-
Indole production[f]	-	-	+	+	-	+	-	-
Malonate utilization[g]	-	+	V	+	+	+	V	+

[a] Data are derived from reports by Iversen et al. (3, 4) and Joseph et al. (5). Negative tests should be incubated for 7 days prior to being discarded, unless otherwise indicated. Results for some of the phenotypic reactions, such as those for VP and ornithine decarboxylation, can be obtained directly by using the API 20E strip (bioMerieux, Inc.) and by using reagents according to the manufacturer's instructions. +, 90 to 100% positive; V, 20 to 80% of members of the group are positive; –, 10 to 20% positive.

[b] The Voges-Proskauer test was performed by adding 40% potassium hydroxide in water and 5% 1-naphthol in 95% ethanol to cultures incubated for 24 h at 37°C in methyl red–Voges-Proskauer broth.

[c] The methyl red test was performed by adding the methyl red reagent (0.1 g per 300 ml of 95% ethanol) to cultures grown for 48 h at 37°C in 4 ml of methyl red–Voges-Proskauer broth.

[d] Ornithine decarboxylation is shown by an alkaline reaction according to the API 20E instructions.

[e] Acid production from carbohydrates was tested in phenol red broth base with the addition of filtered-sterilized carbohydrate solution (final concentration of 0.5% in cultures incubated for 24 h at 37°C).

[f] Determined using Kovac's reagent after growth in peptone broth (CM0009; Oxoid) of cultures incubated for 24 h at 37°C. The James reagent used in conjunction with the API 20E strip is an alternative test.

[g] Determined using sodium malonate broth in cultures incubated for 24 h at 37°C.

Another gene that was initially designed as a species-specific target is the zinc metalloprotease gene, *zpx* (71). However, due to the reclassification of the genus, including the reassignment of *Siccibacter turicensis*, *Franconibacter pulveris*, and *Franconibacter helveticus* from the genus *Cronobacter* (72), these gene targets (and PCR assays) are now considered genus-level targets and should be used as such.

Species-Specific Gene Targets

Currently there are two PCR assays widely used to identify *Cronobacter* which are based on species-specific single nucleotide polymorphisms (SNPs) and are associated with *rpoB* (49, 50) and *cgcA* (51). Qiming et al. (73) reported on a PCR assay (utilizing the genes ESA_02130, ESA_02797, and ESA_04347-ESA_04349) that could detect *C. sakazakii* in broth culture (detection levels: 1.3 pg/µl to 135 fg/µl) from artificially contaminated PIF at detection levels of 5.5×10^1 CFU/10 g.

Molecular Serotyping

In most members of the *Enterobacteriaceae*, the LPS Oantigen biosynthesis-encoding gene clusters are flanked by the *galF* and *gnd* genes, which contain 6 to 19 genes and are generally conserved within a species (74). To aid in the characterization of *Cronobacter* strains isolated from foods and PIF manufacturing environments, a harmonized molecular serotyping (LPS) identification scheme was designed by Yan et al. (74), which is based on information obtained from studies by Mullane et al. (75), Jarvis et al. (76, 77), and Sun et al. (78). Yan et al. (74) studied 409 *Cronobacter* isolates representing the seven *Cronobacter* species using the Mullane-Jarvis and Sun molecular serotyping schemes. PCR analysis revealed many overlapping results obtained by the two serotyping schemes. The proposed identification of isolates should be accomplished using the *rpoB* species-specific PCR-based assay that has been widely used and validated for species identification of *Cronobacter* in many surveillance studies (72, 74, 76, 77, 79–87) prior to applying serotype-specific primers. Interestingly, many of the LPS biosynthetic gene loci contained species-specific SNPs which could be used to identify these O antigens (75).

Molecular Subtyping

Molecular subtyping has long been regarded as a useful approach to help investigate outbreaks, identify food sources, and understand the nature of bacteria colonizing ecological niches. Mullane et al. (88) applied pulsed-field gel electrophoresis (PFGE) to characterize and track *Cronobacter* species in a PIF processing facility. Since that study, the PFGE protocol was validated and added to CDC's PulseNet international epidemiological laboratory network system, which compares the DNA fingerprints of bacteria from patients to find clusters of disease that might represent unrecognized outbreaks (89–91). PFGE is commonly considered the gold standard for epidemiologic studies, and PulseNet has added WGS to the surveillance network. Using WGS for molecular subtyping will enhance PulseNet's ability to detect and solve outbreaks faster and with more accuracy (92).

Sequence Typing

The taxonomic scheme proposing seven *Cronobacter* species as described by Iversen et al. (3, 4) has been supported by data from many WGS studies (46, 72, 93–96), and the taxonomic genomic separation of *C. sakazakii* from *C. malonaticus* has been determined by multilocus sequence typing (MLST) (52). Evidence revealing that *C. malonaticus* and *C. sakazakii* are separate species is based on SNPs of two genes, *rpoB* and *cgcA* (49, 51). Currently, a database containing defined sequence types covering all *Cronobacter* species is maintained at Oxford University. The database and MLST analytics can be accessed at www.pubMLST.org/ cronobacter (96). Several studies using MLST have determined the genetic diversity of *Cronobacter* in PIF production facilities (82, 87, 97), in PIF and FUFs (98–100), in retail foods (101–106), and in a variety of other sources (106). Results revealed there is considerable genomic diversity among *Cronobacter* species. Presently, *C. sakazakii* sequence type 4 (ST4) predominates in neonatal meningitis cases. Other sequence types associated with meningitis are *C. sakazakii* ST8 and ST1. Similarly, a large number of ST12 *C. sakazakii* strains have been isolated from infants with NEC. The MLST typing scheme includes more than 55 sequence types of *C. sakazakii*.

Microarray Analysis

Within the first 2 years of publication of the first *Cronobacter* genome, DNA microarrays were being used as tools to study the genomic diversity of members of this genus. Healy et al. (107) used a DNA

microarray With 276 selected genes from the genome of *C. sakazakii* strain BAA-894 to compare it with strains representing six of the seven *Cronobacter* species (*C. condimenti* had not be discovered prior to that study). Their results supported the reclassification scheme proposed by Iversen et al. (4) in which each species was separated into clusters, except that some of the *C. malonaticus* strains grouped within a larger cluster of *C. sakazakii* strains. Other strains formed a subgroup within a larger cluster that also contained *C. turicensis*, *Cronobacter* genomospecies 1 (now identified as *C. universalis*), and an unknown *Cronobacter* species. A subsequent study by Kucerova et al. (93) featured an array that used the entire genome (387,000-probe oligonucleotide tiling DNA microarray) of *C. sakazakii* strain BAA-894 for comparison with strains representing five of the seven *Cronobacter* species. Among the 4,382 annotated genes identified in *C. sakazakii* strain BAA-894, about 55% were common to all *C. sakazakii* isolates and 43% were common to all *Cronobacter* strains. Many of the genes absent in more than half of the strains tested were identified as phage genes, which emphasizes the importance of the mobilome in driving the genomic diversity of *Cronobacter*. Both studies used a common method of analysis which incorporated the use of direct comparisons of gene probe intensities obtained for the different species (strains) to probe intensities of similar genes from the reference strain, *C. sakazakii* BAA-894.

In contrast to the two microarray approaches described above, a more extensive multigenome array approach containing the annotated pangenome of 15 sequenced strains of *Cronobacter*, representing all seven species, was applied by Tall et al. (83) and was based on the design of Jackson et al. (108) for *E. coli*. The sequencing of these genomes was accomplished by a five-member international consortium in 2008–2009 (46, 72, 94, 109). The microarray was a custom-designed Affymetrix array which provided an opportunity to survey specific and unique genes from different *Cronobacter* species and within each species group. Gene homologs across the seven species were represented by the smallest number of probe sets that theoretically covered all alleles of a gene target. The microarray consisted of 19,287 independent genes representing 15 *Cronobacter* genomes and 18 plasmids and 2,371 virulence factor genes of phylogenetically related Gram-negative bacteria. It was determined that the *Cronobacter* microarray could distinguish the seven *Cronobacter* species from one another and from non-*Cronobacter* species; within each species, strains grouped into distinct clusters based on their genomic diversity. These results also supported the phylogenic divergence of the genus and clearly highlighted the genomic diversity in each member of the genus.

Since the initial description of the pangenomic Affymetrix microarray, Yan et al. (63, 82) have identified two phylogenetic lineages among ST4 strains which differed in the number of phage-related genes and genes associated with the pESA3-harbored type 6 secretion system (T6SS) gene cluster. Strains from lineage 1 differed from those in lineage 2 by 24 genes, of which 7 were phage related. DNA microarray analysis also revealed that most of the isolates representing each lineage possessed the 5′ end of the T6SS gene cluster. However, none of the ST4 lineage 1 isolates possessed the *vgrG* gene, encoding a known T6SS effector protein (67), whereas eight of nine ST4 lineage 2 isolates did. Chase et al. (87) also used the pangenomic microarray as well as WGS to comparatively characterize a group of ST83 *C. sakazakii* strains that had persisted in a Swiss PIF manufacturing facility for more than 4 years, with 109 other *C. sakazakii* strains isolated from a variety of sources. The phylogenetic analysis revealed that the pangenomic *Cronobacter* microarray could clearly separate the strains according to species and respective STs, with minor exceptions. These results further substantiate the idea that the microarray and its >19,000 *Cronobacter* genes as captured on the microarray are more resolving than the seven-allele MLST scheme. Figure 14.3 provides an example of a phylogenetic analysis developed by using microarray data.

Whole-Genome Sequencing

WGS of *Cronobacter* species will likely continue to facilitate the identification of unknown microorganisms, and it is hoped that its implementation as a standardized tool will enable the identification and subtyping of all foodborne bacterial pathogens worldwide, thereby replacing traditional methods such as PFGE as a strategy to strengthen preparedness and response, reduce the global social and economic disease burden, and enhance the protection of human health (41, 67, 110,

111). WGS can also provide detailed information regarding unique geno-and phenotypic features of foodborne pathogens involved in outbreaks, such as their repertoire of antibiotic resistance genotypes. WGS analysis of pure culture isolates obtained from clinical microbiology infections has the potential to revolutionize the fields of food safety and public health microbiology. Additionally, metagenomics and other "-omics" disciplines may provide solutions to a cultureless future in clinical microbiology, food safety, and public health. Information mined from WGS data obtained from metagenomics assays can be particularly useful for the identification of clinical causative agents or foodborne contamination and the detection of antimicrobial resistance and/or virulence factor genes, in addition to providing high-resolution subtyping information about pathogens (112).

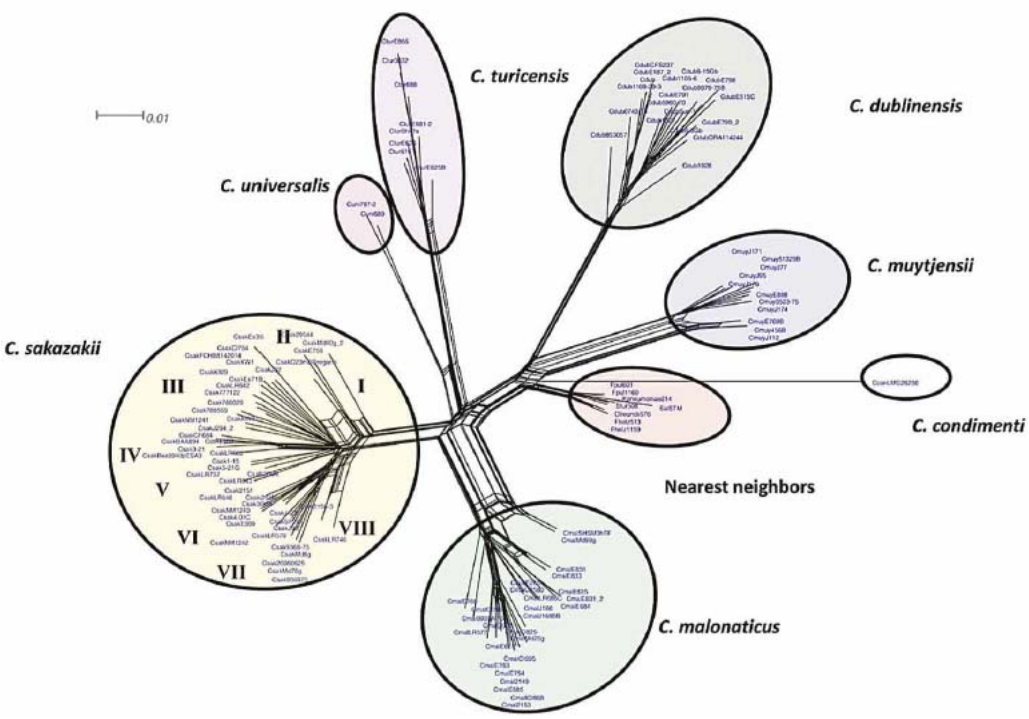


Figure 14.3 Neighbor net (SplitsTree4) analysis of 126 *Cronobacter* and phylogenetically related strains which were generated from the microarray-based gene differences described by Tall et al. (83). The phylogenetic tree illustrates that the *Cronobacter* microarray could clearly separate the seven species of *Cronobacter*, with each species forming its own distinct cluster. *C. sakazakii* subclades are designated with roman numerals I through VII. The scale bar represents 0.01 base substitution per site. Reprinted from reference 83 with permission.

As mentioned above, the first *Cronobacter* species which was sequenced was *C. sakazakii* BAA-894; this was quickly followed by the sequencing of *C. turicensis* strain LMG 23827^T (z3032) (93, 109). There are 174 WGS assemblies currently deposited in the NCBI Genome database for *Cronobacter* (https://www.ncbi.nlm.nih.gov/genome?term=Cronobactercmd=Details Search; accessed 25 September 2017). Table 14.2. summarizes some of the noted genomic features of these genomes from each *Cronobacter* species. An example of a phylogenetic analysis using WGS information is demonstrated in Fig 14.4.

Plasmids

WGS studies have revealed that most *Cronobacter* strains contain plasmids (many possess multiple plasmids), which is consistent with what is known about many other enteric pathogens. One such plasmid group that has been identified is the IncF group of plasmids, which has eight related replicon designations: RepFIA, RepFIB, RepFIC, RepFIV, RepFV, RepFVI, RepFVII, and RepFIIA (79). IncF plasmids range in size from 1 kb to several hundred kilobases and from one copy to several hundred

copies per cell, with some members possessing genes of additional origins of replication, usually RepFIIA. Three highly homologous RepFIB plasmids, pESA3 (131 kb; possessed by *C. sakazakii* BAA-894), pSP291-1 (118 kb; possessed by *C. sakazakii* strain SP291), and pCTU1 (138 kb; possessed by *C. turicensis* strain z3032), contain several putative virulence genes, including two genetic loci encoding iron acquisition systems, namely, an ABC transporter gene cluster and an aerobactin-like or cronobactin siderophore receptor gene cluster, identified as *eitCBAD* and *iucABCD-iutA*, respectively. These genetic components, in addition to the origin of replication gene (defining the incompatibility class; IncFIB or *repA*), together make up a common plasmid backbone. Franco et al. (79) and Grim et al. (81) determined that the *iucABCDiutA* siderophore is the only active siderophore present in *Cronobacter*. Additionally, plasmids pESA3 and pCUNV1 (*C. universalis*) contain a *cpa* (*Cronobacter* plasminogen activator) gene, and plasmid pESA3 also has a 17-kb T6SS locus, whereas plasmid pCTU1 contains a 27-kb region carrying a filamentous-hemagglutinin (FHA)-encoding gene (*fhaB*). The presence of the FHA-specific transporter gene (*fhaC*), of genes for associated putative adhesins (FHA locus), and of *cpa*, T6SS, and FHA loci depends on the species, revealing a strong correlation with the presence of virulence traits, plasmid type, and species. Figure 14.5 demonstrates the sequence homology among pESA3-pSP291-1 and pCTU1-like plasmids.

Table 14.2 Summary of 181 *Cronobacter* genome characteristics[a]

				Cronobacter species			
Characteristic	*C. condimenti*[b]	*C. dublinensis*	*C. malonaticus*	*C. muytjensii*	*C. sakazakii*[c]	*C. turicensis*	*C. universalisc*
No. of genomes	1	5	14	2	156	5	1
Size (Mb)							
Mean	4.35	4.6	4.55	4.34	4.46	4.28	4.43
Range	4.35	4.45–4.76	4.41–4.90	4.32–4.36	3.75–4.99	3.52–4.64	4.43
GC content (%)							
Mean	55.8	57.82	56.7	57.7	56	57.1	57.9
Range	55.8	57.30–58.30	55.8–57	57.6–57.7	55–57.7	56.7–57.3	57.8
No. of CDS Mean							
Mean	4,164	4,526	4,496	4,143	4,152	4,520	4,278
Range	4,164	4,255–4,979	4,219–4,822	4,133–4,152	3,917–5,243	4,441–4,639	4,278
No. of proteins							
Mean	3,926	3,912	4,103	3,928	3,858	4,107	4,078
Range	3,926	3,653–4,225	3618–4589	3,919–3,983	3,274–4,463	3,724–4,250	4,078
Plasmid(s)	pCCO1	pCDU1	pCMA1, pCMA2	None	pESA3, pESA2, pSP291-3	pCTU1, pCTU2, pCTU3	pCUNU1

[a] Values were obtained from data on strains described in NCBI (https://www.ncbi.nlm.nih.gov/genome/genomes/1170; accessed on 9/25/2017) and reference 94.

[b] Values were obtained from only one *C. universalis* and *C. condimenti* strain, as described in NCBI.

[c] Plasmid information was obtained from four *C. sakazakii* strains, as follows: pCSK29544_1p (NZ_CP011048.1/CP011048.1), pCSK29544_2p (NZ_CP011049.1/CP011049.1), and pCSK29544_3p (NZ_CP011050.1/CP011050.1) from *C. sakazakii* strain 29544T; pESA2 (NC_009779.1/CP000784.1) and pESA3 (NC_009780.1/CP000785.1) from strain BAA-894; pSP291-1 (NC_020263.1/CP004092.1); pSP291-2 (NC_020261.1/CP004093.1) and pSP291-3 (NC_020262.1/CP004094.1) from strain SP291; and pCS1 (NZ_CP012254.1/CP012254.1), pCS2 (NZ_CP012255.1/CP012255.1), and pCS3 (NZ_ CP012256.1/CP012256.1) from strain 8155. Plasmid information for *C. condimenti* strain 1330, *C. dublinensis* strain LMG 23823, *C. malonaticus* strain LMG 23826, *C. turicensis* strain z3032, and *C. universalis* was obtained from the following respective accession numbers: NZ_CP012265.1, NZ_CP012267.1, NZ_CP013941.1/CP013941.1 and NZ_CP013942.1/CP013942.1, NC_013283.1/FN543094.1, NC_013284.1/FN543095.1 and NC_013285.1/FN543096.1, and NZ_CP012258.1/CP012258.1 (https://www.ncbi.nlm.nih.gov/genome/plasmids/1170?).

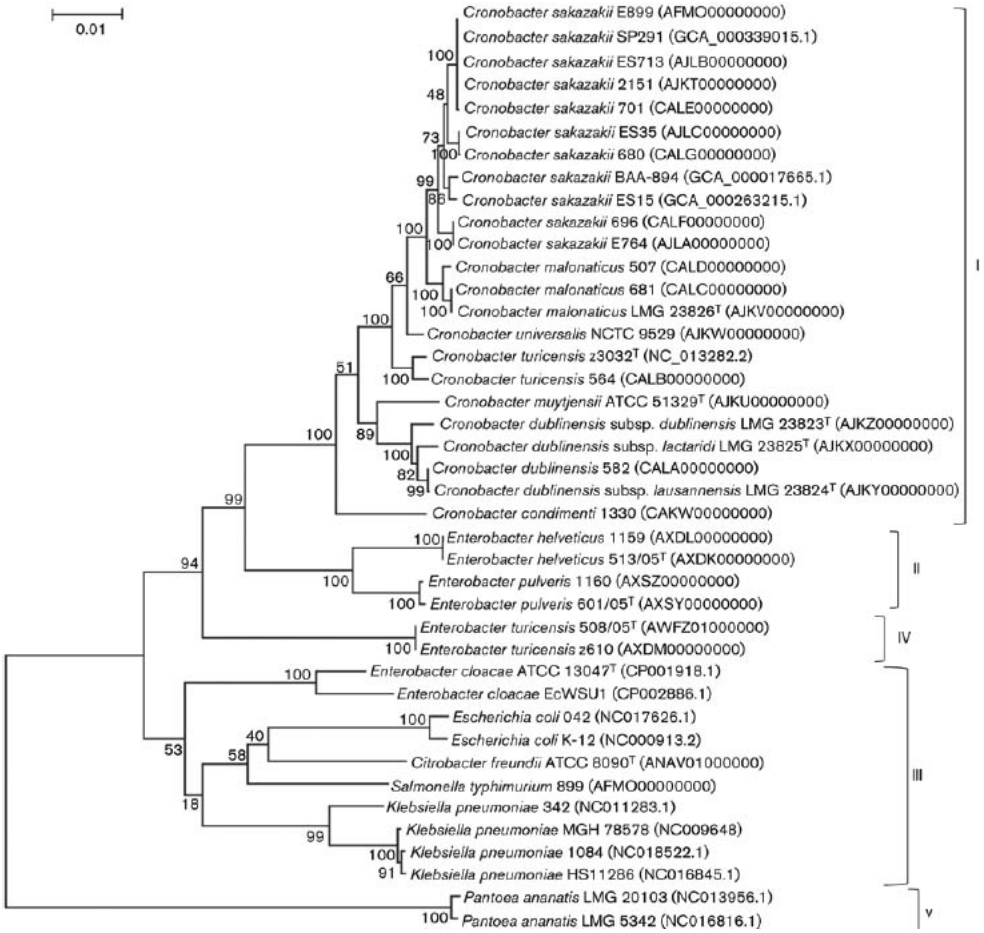


Figure 14.4 Phylogenetic analysis using the neighbor-joining algorithm of 34 *Cronobacter*, 10 *Enterobacter*, and eight *Enterobacter*-related genomes based on the alignment of SNPs from 300 orthologous genes as described by Stephan et al. (72). Clades are represented by roman numerals 1 through V. Clade I represents genomes from *Cronobacter* species. Clade II represents genomes from *F. helveticus* and *F. pulveris* strains. Clade III represents genomes from two *E. cloacae* strains and genomes from *Citrobacter*, *Salmonella*, *Escherichia*, and *Klebsiella* strains. Clade IV represents genomes from *S. turicensis* strains. Lastly, genomes from two *Pantoea* strains are represented in clade V. The scale bar represents 0.05 base substitution per site. Reprinted from reference 72 with permission.

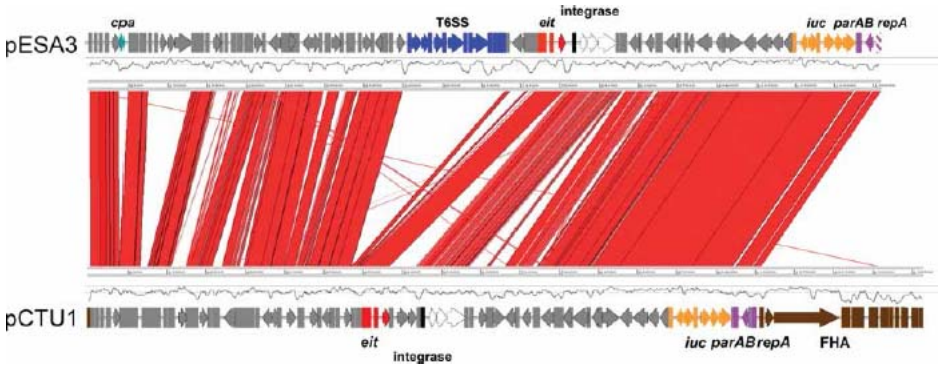


Figure 14.5 Sequence alignment of *Cronobacter* plasmids pESA3 and pCTU1 produced with the Artemis comparison tool as described by Franco et al. (79) and showing regions of homology. In the middle section, the red color indicates significant nucleotide homology, as determined by BLASTn analysis, between pESA3 and pCTU1, and the location on each plasmid, for example, *eit*, *iuc*, *parAB*, and *repA*. White areas indicate regions or loci present on one plasmid and absent on the other, e.g., *cpa*, T6SS genes, and FHA genes. The sequence of pESA3 was modified by rejoining the *repA* gene at the 3′ end, which is split in the GenBank sequence (NCBI RefSeq accession no. NC_009780.1). pESA3 and pCTU1 (NCBI RefSeq accession no. NC_009780.1) possess molecular sizes of 131,196 and 138,339 kb and mean G+C contents of 56.85% and 56.05%, respectively. Selected genes or loci are shown in color as follows: *eit*, red; *iuc*, orange; *parAB* and *repA*, purple; integrase genes, black, and associated genes, white; *cpa*, teal; T6SS genes, blue; and FHA genes, brown. Reproduced from reference 79 with permission.

Other plasmids possessed by many *Cronobacter* species include pCTU2 (*C. turicensis*), pESA2, and pSP291-2 (both possessed by *C. sakazakii*). These plasmids carry a gene cluster responsible for plasmid mobilization and conjugation, which is now considered a T4SS (46, 79, 93, 95, 109). These plasmids contain an IncF2 (incompatibility class) origin of replication site (*repA*). Lastly, another group of plasmids that are represented by plasmid pCTU3 (*C. turicensis*) and pSP291-3 (*C. sakazakii*) possess an IncH1-like origin of a replication gene (incompatibility class). Little is known about this Inc class of *Cronobacter* plasmids except that they possess genes encoding heavy metal resistance. Table 14.2. also describes these plasmids and others which have been identified from closed genomes.

It is interesting to speculate that these heavy metal resistance genes are remnants from when the hypothetical ancestral *Cronobacter* was evolving during the Paleogene geologic period (which occurred approximately 59 million years ago) of the Cenozoic era, when early modern plants appeared and the grassland plants continued to evolve, as proposed by Schmid et al. (113) and Joseph et al. (66). Table 14.3. shows the prevalence and distribution of pESA3 targets which have been identified among 800 strains of *Cronobacter* obtained from diverse food, environmental, and clinical sources throughout the world.

CRISPR-*cas* Subtyping System

In 2016, Ogrodzki and Forsythe (114) described a clustered regularly interspaced short palindromic repeat (CRISPR)-*cas* profiling system for C. *sakazakii*. In general, CRISPR-*cas* systems comprise three genetic components: a *cas* gene cluster and an AT-rich leader sequence, followed by a CRISPR spacer array composed of short (~24-to 48-nucleotide) direct repeat sequences separated by similarly sized, unique spacers which are usually derived from mobile genetic elements such as bacteriophage and plasmids (114). It is thought that CRISPR-*cas* systems provide for a type of adaptive immunity from invasive genetic elements (phages and plasmids), functioning to regulate lysogeny and biofilm formation. It has been determined that the variability of a CRISPR array can provide a greater power of differentiation for genotyping within clonal lineages than other molecular assays such as PFGE (114). This information can also be applied for source attribution of isolates from clinical cases through food sources to a production site. Zeng et al. (115) used CRISPR-*cas* analysis to reveal that prophages

contributed to approximately 16.6% of the genetic diversity of *C. sakazakii* and determined that the gain or loss of prophages and spacers that are associated with CRISPR loci has frequently played a role in the evolution of *C. sakazakii*.

Table 14.3 Plasmidotype patterns observed for 801 *Cronobacter* isolates representative of the seven *Cronobacter* spp.

						No. (%[a]) of isolates with the indicated plasmidotype									
Species	No. of isolates	*repA*	*cpa*	Δ*cpa*	IntL	*vgrG*	R end	IntR	ΔT6SS	*fhaB*	ΔFHA	*dfhaB*	*eit*	*iuc*	*Cc*
C. sakazakii	652	629 (96)	604 (96)	10 (2)	600 (95)	311 (50)	344 (55)	204 (32)	1 (0)	76 (14)	115 (18)	6 (3)	628 (100)	626 (100)	36 (1
C. malonaticus	53	51 (96)	0 (0)	19 (44)[b]	2 (5)	1 (3)	0 (0)	0 (0)	41 (95)	51 (100)	0 (0)	2 (5)	51 (100)	50 (98)	1 (
C. turicensis	21	21 (100)	0 (0)	13 (76)	2 (9)	2 (11)	1 (5)	0 (0)	11 (52)	21 (100)	0 (0)	0 (0)	21 (100)	21 (100)	3 (3
C. muytjensii	16	12 (75)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	12 (100)	12 (100)	1 (11)	12 (1
C. dublinensis	56	55 (98)	0 (0)	7 (21)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (3)	1 (4)	55 (100)	55 (100)	21 (46)	55 (1
C. universalis	2	2 (100)	2 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (100)	2 (100)	0 (0)	0 (0)	2 (100)	2 (100)	0 (
C. condimenti	1	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	1 (1
Total	801	771 (96)	606 (76)	49 (6)	604 (75)	314 (39)	345 (43)	204 (25)	53 (7)	152 (19)	116 (14)	75 (9)	770 (96)	723 (90)	10 (1

[a] Percent PCR positive for each gene locus in relation to the total number of plasmid-harboring strains of that species, except for the data for *repA*, which are in relation to the total number of strains. The number of strains was updated and adapted from a similar table in reference 79.

[b] Only 44 *C. malonaticus* strains were tested by PCR.

Capsule Profiling or Typing (K Typing)

Ogrodzki and Forsythe proposed a new capsular typing scheme and found a previously uncharacterized capsular region (*kps*) in 104 *Cronobacter* strains (116). The region was homologous to the well-described K-antigen gene cluster of *E. coli*. The gene cluster comprises three regions: K-antigen region 1 (*kpsEDCS*) and region 3 (*kpsTM*) are conserved across the genus, and there are two variants of region 2. Genes associated with K-antigen type 1 are present in all seven species of *Cronobacter*. However, the prevalence and distribution of K-antigen type 2 (specifically of interest is the capsular profile K-antigen gene cluster 2–colanic acid gene cluster 2–cellulose positive) are more limited, and this antigen is found only in *C. sakazakii*, *C. malonaticus*, *C. turicensis*, and *C. dublinensis*. It is thought that the presence of this capsular profile may confer a favorable phenotype for desiccation resistance, persistence, and serum resistance as well as increased macrophage survival, resulting in a more physiologically fit pathogenic microorganism.

VIRULENCE FACTORS

Outer Membrane Proteins

Although the genomics and transcriptomics of *Cronobacter* pathogenesis have not been fully elucidated, considerable information has been obtained in recent years that has enabled the identification of several virulence markers and putative genetic loci that may contribute to its pathogenesis. Two well-characterized virulence genes, *ompA* and *ompX* (encoding outer membrane proteins [OMPs] OmpA and OmpX), are involved in adherence and invasion of brain endothelial cells through the binding of the cells to fibronectin (117, 118). Kothary et al. (86) revealed that both OmpA and OmpX are packaged within outer membrane vesicles (OMVs) expressed in stationary-phase-grown cells of *C. sakazakii*, *C. malonaticus*, and *C. turicensis*, and Ye et al. (119) determined that the expression of these OMPs is greater in a virulent *C. sakazakii* strain than in an avirulent strain.

Other OMPs packaged within OMVs include MipA (peptidoglycan synthesis), OmpC, OmpE, and OmpF (porins), CTP (a conjugative plasmid transfer T4SS protein), GroEL (chaperonin), and an autotransporter secretion T5SS protein named outer membrane transporter protein. Furthermore, PCR and microarray analyses of 240 strains representing the seven *Cronobacter* species revealed that these OMPs are highly conserved among all the species. OMVs were once thought to be artifacts, but they are now recognized as structures possessing very diverse functions. Foremost, OMVs are currently regarded as a secretion system used by bacteria to communicate with host cells and other bacterial cells. Kothary et al. (86) also determined that in addition to OMPs, these OMVs contained substantial amounts of LPS. The existence of LPS components in PIF was already known (120), and their presence may lead to a situation where the LPS in PIF causes a proinflammatory response in a susceptible host which could lead to a fatal "cytokine storm." Townsend et al. (120) determined that the presence of endotoxin along with *C. sakazakii* in infant formula enhanced the translocation of *C. sakazakii* from the rat gut lumen to the meninges and through the BBB. One possible mechanism is thought to be the disruption of the tight junctions by the LPS, thus increasing the permeability of host barriers to the pathogen (47). More information is required to elucidate the process regulating the production of OMVs and their role in disease.

Fimbriae

Comparative genomics studies have revealed that *Cronobacter* possesses genes for several colonization factors, or fimbriae (94, 95, 121). These studies revealed that among members of the genus, there are 11 different fimbria types. For example, Grim et al. (94, 122) determined that the *Cronobacter* genome contains genes for type IV pili in addition to a P pilus homologous to that found in uropathogenic *E. coli* that causes meningitis in infants. Interestingly, not all *Cronobacter* species contain the genes for all of these adherence factors. For instance, *C. sakazakii* BAA-894 and *C. malonaticus* LMG 23826 harbor a type 1 fimbria (GR82) gene which is absent in the other genomes. Furthermore, some *Cronobacter* genomes contain genes for curli fimbriae which are homologous to the curli fimbriae of *E. coli* and the *tafi* fimbriae of *Salmonella* (94). However, little is known about their role in pathogenicity.

Exoproteins

Pagotto et al. (123) were the first to describe the presence of putative enterotoxins expressed by *Cronobacter*. Using the suckling mouse assay, these investigators observed an enterotoxin effect in four of 18 *Cronobacter* isolates tested which also were toxigenic to Vero and Y-1 cells, causing both rounding and cell lysis. Raghav and Aggarwal (124) were the first to partially purify and characterize a putative *Cronobacter* enterotoxin. The molecular mass of the toxin was 66 kDa, and it was stable at 90°C for 30 min, implying that it is a moderately heat-stable toxin. This indicates that the enterotoxin could be resistant to commercial milk pasteurization treatments and thus may be active in pasteurized liquid infant formula. The gene encoding this toxin remains to be identified.

As suggested by Pagotto et al. (123), *Cronobacter* also secretes proteolytic enzymes that in mice lyse cells and create tissue damage at the site of infection. Kothary et al. (71) identified a zinc metalloprotease that deforms Chinese hamster ovary cells, causing rounding and cell damage. The genes for a hemolysin have been identified, but there is controversy regarding its activity (93, 95, 121). To better understand the prevalence and phylogenetic relatedness of the putative hemolysin III homolog (COG1272), Lee et al. (125) assayed 389 *Cronobacter* strains by PCR, DNA microarray, and

WGS analyses. PCR analysis using primers reported by Cruz et al. (121) revealed that COG1272 was present in 285 of 298 (95.6%) *C. sakazakii* strains studied, 3 of 7 (42.9%) *C. muytjensii* strains, 13 of 33 (39.4%) *C. malonaticus* strains, 3 of 12 (25%) *C. turicensis* strains, 4 of 37 (10.8%) *C. dublinensis* strains, and 1 of 1 *C. universalis* strain. *C. condimenti* was PCR negative for COG1272. However, microarray analysis and WGS revealed that *C. condimenti* did possess a COG1272 ortholog and that primers were more *C. sakazakii* specific than was previously thought. Using microarray analysis and WGS in parallel, Lee et al. (125) determined that in addition to COG1272, 27 other hemolysin-related genes were present, including homologs of genes for a hemolysin containing a cystathionine beta-synthase domain, a 21-kDa hemolysin precursor protein, and several putative hemolysins within each *Cronobacter* species. Furthermore, Ye et al. (119) provided transcriptomic data that suggest that the hemolysin III gene (COG1272) is upregulated in a virulent strain of *C. sakazakii* compared to a nonvirulent strain.

GENOMICS AND TRANSCRIPTOMICS OF VIRULENCE GENES

There have been several reports of various genes found genomically that may impart to *Cronobacter* species the ability to be a more physiologically fit pathogen. For example, *ibeB* (a gene synonymous with *cusC*), which belongs to a cluster of genes encoding a copper and silver resistance cation efflux system, was reported (93, 94) to be involved with greater invasiveness of HBMECs. Kucerova et al. (93) reported that they too found that a complete cation efflux operon (*cusA*, *cusB*, and *cusC*) and that its regulatory gene, *cusR*, was present in isolates associated with neonatal infections (including *C. sakazakii*, *C. turicensis*, and *C. malonaticus*) and absent in the other tested strains. Ye et al. (119) recently reported transcriptomic data for a virulent strain of *C. sakazakii* which were compared with those for an avirulent strain; differentially expressed genes included flagellum-associated genes, such as *flhD*, *motA*, *flgM*, *flgB*, and *fliC*, OMP or lipoprotein genes (*ompW*, *slyB*, *blc*, *tolC*, and *lolA*), chemotaxis genes, including *cheY* and *cheR*, a gene for the virulence-related factor hemolysin III (*hlyIII*), a hemolysin expression-modulating gene (*hha*), and genes for important regulation factors (*sdiA*, *cheY*, *bss*, and *fliZ*). Jing et al. (126) reported on the transcriptome profile of *C. sakazakii* BAA-894 after interaction with HCT-8 cells. They determined that expression of some flagellar genes (*flgA*, *flgB*, *flgC*, *flgD*, *flgF*, *flgG*, *fliK*, *fliM*, *fliL*, and *fliE*) and adherence factors was upregulated. High osmolarity and osmotic stress-associated genes (*kdpABC*, *proV*, and *betB*) were highly upregulated, as well as genes responsible for the synthesis of lipopolysaccharides and OMPs (*mltD*, *lpxP*, *yidC*, *spr*, *ompA*, and *ompX*), iron acquisition systems (*fepE*, *entC*, *iutA*, *fhu*, *eit*, and *efe*), and glycerol and glycerophospholipid metabolism.

Several bacterial pathogens produce diffusible signal factor-type quorum-sensing signals to control virulence. Suppiger et al. (127) determined that *C. turicensis* LMG 23827^T possesses a functional quorum-sensing system (*rpfF/R* system) that is involved in the regulation of various phenotypes, including colony morphology, biofilm formation, and swarming motility; results from a zebrafish embryo infection model revealed that this regulatory system also controls virulence by modulating bacterial intracellular c-di-GMP levels.

IN VIVO MODELS

To gain insights into the pathophysiology of *Cronobacter*-induced diseases, the selection and/or development of *in vivo* models that mimic the clinical manifestations of infections is required. The following subsection describes animal models that have been used thus far to identify bacterial and host factors that contribute to *Cronobacter* pathogenesis.

Mammalian Models

The first study of pathogenesis employing an *in vivo* model was published by Pagotto et al. (123), who reported the presence of a putative *C. sakazakii* enterotoxin and cytotoxins as well as providing data on the minimal lethal dose required for challenge studies using either the oral or the intraperitoneal

inoculation routes in a suckling mouse assay. Food and clinical isolates were tested for enterotoxin production, and 22% of these strains tested positive. Challenge studies using intraperitoneal injection revealed that all tested strains were lethal at 10^8 CFU per mouse, but most, including one enterotoxin-positive strain, were nonlethal at high oral doses. It was hypothesized that accessory and yet-unidentified bacterial virulence and/or host factors may be required for these strains to exhibit their full virulence. It is noteworthy that the genetic basis of the proposed enterotoxin in *C. sakazakii* has not been determined.

In subsequent experiments, a variety of animals (nonmammalian and mammalian, including chickens [1 day old], rabbits [2.7 to 3 kg, 2 months old], guinea pigs [300 to 400 g, 3 to 4 months old], and young gerbils [40 to 50 g, 1 to 2 months old]) were tested and evaluated for signs of infection and/or death after being fed various *C. sakazakii* strains suspended in reconstituted infant formula. The neonatal gerbils presented as the most promising nonprimate model to study infections via the oral route (16, 128).

Since milk-based infant formulas contaminated with *Cronobacter* have been implicated in outbreaks of NEC, several research groups have attempted to develop an animal model that mimics the manifestations of human NEC (including mucosal edema, enterocyte apoptosis, and vascular thrombosis) caused by *Cronobacter*. A neonatal rat model, using a combination of formula feeding, hypoxia, and administration of *C. muytjensii* (ATCC 51329^T), was evaluated by Hunter et al. (16) to investigate *E. sakazakii* (*Cronobacter*)-induced NEC. Newborn rats fed infant formula including 10^5 CFU of bacteria exhibited clinical symptoms of grade 3 NEC by day 4 postinfection. The rats that were formula-fed and subjected to hypoxia only (controls) presented less severe pathological symptoms and had a mortality rate of 40%, whereas the mortality rate in *Cronobacter*-infected animals was 70%. Histopathological analysis of intestinal sections from infected rats revealed macroscopic and microscopic features analogous to those observed in human NEC. Infections increased binding of the bacteria to the villus tips as well as enterocyte blebbing and gap formation in the epithelium. It was concluded that epithelial damage was mediated through enterocyte association without direct invasion by bacteria. *C. muytjensii* may cause barrier damage by inducing enterocyte apoptosis and stimulating expression of the inflammatory cytokine IL-6. These findings as well as the role of NO in mediating apoptosis were confirmed by follow-up studies using intestinal epithelial cells (Caco-2) (16, 23).

Emami et al. (30) evaluated a mouse model in which oral feeding of 10^3 CFU of *C. muytjensii* to 3-day-old mice induced NEC-like pathology. It was determined that *C. muytjensii* infection causes epithelial damage by recruiting greater numbers of dendritic cells than macrophages and neutrophils in the gut and suppresses dendritic-cell maturation. This requires OmpA expression in *C. muytjensii*. Interaction of OmpA with dendritic cells mediates epithelial barrier disruption through secretion of pro-or anti-inflammatory cytokines. Destruction of the intestinal epithelium was due to increased production of transforming growth factor β and IL-10. Depletion of dendritic cells in newborn mice protected against *C. muytjensii*-induced NEC, whereas depletion of either neutrophils or macrophages from 2-day-old mice followed by infection rendered the animals susceptible to infection earlier than normally infected littermates (30).

Translocation through the gastrointestinal barrier is a prerequisite to facilitate systemic spread into the central nervous system and penetration of the BBB. Studies to determine the ability of *E. sakazakii* (*Cronobacter*) to penetrate the BBB and cause meningitis in orally induced infections were hindered due to lack of a suitable animal model.

One of the first studies using an animal model to address *Cronobacter*-induced meningitis and brain abscess formation was conducted by Townsend et al. (29). They utilized intracranial inoculation of neonatal rats with *E. sakazakii* (*Cronobacter*) to observe the progression of brain inflammation and the ensuing host inflammatory response. Infiltrating macrophages and neutrophils comprised the inflammatory foci and contained phagocytosed bacteria. In the same study, it was determined that several *Cronobacter* strains were capable of invading rat brain capillary endothelial cells (rBCEC4) and that these could persist in macrophages for up to 96 h. It was noted that intracellular survival may be modulated by the presence of superoxide dismutase (encoded by the *sod* gene), which may influence

survival under acidic conditions and/or in the presence of macrophage oxidase. In addition, it was determined that *Cronobacter* may bias early IL-10/IL-12 cytokine expression in macrophages, suggestive of a type 2 immune response, which is inefficient in fighting intracellular infections, and this may explain the opportunistic nature of this infection.

Since the natural route of infection of this pathogen is via gut colonization, there may be limits to the extrapolation of information from the intracranial infection model to human disease. Mittal et al. (129) used a newborn rat model to identify and characterize the role of *E. sakazakii* (*C. muytjensii*) OmpA protein in the onset of meningitis. An orally administered OmpA-expressing strain translocated the intestinal barrier, multiplied in the blood, and subsequently penetrated the BBB. Cronobacters were present in large numbers in the brains of infected animals and were associated with neutrophil infiltration, hemorrhages, and gliosis. OmpA expression was also involved in the resistance to blood and serum complement killing. Thus, this newborn-rat model without hypoxia was a suitable model for observing translocation of cronobacters into the system, resulting in meningitis (129).

There are, however, also disadvantages in the use of mammalian models, including high monetary costs, ethical issues, and the need for laborious and invasive sample analysis. An alternative infection model using insects has been used in pathogenesis studies.

Nonmammalian Models

Among the invertebrates used, *Galleria mellonella* (greater wax moth) is of special interest because this model can be easily and inexpensively obtained in large numbers and is easy to manipulate. There are no ethical constraints, and their short life cycle makes them ideal for large-scale studies. Although insects lack an adaptive immune response, their innate immune response is remarkably similar to the immune response in vertebrates. In addition, *G. mellonella* can survive at 37°C, which is important in studying temperature-sensitive virulence of pathogens.

G. mellonella larvae were used by Carranza et al. (130), who determined that injection of 7×10^6 CFU of *C. turicensis* 3032 (LMG 23827T) could kill the larvae after 72 and 96 hours postinfection, whereas injection of *E. coli* JM83 was nonpathogenic. More recently, the model was used to test the efficiency of phage therapy against *C. sakazakii* infections (131).

The wax moth model proved useful for prescreening of potential virulence factors and for testing the efficacy and safety of phages prior to confirmation or clinical evaluation in mammalian models. However, in many cases, assays in which death and survival are the only measurements are not sufficient for studying the progression of an infection. There is a need for models in which the interaction between invading bacteria and the individual host immune cells can be followed *in vivo* and, ideally, in real time.

The *Caenorhabditis elegans* model has some advantages, such as ease of cultivation and transparency. It seems particularly efficient in detecting virulence traits in opportunistic bacterial pathogens, because virulence in strict pathogens is often host specific and cannot be universally modeled in the nematode infection system (132).

Sivamaruthi et al. (133, 134) determined that *C. sakazakii* BAA-894, a strain of clinical relevance, can kill *C. elegans* with a 50% lethal dose at 133+3 hours, whereas *E. coli* OP50 (food source of *C. elegans*) had no effect. A gradual multiplication of bacteria was observed during infection of *C. elegans* with *C. sakazakii*, with *Cronobacter* populations at 24 h reaching 3.25×10^4 CFU per worm. These findings suggest that the pathogenicity of *C. sakazakii* was mainly due to bacterial accumulation and persistent infection inside the host. There was notable damage within the infected nematode, particularly in the intestinal region, with induction of apoptosis (cell death confirmed by Sytox staining) identified as the major event. This mechanism is similar to that of human NEC pathogenesis. The study also elucidated the role of genes known to be associated with *C. elegans* innate immune response to pathogenic infections. Subsequent studies by this group revealed that exposure to *C. sakazakii* LPS led to paralysis and death of *C. elegans* (135). Analysis of Fourier transform infrared spectra of LPS revealed that *C. sakazakii* modifies its LPS to escape recognition by the host immune system. Studies using qPCR

revealed that LPS modulated the expression of selected host immune and aging-related genes, and it was determined that the p38 MAPK pathway has a significant role in host immune response against LPS-mediated challenges (135).

One of the drawbacks of this model is that these organisms are genetically not closely related to humans, and their immune system has many differences from that of humans. In addition, in this model, *C. elegans* is typically infected by feeding the bacteria to the worms; hence, it is not possible to determine the exact infective dose.

The zebrafish (*Danio rerio*) embryo model is a hybrid between the mouse and invertebrate infection models. The most impressive feature of this model is the possibility of performing noninvasive, high-resolution, longterm, time lapse and time course experiments in the transparent embryo to visualize infection dynamics with fluorescent markers. Its small size, ease of breeding, high fertility, and genetic tractability are additional features that make the zebrafish embryo an attractive model for high-throughput infection studies. Furthermore, the zebrafish immune system displays many similarities to that of mammals, with counterparts for most of the human immune cell types. The zebrafish's innate immune system begins to develop as early as 24 hours postfertilization; however, the development of the adaptive immune system lags. This fact makes the model especially attractive for studying a pathogen causing disease in neonates and premature babies.

The development and adaptation of the zebrafish embryo model to study *Cronobacter* pathogenesis were first described in 2015 (136). Various parameters, such as infecting embryos by bath immersion versus microinjection of bacteria as well as infection dosages and sites of injection, were evaluated for the ability to model systemic *Cronobacter* infections. Microinjection of 50 CFU of *Cronobacter* into the yolk sac resulted in rapid multiplication of bacteria and dissemination into the bloodstream at 24 h postinfection, followed by the development of severe bacteremia and larval death within 3 days. In contrast, the innate immune response of the embryos was sufficiently developed to control infection after receiving an intravenous injection of up to 10^4 CFU of *Cronobacter*. Infection studies using an isogenic mutant incapable of surviving and replicating in human macrophages (Δ*fkpA*) revealed that this strain was highly attenuated in its ability to kill the larvae. In addition, the suitability of the zebrafish model to study the effectiveness of antibiotics to treat *Cronobacter* infections in zebrafish embryos was confirmed. In this study, light and fluorescence microscopy, as well as CLSM and TEM analysis, were employed to visualize progression of infection and the pathology of infection.

Several studies were published by the same group in which the zebrafish embryo model was used to investigate the divergent virulence potential among species and strains within the genus *Cronobacter*. Eshwar et al. (85) used the zebrafish embryo model to study the role of RepF1B-like plasmids as virulence plasmids in *Cronobacter* and determined that two virulence factors—the *Cronobacter* plasminogen activator (*cpa*) and the zinc metalloprotease (*zpx*)—were involved in *in vivo* pathogenesis. Another study focused on the description and characterization of a novel diffusible signal factor-type quorum-sensing-dependent signaling system in *C. turicensis*. It was determined that this signal/sensor system is functional, is involved in the regulation of various phenotypes, and plays a role in virulence (127). Chase et al. (87) used this model to study *C. sakazakii* strain H322, a highly persistent ST83 strain obtained from PIF. This and other ST83 strains investigated in the study produced substantial mortality in the infection experiments, suggesting an elevated risk for infections and illness in neonates exposed to PIF contaminated with isolates of ST83.

The zebrafish embryo model has great potential for studying systemic *Cronobacter*-mediated infections. It combines many of the advantages present in the above-mentioned models and makes it possible to study infections in real time. In addition, it may be used as a prescreen for bacterial virulence factors and potential host factors involved in the immune response. Such experiments may also be regarded as meeting ethical standards in animal welfare, as up until 5 days postfertilization the central nervous system of the zebrafish embryo is only partially developed and complex behaviors of the juvenile or adult are absent. This is recognized by licenses for fish experimentation, which are necessary only from the stage at which fish can independently feed (i.e., >120 hours postfertilization). Thus, by using the zebrafish embryo model as a prescreen, it will be possible to reduce the number of

experiments with nonhuman mammal models, which will still be necessary for confirmation purposes. Finally, the zebrafish model poses no challenge to the position of rodent models: it is complementary to them, especially in the initial stages of infection.

ANTIBIOTIC RESISTANCE

Prior to 2000, most infections caused by *Cronobacter* were frequently treated with ampicillin, gentamicin, and/or chloramphenicol or a combination of gentamicin and ampicillin (137). However, as early as 2001, *Cronobacter* strains were uniformly found to be resistant to ampicillin, cefazolin, and extended-spectrum β-lactams, whereas they were uniformly susceptible to trimethoprim-sulfamethoxazole and aminoglycosides. By 2015, antimicrobial susceptibility testing by Xu et al. (105) revealed that 84.5% and 46.5% of isolates obtained from ready-to-eat foods in China were resistant to penicillin G and cephalothin, respectively; in contrast, all tested isolates were susceptible to cefotaxime, ciprofloxacin, tetracycline, and nalidixic acid. In another study, multidrug resistance was associated with *C. sakazakii* isolates from domestic kitchens (138), with the greatest percentage of resistance in these strains to penicillin (76.1%), followed by tetracycline (66.6%), ciprofloxacin (57.1%), and nalidixic acid (47.6%). None of the *C. sakazakii* isolates was resistant to gentamicin.

Surveillance has revealed that antimicrobial resistance in *Cronobacter* is changing from most strains being pansusceptible to more strains becoming increasingly resistant. To illustrate this trend, there was a recent report from China of two cases involving multidrug resistance (139). The Cronobacter strains identified in this study were resistant to third-generation cephalosporins, aminoglycoside, and/or trimethoprim-sulfamethoxazole. This was followed by a second report of multidrug resistance in a *C. sakazakii* strain isolated from a sputum sample. The isolate possessed three plasmids, i.e., IncHI2, IncX3, and IncFIB incompatibility groups, which acquired several plasmidborne accessory modules carrying a large number of genes involved in resistance to carbapenems, aminoglycoside, tetracyclines, phenicols, and sulfonamide-trimethoprim. These plasmid-borne antibiotic resistance genes were associated with insertion sequence elements, integrons, and transposons, indicating that the assembly, mobilization, and acquisition of these corresponding accessory modules with complex chimera structures were facilitated by transposition and/or homologous recombination (140).

FUTURE DIRECTIONS

The viewpoint that *Cronobacter* is just a neonatal foodborne pathogen has changed remarkably in the 10 years since its reclassification, and perception of its role will likely continue to evolve. Once considered a harmless inhabitant of the human intestinal tract or a contaminant of dried foods, *Cronobacter* is now seen as an opportunistic group of pathogenic species with remarkable versatility in their abilities to cause disease in humans. It is now realized that infections due to *Cronobacter* can affect neonates, infants, and elderly individuals, and this organism continues to generate national and international attention. Pathogen-specific virulence factors have been discovered that adversely affect a wide range of eukaryotic cell and host processes, including protein synthesis, cell division, and proinflammatory host responses. What little we know about these virulence factors is related to acquisition of a variety of mobile genetic elements, such as plasmids, transposons, and pathogenicity islands. This genomic plasticity implies ongoing reassortment (microevolution) and possibly acquisition of new virulence factors that will challenge our efforts to classify these organisms into various subgroups or into sharply delineated genomopathotypes. Additionally, these results suggest that other unidentified sources or vectors will be involved in the transmission of *Cronobacter*. Thus far, these sources have been difficult to identify. However, the observations of Friedemann (28) and those of Farmer (141) and Jason (7) revealed that environmental sources of infection should be considered and that consistent and sufficiently informative data from *Cronobacter* infections should be recorded in a centralized reporting system. Two such systems are the National Notifiable Diseases Surveillance System, which is maintained by the Centers for Disease Control and Prevention (https://wwwn.cdc.gov/nndss/data-and-statistics.html), and the European Centre for Disease Prevention and Control's Surveillance System (https://ecdc.europa.eu/en/publications-data/european-

surveillance-system-tessy). The dynamic nature of *Cronobacter* will continue to present additional challenges for the global food safety and public health communities in the diagnosis, treatment, and prevention of infections caused by this group of highly diverse, versatile, and opportunistic pathogens.

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15
Aeromonas

HISTORY AND BACKGROUND

The genus *Aeromonas* contains chemoorganotrophic bacteria that are Gram-negative, non-spore-forming, facultatively anaerobic, oxidase-positive (with rare exceptions), glucose-fermenting, rod-shaped bacteria (occasionally shortened into coccobacilli). This genus comprises both mesophilic and psychrophilic species. The history of *Aeromonas* dates back to 1891, when it was linked to red leg disease in frogs and described as *Bacillus hydrophilus fuscus* (1). Over 50 years later, it was reclassified as *Aeromonas hydrophila* (2). The first description of psychrophilic nonmotile aeromonads was for isolates from diseased trout, which were named *Bacillus der Forellenseuche* (3). Later, Chester proposed that this organism be named *Bacterium salmonicida*, which today is known as *Aeromonas salmonicida* (4).

CLINICAL MANIFESTATIONS

Aeromonads were discovered more than 100 years ago, and their role in human disease was first described in 1954, when the organism was associated with the death of a 40-year-old Jamaican woman with acute fulminating metastatic myositis (5). Diarrhea was later associated with aeromonads isolated from a 10-year-old girl (6). Subsequently, aeromonads have been implicated in various human and animal diseases. Although it is generally accepted that some *Aeromonas* spp. can cause serious clinical manifestations, their status as relevant clinical pathogens has been controversial for decades, especially with regard to human gastrointestinal tract infections. Based upon frequency, the most typical *Aeromonas*-associated diseases involve diarrheal cases (with different manifestations) among young children and immunocompromised populations. Also common are soft-tissue and wound infections subsequent to traumatic injuries, usually in immunocompetent individuals. Other intraabdominal infections and extraintestinal infections are rare and usually occur in individuals with underlying medical conditions (7–10).

A wide spectrum of diseases have been attributed to aeromonads, with four species being predominant in most clinical infections: *A. hydrophila*, *A. caviae*, *A. veronii*, and *A. dhakensis* (10). Table 15.1. lists all *Aeromonas* spp. linked to human diseases. It is important to note some taxonomic nuances regarding clinical *Aeromonas* spp. First, *A. veronii* bv. sobria (*A. veronii*), one of the main species of clinical importance, is often misidentified as *Aeromonas sobria* (9). In fact, *A. sobria sensu stricto* is described as a typical environmental species frequently isolated from waters, sewage, and diseased fish without relevance to clinical microbiology (11). Second, *A. dhakensis* was previously designated *A. hydrophila* subsp. *dhakensis* and/or *A. aquariorum* but is currently identified as a new clinical species. Its frequency in clinical samples is higher than previously thought, due to previous biochemical misidentifications as *A. hydrophila*, *A. caviae*, or *A. veronii* (12–14).

Table 15.1 Clinical diseases associated with *Aeromonas* species[a]

Species	Gastro-enteritis	Wound and soft tissue infection	Bacteremia	Intra-abdominal infection	Respiratory tract infection	Ocular diseases	Urinary tract infection	Other
A. allosaccharophila			+					
A. bestiarum			+					
A. caviae	+	+	+	+	+	+	+	+
A. dhakensis	+	+	+	+	+		+	+
A. diversa		+						
A. hydrophila	+	+	+	+	+	+	+	+
A. jandaei	+		+				+	
A. media	+	+	+				+	
A. popoffii							+	
A. salmonicida			+					+
A. sanarellii		+						
A. schubertii		+						
A. taiwanensis	+	+						
A. tecta	+							
A. trota	+	+		+				
A. veronii	+	+	+	+	+	+		+
A. enterica	+							
A. intestinalis	+							

[a] Data are derived from references 12, 13, 39, 42, 43, 149 and 161–181.

Gastroenteritis

Worldwide enteric infections are commonly caused by *Aeromonas* spp. and affect young children, elderly persons, patients suffering from underlying maladies (including immune disorders) and, less frequently, healthy individuals (7, 10). *Aeromonas*-associated gastroenteritis is underestimated due to the absence of routine testing for it in diarrheal stools, and the identification of these organisms is sometimes accidental (7, 10). This gastroenteritis presents in various forms, ranging from asymptomatic enteritis to the most common secretory (watery) enteritis and to severe cholera-like or dysentery-like diseases (7). In addition, rare cases of chronic diarrhea (15), traveler's diarrhea (16), and complications such as small bowel obstructions (17), enterocolitis (18), segmental enteric necrosis (19), and hemolytic-uremic syndrome (20, 21) have been described.

Recent decades have been marked by the uncertain and controversial status of *Aeromonas* as a true enteropathogen. Perhaps the strongest argument is that it does not fulfill Koch´s postulates. However, an immunocompetent laboratory worker accidentally ingested an *Aeromonas trota* culture from a clinical isolate and developed "rice-water" stools (22). The same strain was isolated from his stool, thus supporting Koch's postulates, though the extent of illness from the original isolate was unknown. Another case study ribotype matched an *Aeromonas* isolate from a ready-to-

eat shrimp cocktail to the corresponding diarrheal stool from a previously healthy male (23). Challenges to the true enteropathogenic nature of *Aeromonas* spp. still confound scientists, due to its being identified in 1 to 4% of asymptomatic individuals' stool samples (7), indicating that it can colonize without triggering pathogenic processes.

The lack of a perfect animal model to depict aeromonad-induced enteritis presents the next problem in elucidating this organism's pathogenic nature; however, the murine and rabbit ileal-loop models have provided some support. To characterize the role of the enterotoxins Act, Alt, and Ast in virulence, the murine model was used with *A. dhakensis* SSU (previously designated *A. hydrophila* isolate SSU [24]) (25), and this model is depicted in Fig 15.1. A secretory fluid response was quantified in enterotoxin mutants, with Act accounting for the strongest (64%) fluid secretory response, followed by Alt (48%) and Ast (43%). Mutating all three enterotoxins resulted in no detectable fluid response, confirming their roles as virulence factors. The rabbit ileal-loop model was utilized more recently to characterize the pathogenic behavior of a clinical *A. caviae* strain (26). Microscopy revealed massive bacterial colonization in the form of stacked bricks with biofilm formation, accompanied by pathological changes of vacuolization and villus destruction in enterocytes, as well as anatomical cellular disruptions.

Last, a lack of multiple large outbreaks, considering the ubiquitous nature of aeromonads, argues against their being true enteropathogens. However, misidentification and lack of clinical lab work, especially considering that the predominant manifestation is secretory enteritis, could explain the minimal number of reported outbreaks. Investigators of one outbreak did identify *A. hydrophila* from 33 of 55 villagers presenting with loose stools two to four days after beef consumption, but they failed to analyze the meat carcass for the same isolate (27). In Sweden, 24 of 27 guests at a funeral developed diarrhea after consumption of a traditional Swedish shrimp dish, which later tested positive for *A. hydrophila*, but unfortunately, no fecal samples were assayed (28). Similarly, more than 200 students developed gastroenteritis at a college in China, but only 15 fecal samples were collected and only three were positive for *A. hydrophila*, though they were negative for any of the other enteropathogens tested (29). No direct link to food was identified, due to a lack of food source to test. Regarding these studies, it is imperative not only to determine the species of *Aeromonas* associated with gastroenteritis but also to identify the same strain from a food or water source. A common argument against the pathogenicity of aeromonads is based on a human volunteer study conducted by Morgan et al. (30). Only 2 of 57 volunteers challenged with a virulent strain of *A. hydrophila* developed acute enteric symptoms. Given the high challenging dose used (10^4 to 10^{10} CFU), the low infection rate was surprising. Possible explanations include the following: (i) the volunteer study selected healthy individuals (to avoid severe complications), which might have influenced the final outcome; (ii) the challenge strains are often repetitively subcultured under laboratory conditions, which might affect virulence (31).

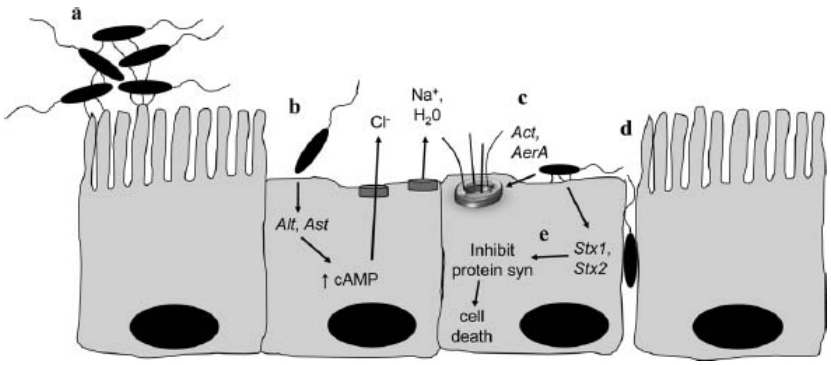


Figure 15.1 Virulence factors associated with *Aeromonas* spp. causing gastroenteritis. Aeromonads encode numerous virulence factors, though their pathogenic roles vary with disease. The image presents the most influential virulence factors associated with gastroenteritis. Adherence to intestinal epithelial cells and mucosa to initiate infection is mediated via lateral flagella. Additionally, adherence is involved in aggregative behavior with other aeromonads, resulting in a "stacked-brick" formation and biofilm formation (a). Once bound to the epithelial cells of the small intestine, *Aeromonas* spp. have an arsenal of enterotoxins they can secrete. Two of the enterotoxins, Alt and Ast, act in a cholera toxin-like fashion, elevating adenylate cAMP in the host. The exact mechanisms are not fully elucidated, but this induces an efflux of chloride ions (b), leading to osmotic leakage of water into the intestinal lumen and causing diarrhea. Other virulence factors associated with gastroenteritis include pore-forming aerolysin-related cytotoxic enterotoxin (Act) and aerolysin (AerA). These enterotoxins cause direct damage to the intestinal wall via enterocyte toxicity (c). The release of enterotoxins during disease distorts enterocyte morphology, including loss of microvilli (c) as well as detachment, providing passages through submucosa and tight junctions (d) (26). Some clinical strains of *Aeromonas* produce Shiga toxin homologs (encoded by *stx1* and *stx2*), causing a ribotoxic stress response, which in turn leads to inflammation and apoptosis (e), though the exact mechanisms of action of these *Aeromonas* homologs have yet to be determined.

The lack of disease correlation highlights the potential importance of host genetic susceptibility and the influence of gut microbiome composition. Teunis and Figueras compared the results of the human volunteer study with results from previous natural outbreaks and determined that the infectious doses for all natural outbreaks were more than a 100-fold lower than those for the volunteer study (31), suggesting a dose-response explanation. In developing countries, rates of isolation of *Aeromonas* spp. from diarrheal children vary between 1 and 88%, compared to a 1 to 48% isolation rate from asymptomatic patients' stools (32). These numbers further support the idea that not every gastrointestinal colonization with *Aeromonas* leads to infection. More studies are needed to determine key factors involved in hostpathogen interactions triggering gastrointestinal maladies.

Wound and Soft-Tissue Infections

Skin and soft tissues are the most common sites of extraintestinal infections caused by aeromonads. Typically, these wound and soft-tissue infections occur in immunocompetent populations, usually upon posttrauma exposure to contaminated water or soil (7–9). However, patients suffering from other injuries, such as burns, surgical wounds, crush injuries, or animal bites, are endangered as well (9, 10). Clinical manifestations include cellulitis, furunculosis, skin nodules, abscesses, necrotizing fasciitis, myonecrosis, and gangrene or osteomyelitis (7, 33). Immunocompetent individuals usually have localized infection with favorable outcomes, while fulminant fatal consequences occur predominantly in immunocompromised hosts (8). The pathogenesis of soft-tissue infections caused by aeromonads remains equivocal, though

degradative enzymes are leading virulence candidates. A new animal model using *Caenorhabditis elegans* to study *A. dhakensis* pathogenicity during necrotizing fasciitis might provide new insights (34) in the future.

Bacteremia and Septicemia

While bacteremia is characterized by the presence of bacteria in the bloodstream and can be eliminated asymptomatically, septicemia represents inflammatory processes associated with multiplication of bacteria in bloodstream, often accompanied by toxin release and a systemic response triggering sepsis (Fig 15.2). Underlying diseases, such as hepatobiliary disease, malignancy, immune disorders, and diabetes, can play a key role in the acquisition and outcome of bacteremia and septicemia caused by aeromonads (7, 10). Immunocompetent populations are also at risk for bacteremia or septicemia when colonization by aeromonads is preceded by injury or trauma or occurs subsequent to specific treatments, such as surgery or leech therapy (10). *Aeromonas* bacteremia/septicemia presents predominantly as a monomicrobial, community-acquired infection and occurs mainly in male populations (10), with high mortality rates (23 to 70%) (35, 36).

Other Infections

A rare presentation of *Aeromonas* infections is pneumonia or other respiratory tract infections. They occur in both healthy and immunocompromised individuals subsequent to bacteremia (37). Near-drowning incidents also result in an increased risk for pneumonia, which presents within 24 h (38). Because members of this genus do not typically cause pneumonia, it may be underdiagnosed by clinicians and laboratory technicians. Ocular diseases are also caused by aeromonads posttrauma, being spread endogenously or via exposure to contaminated environmental sources (7, 39). These infections are usually mild, but some severe cases of endophthalmitis, keratitis (or lens-related keratitis), and corneal ulcerations (7, 8) have been reported.

Intra-abdominal *Aeromonas* infections are typically community acquired and affect patients with malignancy of the hepatobiliary system (often cirrhosis), pancreas, or immune system (40), with subsequent 30% mortality (41). These infections extend beyond the hollow viscus organ into the peritoneal space and include hepatobiliary tract infections, peritonitis, pancreatitis, cholangitis, and hepatic abscesses.

Only a few cases of *Aeromonas* urinary tract infections have been documented, and they were associated with *A. caviae* (42) or *A. popoffii* (43). *A. hydrophila* was identified as the etiologic agent in an acute epididymitis and pyelonephritis case in an immunocompetent 42-yearold patient 24 h after engaging in sexual intercourse in a swimming pool (44), reemphasizing the potential risks associated with water sources containing aeromonads.

CLASSIFICATION

There have been many changes in the taxonomic structure of the genus *Aeromonas* in the last few decades. In the first edition of *Bergey´s Manual of Systematic Bacteriology*, the genus *Aeromonas* was assigned to the family *Vibrionaceae* based on phylogenetic properties (45). Later, based on various studies using DNA hybridization, analyses of 5S and 16S rRNA, and DNA-rRNA hybridization, supported by rRNA gene and rRNA sequencing, *Aeromonas* was confirmed as being evolutionarily distinct from *Vibrionaceae* and *Enterobacteriaceae*. Since the second edition of the *Bergey´s Manual of Systematic Bacteriology*, the family *Aeromonadaceae* has been established within the order *Aeromonadales* and class *Gammaproteobacteria* (46).

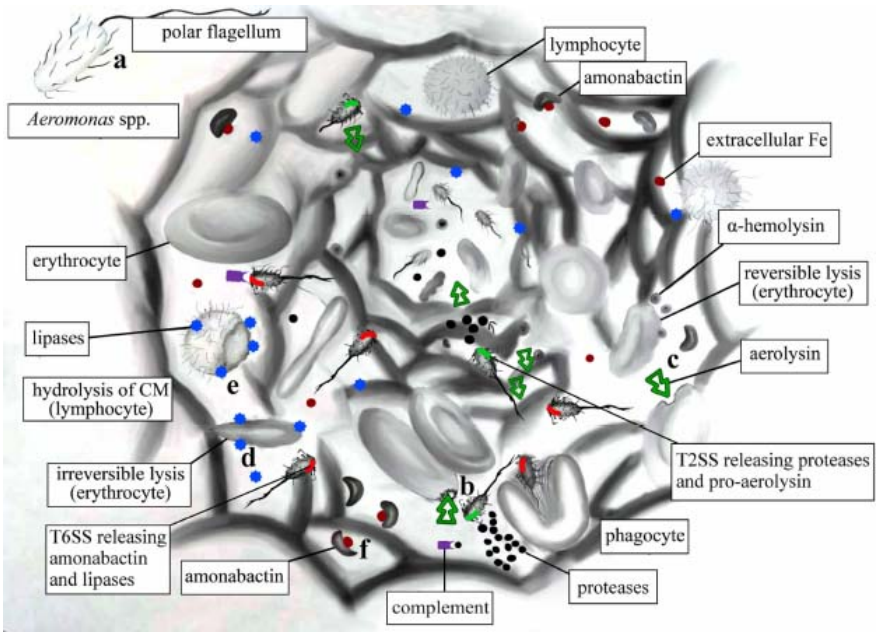


Figure 15.2 Potential role of virulence factors in *Aeromonas*-associated bacteremia. Aeromonads produce a single polar flagellum to aid in motility (a). T2SS releases proaerolysin and proteases, which may either act as activators of aerolysin or break down complement proteins (b). Mature aerolysin is a pore-forming enzyme causing complete lysis of red blood cells (c). Hydrolysis of lymphocytic membranes occurs via secreted lipases mediated by T6SS (d) and can cause irreversible erythrocyte lysis (e). Additionally, T6SS secrete iron-binding proteins (e.g., amonabactin) that enable acquisition of extracellular iron (f). Reversible lysis of erythrocytes may occur following α-hemolysin production. CM, cell membrane. Illustration by Cody Rasmussen-Ivey, Tibor Král, and S. Králová.

Originally, the genus was divided into two main categories (phenotypically and genotypically corresponding). The first category includes mesophilic and motile aeromonads, and the second, a more homogenous group, includes nonmotile psychrophilic species. DNA-DNA hybridization studies later differentiated at least 14 distinct hybridization groups (47). However, the term "hybridization group" is outdated, and its usage is relevant only in specific cases (e.g., unnamed taxonomic groups) (48). With regard to taxonomy, 16S rRNA analysis should be used cautiously due to the high degree of homology (98 to 100%) within 16S rRNA gene sequences among aeromonads, leading to discrepancies in *Aeromonas* phylogeny (47, 49). Additionally, a small number of 16S rRNA microheterogeneities (nucleotide polymorphisms) were detected, leading to inconclusive identification or misidentification, thus affecting *Aeromonas* species classification and taxonomic position (50).

A promising molecular tool for identifying novel *Aeromonas* species (e.g., *A. popoffii*, *A. molluscorum*, *A. bivalvium*, *A. tecta*, and *A. diversa*) is amplified fragment length polymorphism analysis (AFLP). Although AFLP groupings do not always correspond to those obtained with DNA-DNA hybridization studies (48), AFLP application can determine the taxonomic identities of *Aeromonas* isolates with different origins. To establish a more sensitive taxonomic approach regarding *Aeromonas* phylogeny, several housekeeping genes having greater divergence, such as *gyrB*, *rpoD*, *dnaJ*, *cpn60*, *recA*, and *gyrA*, are used either alone or in combination as a more powerful taxonomic tool (51). Currently, *Aeromonas* contains 31 validly described species (Table 15.2.), with three subspecies of *A. hydrophila*, five subspecies of *A. salmonicida*, and two biovars of *A. veronii* (52).

Table 15.2 Origins and dates of identification of *Aeromonas* species

Species	Year	Source
A. allosaccharophila	1992	Diseased eel
A. aquatica	2015	Lake water
A. australiensis	2013	Treated effluent used for irrigation
A. bestiarum	1996	Diseased fish
A. bivalvium	2007	Bivalve mollusks
A. cavernicola	2013	Cavern water
A. caviae	1988	Guinea pig
A. dhakensis	2013	Diarrheic feces of child
A. diversa	2010	Leg wound
A. encheleia	1995	Healthy eel
A. eucrenophila	1988	Fish
A. finlandensis	2015	Lake water
A. fluvialis	2010	River water
A. hydrophila	1943	Tin of milk with fishy odor
A. hydrophila subsp. *anaerogenes*	1964	
A. hydrophila subsp. *hydrophila*	1969	
A. hydrophila subsp. *ranae*	2003	
A. jandaei	1991	Diarrheic feces of male patient
A. lacus	2015	Lake water
A. media	1983	Effluent water from a fish farm
A. molluscorum	2004	Bivalve mollusks
A. piscicola	2009	Diseased salmon
A. popoffii	1997	Drinking water production plant
A. rivipollensis	2016	River water
A. rivuli	2011	Karst water rivulet
A. salmonicida	1953	Salmon
A. salmonicida subsp. *achromogenes*	1967	
A. salmonicida subsp. *masoucida*	1969	
A. salmonicida subsp. *pectinolytica*	2000	
A. salmonicida subsp. *salmonicida*	1967	
A. salmonicida subsp. *smithia*	1989	
A. sanarellii	2010	Elbow wound
A. schubertii	1988	Forehead abscess injury
A. simiae	2004	Feces of monkey
A. sobria	1976	Fish
A. taiwanensis	2010	Burn wound
A. tecta	2008	Diarrheic feces of child

A. trota	1991	Human feces
A. veronii	1987	Sputum of a drowning victim
A. veronii bv. sobria		
A. veronii bv. veronii		
Effective publication only (not yet validly named)		
A. aquatilis	2017	Lake water
A. enterica	2017	Human feces
A. intestinalis	2017	Diarrheic feces of female patient
A. crassostreae	2017	*Crassostrea gigas*

ISOLATION AND IDENTIFICATION

Many different transport media (e.g., Cary-Blair medium, Amies medium, modified Stuart's medium, and buffered glycerol in saline) for presumptive samples containing aeromonads (mostly stool) are available with good recovery at room temperature (7, 46). Aeromonads grow well for subsequent isolation on common laboratory media (e.g., Luria broth, tryptic soy broth, and Mueller-Hinton broth) and most enteric differential media, except for thiosulfate-citrate-bile salts-sucrose agar (11). However, growth on enteric media can be overlooked due to common microflora presenting as lactose-fermenting strains. Aeromonads can be recovered from nonenteric clinical samples by using various media based on the origin of the sample (e.g., Rimler-Shotts medium, starch-glutamate-ampicillin-penicillin-based medium, mAeromonas medium, *Aeromonas* agar, ampicillin-dextrin agar [ADA], and starch ampicillin agar); however, the use of enrichment procedures for recovery of *Aeromonas* spp. is not recommended (11). Ideal incubation temperatures vary between 25 and 30°C for environmental samples and between 25 and 37°C for clinical strains of *Aeromonas*. Isolation of *Aeromonas* spp. from food and water samples uses more standardized procedures utilizing starch-ampicillin agar and ADA for recovery of aeromonads after 24 h of cultivation at 30°C (53). Environment Protection Agency (EPA) standards under the Safe Drinking Water Act employ either direct plate count methods or membrane filtration techniques (54) in combination with selective and differential media such as ADA with vancomycin.

As a genus, *Aeromonas* is easily distinguished from other Gram-negative, facultative anaerobes (*Enterobacteriaceae*, *Plesiomonas*, and *Vibrio*) by a few simple phenotypic tests (Fig 15.3). Presumptive identification on selective and differential media is routinely followed by biochemical tests for species identification. Conventional tests, as well as miniaturized identification kits (API 20NE and 50E, Biolog, and MicroScan), are commonly used, although misidentification at the species level can occur (48). This is partially due to the increased number of new *Aeromonas* species and the lack of precisely determined physiologic traits among all species to ensure comparable results between different laboratories (7, 48).

Of the analytic, chemotaxonomic methods, fatty acid analysis (FAME) has been successfully used for *Aeromonas* identification from food (53). Another chemotaxonomic approach, matrix-assisted laser desorption ionization–time of flight mass spectrometry, successfully identified species with accuracy greater than 90% (55) and is currently one of the most rapid and accurate identification methods in routine laboratory use. However, only identification of biochemically typical strains has high accuracy (56).

The evolution of molecular methods (e.g., multiplex PCR, restriction fragment length polymorphism analysis-PCR, AFLP, quantitative PCR [qPCR], ribotyping, and the use of species-specific probes and primers) to identify either *Aeromonas* spp. or their virulence factors has

decreased the time for analysis; however, their accuracy, discriminatory power, and reproducibility differ substantially, and application of these approaches is more common for taxonomic purposes than for routine identification.

TOLERANCE TO ENVIRONMENTAL FACTORS ASSOCIATED WITH FOOD PROCESSING AND PRESERVATION

Bacterial contamination of food products can occur in multiple locations within a food processing plant and can have profound effects, including causing food spoilage as well as food-borne illnesses. A variety of measures affecting environmental conditions have been implemented within food processing and storage facilities to help minimize bacterial growth and biofilm formation.

Temperature, Osmotic Pressure, and pH

One of the easiest methods to control microbial contaminants in foods is heating and chilling of food, although *Aeromonas* spp. have demonstrated resilience, especially at cold temperatures. The ideal temperature for growth of the mesophilic species is ca. 25°C (57), and heat can kill *Aeromonas* spp. in culture media at 45°C and above. However, *A. hydrophila*'s thermal resistance in food, such as raw liquid eggs, is substantially greater than that of laboratory strains in culture media (58). Being resilient to colder temperatures, *A. hydrophila* retained viability for 14 days in milk, yogurt, and cheese at 4°C, even when cocultured with probiotics (59). This cold environment had minimal effects on expression of adhesion properties (HEp-2 cells) and virulence factors (e.g., cytotoxins, enterotoxins, and hemolysin) by *Aeromonas* isolates from various foods (e.g., chicken, lamb, and milk) (60). Although bacteria like vibrios may not survive freezing, *A. hydrophila*, *A. caviae*, and *A. veronii* have repeatedly been obtained from frozen foods, including 2 to 6% of beef (61), 10% of sausages (62), and 2 to 6% of fish (63), with *A. hydrophila* surviving in frozen tilapia for one year (64). Another treatment that can affect bacterial viability is providing a starvationlike state by methods such as washing bacterial cells with minimal nutrients. This stressful environment rendered *A. hydrophila* less susceptible to the effects of exposure to 10 to 15% ethanol and the bactericidal effects of prompt temperature fluctuations after being starved for 50 days in sterilized distilled water (65).

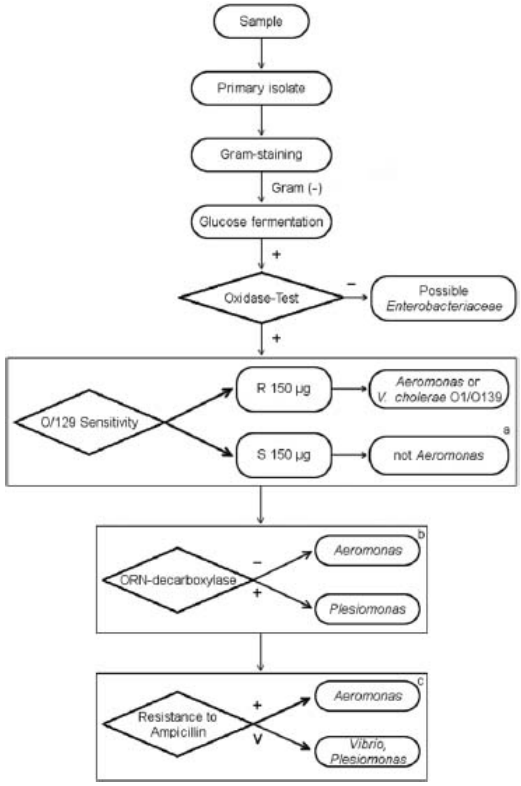


Figure 15.3 Preliminary identification scheme for *Aeromonas*. This flow chart can be used for rapid preliminary identification of aeromonads. Aeromonads are Gram-negative, facultative anaerobes, ferment glucose, and are positive for oxidase, which differentiates them from oxidase-negative *Enterobacteriaceae*. Distinguishing *Aeromonas* spp. from other oxidase-positive Gram-negative bacilli (i.e., *Vibrio* and *Plesiomonas*) is possible with the following test results: resistant to vibriostatic compound O/129 (150 µg 2,4-diamino-6,7-diisopropylpteridine), negative for ornithine decarboxylase (ORN-decarboxylase), and resistant to ampicillin (10 µg). +, positive reaction; –, negative reaction; v, variable result. a, some strains are sensitive to O/129 (150 µg) (161); b, except for rare ornithine decarboxylase-positive strains (162); c, except for *A. trota* (22).

Elevated NaCl concentrations can subject microbes to osmotic stress, with bactericidal effects. For aeromonads, up to 6% NaCl at 4°C is bacteriostatic, with lytic effects occurring as the temperature increases (66). NaCl at 2% impedes biofilm formation of aeromonads (67). Mesophilic *Aeromonas* spp. are sensitive to pH levels less than 6 or greater than 10 (68) and are even more sensitive to pH changes in the presence of sodium nitrite (69).

UV Light, Gamma Irradiation, Cold Plasma, and Hydrostatic Pressure

One of the limitations of typical PCR is differentiating viable from nonviable microorganisms when nucleic acid is amplified. However, new PCR modifications have resulted in viability PCR, which uses propidium monoazide to prevent PCR amplification from any free DNA or accessible DNA through damaged membranes. Another alternative molecular viability technique utilizes bacterial rRNA precursors to test viability through reverse transcription-qPCR (RT-qPCR), enabling species specificity and high sensitivity. Results obtained with RT-qPCR agree with those of culture techniques, demonstrating that 41 mJ/cm^2 of UV light (254 nm) resulted in complete inactivation at 30 seconds of an *A. hydrophila* culture of 1×10^8 CFU/ml (70).

A major challenge in the food processing industry is minimizing bacterial contamination that causes food spoilage. One potential treatment postpackaging is gamma irradiation, which is highly

bactericidal to both planktonic and biofilm growth of *A. hydrophila*, with decimal reduction values observed with application of less than 0.5 kGy (71). This treatment provided a 5-log reduction of aeromonads in mixed sprouts, chicken, fish (72), and frozen shrimp (receiving 3 kGy), supporting its efficacy postprocessing (73). Cold plasma technology can be used as a disinfectant, as a 15-second exposure reduced the population of planktonic *A. hydrophila* by 5 logs and a 15-min exposure inactivated *Aeromonas* biofilms on lettuce (74). Aeromonads are also quite sensitive to high hydrostatic pressure (i.e., 100 MPa) compared to other common foodborne pathogens (e.g., *Escherichia coli*, *Shigella*, *Listeria*, and *Salmonella*) (75).

Halogens, Ozone, and Modified Atmosphere

The World Health Organization has determined that most chlorinated drinking water contains free-chlorine levels between 0.2 and 1.0 mg/liter, with a maximum residual disinfectant safety level of up to 4 mg/liter (4 ppm) according to the EPA. Within U.S. public water systems, aeromonads were detected in 2.6% of 5,042 water samples, with *A. salmonicida* (28.2%) and *A. hydrophila* (24.6%) being the most prevalent species (76). *Aeromonas*-positive samples were more significantly associated with lower free-chlorine concentrations (0.02 mg/liter), whereas *Aeromonas*-negative samples correlated with higher free-chlorine levels (0.4 mg/liter) (76), further supporting the use of chlorine as a disinfectant in drinking water systems if adequate concentrations are maintained. Compared to *E. coli*, *Aeromonas* was more resilient to free chlorine (0.25 mg/liter), especially at 20°C, where there was more than 50% viability after 90 minutes of exposure, although lower temperatures (4°C) did increase sensitivity (77).

Ozone remains an effective treatment for treating both recirculating water from aquaculture ponds and wastewater as well as for preservation of food products. *A. salmonicida* populations in both lake water and seawater were reduced by 4 logs within 60 seconds by a treatment of 0.2 mg of ozone per liter (78). However, its use directly in aquaculture ponds may present a health risk to fish through oxidative stress (79); hence, its application would predominantly be restricted to water recycling and food processing. Packaging atmospheres of foods, especially fresh meat and poultry, can affect the level and diversity of microbial populations and thereby also affect the shelf life of food. Considering the ubiquitous nature of aeromonads and their proteolytic activities on foods (80), optimizing the packaging atmosphere to minimize their growth is critical, although the meat source, temperature, and presence of other microbial populations need to be considered.

RESERVOIRS AND LINKS TO WATER-AND FOODBORNE ILLNESS

With an estimated 48 million foodborne illnesses and 3,000 deaths per year (81), it is important to address the association of *Aeromonas* spp. with foodborne outbreaks. Considering its widespread nature, it is not surprising that *Aeromonas* spp. have been isolated from most types of foods, as well as drinking water.

Drinking Water

The primary reservoir for aeromonads is aquatic environments, including rivers, ponds, groundwater, lakes, estuaries, and seawater. Many aquatic aeromonads are psychrophiles, typically associated with poikilothermic animals. However, aquatic mesophilic aeromonads have been associated with cytotoxicity and clinical infections (82), thereby posing a risk to humans through aquatic recreational activities as well as drinking water. Associations with aquatic recreational activities involve near drowning events, with subsequent pneumonia caused by *Aeromonas* spp. (37, 38), as well as subclinical pediatric diarrhea and skin diseases. With regard to U.S. public water systems, *Aeromonas* spp. remain on the EPA's Contaminant Candidate List. When more than 3,000 human fecal specimens associated with gastroenteritis were analyzed for

pathogens, *Aeromonas* spp. were the most frequently recovered enteropathogen, with ingestion of treated and untreated water being the most probable source (83). As mentioned above, aeromonads are more resilient to free chlorine than most other aquatic enteropathogens (84). One of the challenges in controlling aeromonads is their ability to adhere to pipes and produce biofilms (85–87), making them more resistant to disinfectants, with the presence of the organic material of the biofilm reducing the disinfectant's efficacy. Biofilm formation within the water system presents an additional risk, namely, the exchange of antibiotic resistance genes via horizontal gene transfer (88), which creates increased challenges in clinical cases. Ma et al. determined that there were 84% more antibiotic-resistant genes in drinking water samples than in environmental samples (89). However, wastewater treatment plants harbor the highest prevalence of antibiotic resistance genes (89, 90), contributing to a growing global threat. Chlorinated effluent is rich in antibiotic-resistant isolates as well as aeromonads, with more than 25% of microbial isolates from effluent being resistant to five or more antibiotics (91). Considering that horizontal gene transfer between genera of *Enterobacteriaceae* and *Aeromonas* has already been demonstrated, aeromonads may serve as reservoirs of antibiotic resistance genes (92). Wastewater effluents are returned back to natural bodies of water, thereby allowing the resistant bacteria to colonize aquatic species as their next host (Fig 15.4).

Seafood

China, the United States, and Japan remain the leading consumers of seafood in the world, with the United States consuming 14.4 to 15.8 pounds per person per year from 2010 to 2015, of which 74% was fresh or frozen fish (93). As the world population increased 1.6% annually over the past 50 years, the rate of fish consumption doubled to 3.2%, leading to 31.4% of fish stocks being at biologically unsustainable levels. These trends have driven aquaculture to production of 73.8 million tons of fish and seafood in 2014, and the rate is growing at 5.8% annually (94).

One of the greatest risks for acquiring *Aeromonas* gastroenteritis comes from the consumption of raw and ready-to-eat seafood products. *Aeromonas* contamination of seafood most likely results from intestinal colonization while fish and shellfish reside in aeromonad-infested waters, which could lead to cross-contamination within food processing facilities. At seafood markets, *A. hydrophila* was isolated from 28 of 33 different table fish and 34% of 536 fish, which was a higher rate than the rate of isolation from shrimp (17%) (95). The predominant species, *A. salmonicida* (not a typical human pathogen), was isolated from 74% of sushi (74%), with *A. hydrophila*, *A. caviae*, and *A. dhakensis* each contaminating 2 to 5% of sushi. Most of these *Aeromonas* isolates encoded at least four of the five virulence factors associated with gastroenteritis (i.e., Act, Alt, Ast, HlyA, and AerA) (96). *Aeromonas* spp. pose a risk not only with finfish but also with oysters, crabs, and shrimp, where they reside in biofilms that create a greater safety hazard for consumption because of the difficulty in removing the aeromonads from the seafood (97).

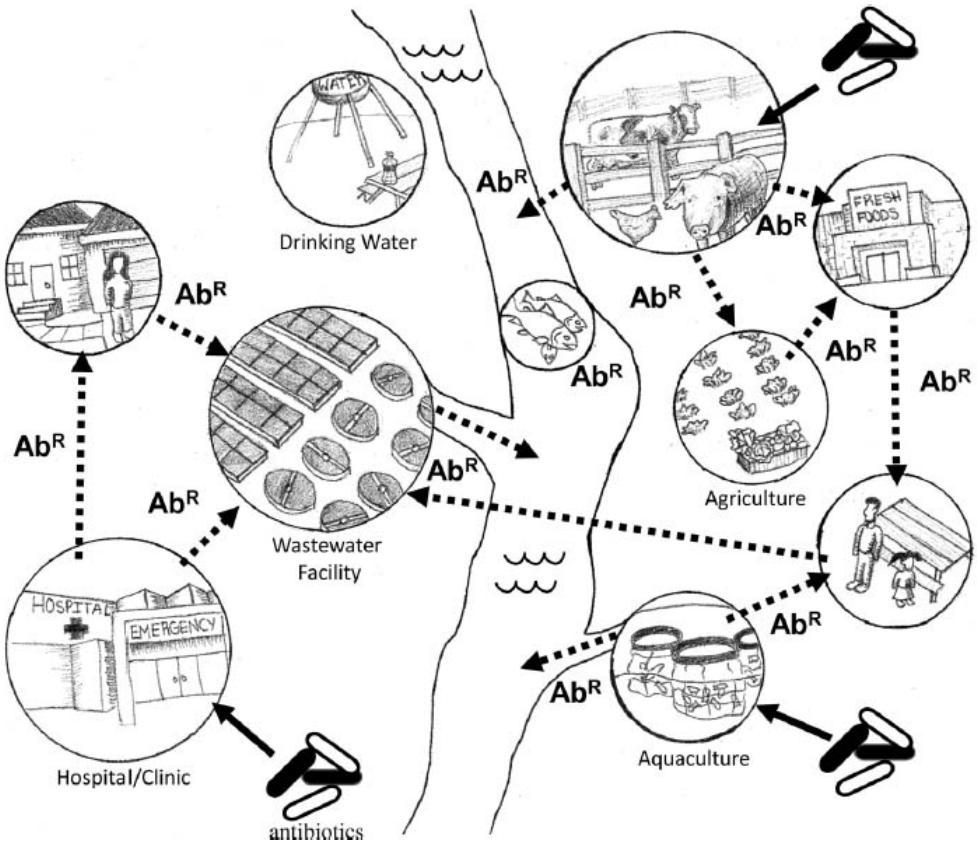


Figure 15.4 Cycle of antibiotic resistance within aeromonad reservoirs. The widespread occurrence of aeromonads contributes to their ability to colonize cold-and warm-blooded animals, both aquatic and terrestrial. The use of antibiotics prophylactically and clinically provides environments with subinhibitory concentrations of antibiotics, creating stress that can lead to resistance. Solid arrows represent the entrance of antibiotics, and dashed arrows represent pathways of transmission of antimicrobial resistance (Ab^R). Illustration by Andrew Albers and T. Skwor.

The innate immune response of teleosts is more elaborate and effective than that of mammals; however, stresses predispose fish to bacterial diseases. Considering that *Aeromonas*-associated fish diseases are opportunistic infections, it is critical to minimize changes in water temperatures and pH and concentrations of ammonia and organic matter, as well as overcrowding. *A. salmonicida* subsp. *salmonicida* is the most prominent species associated with furunculosis, which predominantly affects salmonids, although the disease also presents in nonsalmonids such as Atlantic cod, eel, halibut, carp, and goldfish (98). Infection initiates through colonization of the gills, the dorsal and pectoral fins, and possibly the oral and nasal cavities, with subsequent spreading in the internal organs (99). For more than 100 years, this species has been recognized as a cause of furunculosis, and it has plagued fish farms since the 1950s. Even though protective vaccines are available, the absence of their widespread use and varying degrees of efficacy for different fish strains render them problematic. Instead, aquaculture utilizes antibiotics as a prophylactic treatment, leading to increased levels of antibiotic resistance. In fish farms, 10% of *A. salmonicida*-associated furunculosis isolates were resistant to the antibiotics trimethoprim and sulfonamide, which are used clinically in humans to treat furunculosis (100). The resistance genes from freshwater farm aeromonads are already evident in recipient bay seawater farms, highlighting the risks associated with antibiotic use in aquaculture. Furthermore, these resistant strains have been identified in fish sold in fish markets, revealing their link to the human gut

microbiome (101) (Fig 15.4). Acquisition of antibiotic resistance genes escalates when aeromonads thrive in subinhibitory concentrations of antibiotics. Oxytetracycline, the most common drug utilized in aquaculture, is sensitive to prompt photodegradation, with only a 25-min half-life (102, 103), thus potentially exposing aeromonads and other fish gut microflora to subinhibitory concentrations. Food processing poses yet another environmental risk for the transmission of antibiotic resistance genes. Kruse et al. determined that *E. coli* on a cutting board underwent transconjugation with an R-plasmid-containing *A. salmonicida* strain from an infected salmon after processing (104). Some alternative treatments in lieu of prophylactic antibiotic treatment to protect fish stocks in farms include biofiltration utilizing oysters (105), use of probiotics (106), immunostimulation and vaccination (107), and phage therapy (108).

Meat

There are three risks associated with *Aeromonas* spp. and land animal meat: (i) the potential of contaminated food to cause gastroenteritis, (ii) the possibility of increased food spoilage, and (iii) the creation of a reservoir for antibiotic resistance genes. Animals carry *Aeromonas* spp. as part of their intestinal flora; hence, good sanitation during meat processing as well as food preparation is important. Frequently, contamination is introduced by *Aeromonas*-contaminated water that is used for carcass washing, although in developed countries, inadequate sanitation of household cutting boards predominates (109). *Aeromonas* spp. commonly occur on retail meats, including beef, chicken, lamb, duck, buffalo, sheep, pigs, and sausage (61, 109, 110). Bacterial contamination can play a role in meat spoilage, including producing foul odors and proteolytic and lipolytic activities. Wang et al. identified *A. salmonicida* and *A. hydrophila* as contaminants on chicken breast meat, contributing to sarcoplasmic and myofibrillar proteolytic degradation (80) as well as producing lipolytic activity, which adversely affected the organoleptic properties of meat (111). Overall, *A. hydrophila* is the predominant aeromonad contaminant associated with meat and meat products (109).

Aeromonads as part of the microflora in animals are at risk for the acquisition of antibiotic resistance. In the 1950s, it was discovered that antibiotics can serve as growth promoters of livestock, initiating an era of overuse and increasing antibiotic-resistant bacterial populations within livestock (109), including some that are resistant to last-resort antibiotics (e.g., colistin) (110). Using metagenomics, Ma et al. identified antibiotic resistance gene arrangements shared between pig, chicken, and human fecal isolates (112), revealing an interplay between agriculture and humans (Fig 15.4). Direct interactions with animals, consumption of contaminated meat, and exposure through other environmental factors indirectly mediate the link between these two groups.

Milk and Dairy Products

Raw milk continues to be analyzed for coliform numbers to evaluate safety measures, as *A. hydrophila* and *A. caviae* are frequent contaminants (113), and their lipase production accelerates spoilage (114). Melas et al. detected *Aeromonas* spp. in 41% of raw cow and 35% of ewe's milk samples, as well as in 10% of cheese samples, with the predominant species being *A. hydrophila*. Importantly, pasteurization did eliminate the *Aeromonas* contamination (113). All dairy products can potentially be contaminated, including yogurt (59) and ice cream (115), from which *A. hydrophila* and *A. caviae* have been isolated, respectively.

Most *Aeromonas*-associated dairy contamination is with cow's milk. The association of *Aeromonas* spp. with human breast milk is rarely studied. Considering that breast milk is recommended as the sole nutritional source for infants subsequent to birth, the microbial communities in breast tissue and milk are important in preventing dysbiosis. More than 20% of women are at risk of acquiring mastitis, supporting the importance of identifying microbial

populations associated with acute and subacute infections. Using culture-independent techniques, Patel et al. (116) detected elevated *Aeromonas* spp. in breast milk from women suffering from acute mastitis (16% of sequences obtained) compared to levels in healthy lactating women. The species in mastitic samples included the human pathogenic species *A. veronii*, *A. hydrophila*, and *A. caviae*, whereas *Acinetobacter baumannii* predominated in the milk from healthy women (116). The risk of disease in infants of mothers producing mastitic milk was not determined in this study; however, Qamar et al. (117) conducted a casecontrol study that revealed that 18.7% of diarrheal stool samples from infants (0 to 11 months) were positive for *Aeromonas* spp., which was significantly higher than the rate in healthy controls. This study also determined that infantile *Aeromonas*-associated diarrhea was inversely proportional to breastfeeding (odds ratio, 0.4), further supporting the importance of infants' receiving healthy breast milk (117).

Fruits and Vegetables

Many factors during pre-and postharvest periods contribute to the risks of microbial contamination of fruits and vegetables. During the preharvest period, spray irrigation with nonpotable water can present major risks of bacterial contamination (118). Additionally, abrasions of fruits and vegetables after harvest or during processing increases the sites for bacterial colonization and the potential formation of biofilms; hence the emphasis on postharvest washings. To minimize microbial contamination, washing with chlorine is a common practice. Produce that has been determined to be contaminated by *Aeromonas* spp. includes alfalfa sprouts, asparagus, celery, broccoli, peppers, redand green-leaf lettuce, and spinach (118). Most of these isolates were *A. hydrophila* and *A. caviae*, with 100% of the *A. hydrophila* strains being cytotoxic. These isolates grew between 5 and 35°C (119), highlighting the importance of properly cleaning produce before it is shipped to restaurants and grocery stores.

VIRULENCE FACTORS

Microbial pathogenesis depends not only on being genetically equipped but also on the expression of virulence factors during the host-pathogen interaction. While some virulence factors are present constitutively (e.g., lipopolysaccharide), others are induced only under certain biological conditions (e.g., specific temperatures, tissues, and nutrient concentrations), often resulting in specific pathogenic processes (diarrhea, septicemia, wound infection, etc.). *Aeromonas* spp. are equipped with many virulence factors that have various roles in their pathogenesis (Fig 15.1 and 15.2)

Flagella and Pili

Pathogenic bacteria typically use flagella to promote colonization and invasion of the host tissue. Mesophilic aeromonads possess genes for both polar and lateral flagellar systems, whereas psychrophilic aeromonads are frequently nonflagellated. Motility through liquid is accomplished by a single polar flagellum, whereas an inducible flagellar system with multiple lateral flagella is essential for swarming over surfaces (120). Both systems are essential for enterocyte adherence and biofilm formation (120).

Another filamentous structure, the pilus, serves as a potential colonizing factor, enhancing adherence to host tissues, and is involved in bacterial conjugation, enabling horizontal gene transfer. *Aeromonas* pili have two morphotypes. The predominant form is short and rigid and is involved in autoaggregation between bacteria (121). The other morphotype is a thin, long, and flexible pilus, which mediates adherence to enterocytes; this feature is common among all clinically relevant *Aeromonas* strains associated with diarrheal infections (122).

Degradative Enzymes

Most aeromonads elaborate a variety of extracellular enzymes, such as proteases, lipases, collagenase, nucleases, amylases, chitinases, and elastase. These degradative enzymes may contribute to overall virulence; however, their specific roles in pathogenesis have yet to be determined.

Aeromonads secrete a wide range of proteases that are associated with tissue damage, enhanced invasivity, complement inactivation, and nutrient recycling (123, 124). They include heat-labile serine protease, heatstable metalloprotease, EDTA-sensitive and -insensitive metalloproteases, and fibrinolytic protease (123). Additionally, aminopeptidases can degrade exogenous peptides, activate cytotoxic enterotoxin (Act) and aerolysin (AerA), cleave the terminal methionine from newly synthesized peptides, and stabilize plasmids (123, 125). Serine protease (68 kDa; caseinolytic) and metalloprotease (60, 44, and 31 kDa; elastolytic and caseinolytic) are secreted predominantly during the late stationary phase and are lethal for fish (126). Nitta et al. successfully purified and characterized serine protease from *A. veronii* (65 kDa; proteolytic) (127). Its bioactivity produced α-thrombin from prothrombin, which converts fibrinogen to insoluble fibrin, thus establishing direct involvement in septic shock by promoting disseminated intravascular coagulation. Additionally, it induces vascular leakage, thereby lowering blood pressure with subsequent edema and septic shock (128).

In addition to proteases, aeromonads produce various lipases. These lipolytic enzymes provide bacteria with nutrients, destroy leukocyte membranes (Fig 15.2) and other eukaryotic cells, and generate free fatty acids affecting the immune system (124). Additionally, a glycerophospholipid-cholesterol acyltransferase isolated from *A. hydrophila* and *A. salmonicida* lyses red blood cells through membrane disruption (123, 129).

Collagenases enhance the invasivity of bacterial pathogens through the degradation of collagen. *A. hydrophila* and *A. veronii* both encode these enzymes, which play roles in their pathogenesis (130). Another important virulence factor of *A. hydrophila* is elastase, which hydrolyzes elastin and casein (131). Insertional inactivation of the elastase gene *ahpB* increased by a hundred times the 50% lethal dose for rainbow trout compared to wild-type controls. Deleting the nuclease gene *ahn* from strains of *A. hydrophila* attenuated their pathogenicity in both fish and mice. The biological effects of the *ahn* protein include nucleic acid recycling, protection against foreign exogenous DNA (i.e., bacteriophage) (123), and immunologic evasion, supporting an involvement in systemic dissemination (132).

Hemolysins and Aerolysin

Hemolysins are a group of multifunctional enzymes contributing to pathogenicity (123). Aeromonads possess several hemolysins (AerA, HlyA, Ahh1, and Asa1) that are divided into two classes, α-and β-hemolysins. The α-hemolysins cause incomplete or reversible lysis of erythrocytes, whereas the β-hemolysins (such as aerolysin) are pore formers in cell membranes and lead to complete erythrocyte lysis (Fig 15.2) (123). The most abundant aeromonad hemolysin is a heat-labile hemolysin (encoded by *ahh1*) which has increased hemolytic activity when coexisting with aerolysin A (encoded by *aerA*) (133).

Aerolysin, identified in the early 1970s, is one of the best-characterized aeromonad virulence factors (134). It is initially produced as an inactive preproaerolysin form with a protein signal sequence that is subsequently eliminated upon translocation through the inner cytoplasmic membrane. Proaerolysin (52 kDa), the form without the signal sequence, is secreted as an inactive soluble monomeric or dimeric form (134). Subsequent removal of the C-terminal peptide (135) produces a mature form which binds to a glycosylphosphatidylinositol-anchored protein receptor on red blood cells or enterocytes (136). Once it is bound, aerolysin heptamers form aqueous β-

barrel channels into cell membranes, leading inevitably to cell death (137). In mice, *aerA* mutations in strains of *A. hydrophila* significantly impaired pathogenicity, resulting in >100-fold-higher lethal doses (138). Mechanisms of action can be attributed to its apoptotic induction in murine T cells, aiding in immune evasion (139), to endoplasmic reticulum calcium release within granulocytes through the G-protein signaling pathway (140), or to severe vacuolation of endoplasmic reticulum in enterocytes (141). Together, these observations indicate that aerolysin seems to be required for establishment as well as subsequent maintenance of systemic infections (141).

Enterotoxins

Aeromonads encode three major enterotoxins, namely, cytotoxic enterotoxin (Act), heat-labile cytotonic enterotoxin (Alt) and heat-stable cytotonic enterotoxin (Ast). Although numerous *Aeromonas* spp. carry these genes, their production is limited due to regulation dependent on growth conditions. Cytotoxic enterotoxin (Act) is a single-chain, cholesterol-binding, pore-forming 52-kDa toxin exhibiting cytotoxicity in red blood cells and intestinal epithelial cells (121, 142). As is typical for enteric pathogens, Act evoked a secretory fluid response in a rabbit ligated intestinal loop model and induced lethality in mice (143). Act production is growth condition dependent, with calcium elevating promoter activity, while glucose and iron induce attenuation (130).

Cytotonic enterotoxins primarily act on intestinal epithelium in a cholera toxin-like fashion, increasing cyclic AMP (cAMP) levels and prostaglandins (124). *Aeromonas* spp. contain a variety of cytotonic enterotoxins, including Alt and Ast, although these toxins differ in their cross-reactivity with cholera antitoxins (144). The heatstable enterotoxin of *A. veronii* and Ast of *A. dhakensis* cross-react with cholera antitoxin, whereas *A. hydrophila* heat-stable (15-to 20-kDa) and heat-labile (40-to 44-kDa) Alt enterotoxins lack this interaction. Immunization with heat-labile Alt (56°C for 10 min) reduced fluid secretion after *A. hydrophila* infection in a mouse ligated ileal-loop model (144). Clinically, 23 of 31 aeromonad isolates from a foodborne outbreak after consumption of raw, fermented fish encoded Alt (145). Similar to Alt, the heat-stable (100°C for 30 min) cytotonic enterotoxin Ast increased cAMP levels (146). Together, these two toxins act synergistically, strongly impacting the overall virulence of aeromonad-induced gastroenteritis (147).

Shiga-like toxins (encoded by s*tx1* and *stx2*), which were analyzed in only a few studies, have been identified in over 60% of clinical strains of aeromonads, with 64 of 66 strains carrying at least one *stx* gene. Coculturing cell-free aeromonad supernatants with neutralizing STX1 or STX2 antibodies reduced or completely inhibited cytotoxic phenotypes in Vero cells, thereby establishing their pathogenic role (148). However, other studies revealed a substantially lower prevalence of *stx* genes among *Aeromonas* isolates (149), suggesting that additional studies are needed.

Biofilms

Biofilms are a major problem in the food industry because of their resilience to disinfectants. Their development consists of three steps: (i) irreversible microbial adhesion to a surface or substrate; (ii) proliferation with extracellular polysaccharide production and subsequent genomic regulation, progressing to three-dimensional biofilm maturation; and (iii) dispersal of biofilm. Foodrelated environmental stresses can influence biofilm formation: *A. hydrophila*, *A. caviae*, and *A. veronii* produce substantially more biofilms in colder (10°C compared to 37°C) and more acidic (pH ~5) environments. Food preservatives, such as 0.4% potassium sorbate, significantly increase biofilm formation as well (67). Once a mature biofilm is formed, it becomes more resilient to desiccation, starvation, and disinfectants. The ability to form a biofilm on stainless steel in a food processing facility may threaten the entire food stock associated with the factory. *Aeromonas* spp. have been isolated from a slaughterhouse and a cheese-making factory, where they synthesized

biofilms on stainless steel disks (150), both at room temperature (20°C) and at 4°C, with *A. caviae* having the greatest resistance to treatment with amphoteric surfactants and diamines (86).

When a rabbit ileal-loop method was used, human diarrheal isolates of *A. caviae* were shown to produce thick biofilms within the ileal and colonic mucosa. Initial adherence was mediated via lateral flagella, with subsequent bacterial aggregation leading to biofilm formation, which strengthens tissue adherence, traps more nutrients, accumulates more bacteria, attenuates phagocytic activation and migration by masking pathogenassociated molecular patterns, and alters virulence gene regulation through quorum sensing.

Secretion Systems

Due to the difficulty of crossing two phospholipid bilayers in Gram-negative bacteria, some bacteria have evolved multiple secretion systems that transport virulence factors outside the cell. These proteins are involved in a diverse array of activities, including host immune evasion, invading mammalian cells (potentially resulting in tissue damage), injecting proteins into other bacteria or eukaryotic cells, and adapting to and acquiring nutrients from the environment. *Aeromonas* spp. possess type II, type III, type IV, and type VI secretion systems (T2SS, T3SS, T4SS and T6SS), although there is species variation (151). These secretion systems are required for the release of many of the virulence factors mentioned in this chapter.

Proteins secreted via T2SS must first pass through a Sec or Tat secretion system in the inner membrane. Once in the periplasm, folded virulence factors are passed through the T2SS to its extracellular space (152). *Aeromonas* T2SS secrete aerolysin, the aerolysin-related cytotoxic enterotoxin Act, amylases, DNases, and proteases (151).

Unlike T2SS, T3SS has an injectisome that secrets proteinaceous substrates from the bacterial cytoplasm to the eukaryotic cytoplasm, making it possible for them to cross the inner and outer membranes of bacteria and the eukaryotic cell membrane. There is a high prevalence of T3SS among clinical *Aeromonas* isolates (153), although T3SS is regulated by environmental factors such as the host cell, temperature, and quorum sensing. Mutations within T3SS subunits result in attenuated pathogenic phenotypes. Virulence factors secreted through T3SS include the ADP-ribosylating toxin AexT, the actin disrupter and apoptosis inducer AopO, the inflammatory suppressors AopN and AopP, and the pore formers AopB and AopD (154). Other T3SS-secreted proteins of unknown virulence status include the putative tyrosine phosphatase AopH, inositol polyphosphate 5-phosphatase (Ati2), the putative serine/threonine kinase AopO, and the regulator of T3SS genes ExsE (154). ExsE upregulates lateral flagella; thus, it is involved in host cell adhesion (155).

Similar to T3SS, the T4SS apparatus can transfer molecules through bacterial and eukaryotic cell membranes; however, it is ancestrally related to bacterial conjugation and thus is capable of transferring plasmid DNA containing antibiotic resistance and virulence genes as well as protein. T4SS are unique secreting proteins that remodel host cells to aid in bacterial survival and replication, as well as uptake or transfer of DNA in a contact-dependent or -independent manner (152). They have been identified only in *A. veronii* and *A. caviae*, with the latter having been obtained from hospital effluent (98).

T6SS nanostructures, like T3SS, are more evident in clinical strains than environmental isolates, suggesting a role in virulence (153); however, similar to all of the secretion systems discussed above, they are not obligatory for disease. Additionally, many strains do not carry all 13 subunits needed for a functional T6SS apparatus. Their effector proteins target a range of different molecules, including peptidoglycan on other bacteria, lipases, amidases, glycoside hydrolases, pore-forming toxins, and proteins aiding metal ion uptake. T6SS aids cell-cell communication, therefore playing a role in biofilm formation and responsiveness to quorum sensing (156).

The critical role of secretion systems in disease highlights them as potential targets for new alternative treatments inhibiting them, such as curcumin (157).

Other Virulence Factors

Unique to Gram-negative bacteria, lipopolysaccharide consists of a core lipid A structure and O-specific polysaccharide. The lipid A portion acts as an endotoxin, eliciting local inflammation, which can lead to sepsis (124), whereas O antigen is an important colonizing factor and the basis for *Aeromonas* sp. serotyping (151). Some strains of *A. hydrophila* (e.g., serovars O:11 and O:34) and *A. salmonicida* are capable of capsule production, but only under certain conditions, such as glucose enrichment (158, 159). These capsules serve as virulence factors due to their complement resistance, ability to aid in adherence and biofilm formation, and ability to mask potential cell wall receptor ligands, thereby promoting antiphagocytic activity (158). Additionally, psychrophilic as well as mesophilic aeromonads produce a surface protein layer (S-layer) augmenting similar virulence phenotypes (124).

Iron acquisition is essential for both the host and pathogen. Inside the host organism, most iron is sequestered intracellularly and is bound within iron-carrying proteins or in metal-protein complexes. To overcome iron starvation, mesophilic *Aeromonas* spp. produce two types of low-molecular-weight peptides called siderophores to acquire free iron within the host organisms. These are enterobactin (also produced by other Gramnegative bacteria) and amonabactin (found only in aeromonads) (124). Psychrophilic strains, in contrast, utilize iron-specific outer membrane receptors in a siderophore-independent manner (160).

CONCLUSIONS

Aeromonas as a genus represents many challenges for food and medical microbiologists, diagnostic laboratories, and clinicians. Their ubiquitous nature, adaptation to stressful environmental conditions, ease of acquiring antibiotic resistance, and possession of various virulence factors make *Aeromonas* spp. important contaminants and opportunistic pathogens. Although there has been some debate in the past as to whether *Aeromonas* spp. are true enteropathogens, this chapter provides support for this role by noting many gastroenteritis outbreaks from water and a variety of foods, as well as describing many virulence factors that have been characterized through animal models. Food and water are the two major sources of contamination that pose a risk of acquiring gastrointestinal infections.

Pathogenic strains of *Aeromonas* have been obtained from many foods, including milk, chicken, and seafood. Their contamination is not only a public health concern, because of the potential for acquiring a gastrointestinal infection, but also a food spoilage concern, because of their production of lipases and proteases, which substantially shorten food shelf life. The persistence of aeromonads on refrigerated and frozen foods challenges this type of food preservation. Additionally, this microbe's ability to form biofilms provides an additional level of resistance to disinfectants, desiccation, and starvation. Within food processing environments, aeromonad biofilms that develop on surfaces can also serve as sources of recontamination.

The natural habitat of *Aeromonas* spp. is the aquatic environment, including freshwater, seawater, and brackish waters. Contaminated drinking water, especially when untreated, is the most direct source of infection. However, the resilience of aeromonads to low levels of chlorine and their ability to form biofilms within water treatment systems pose challenges to maintaining safe drinking water. Their ubiquitous presence in rivers, lakes, and seawater has an indirect effect on foods, where psychrophilic species can cause disease in fish in aquaculture or their native environment, thus adversely affecting food economics.

Whether the organisms are present in food or water or in human microflora, the antibiotic resistance acquired by aeromonads merits special attention. An example is when members of the genus *Aeromonas* acquired chromosomal β-lactamase genes, resulting in resistance to the broadspectrum antibiotic ampicillin, now used as a selective agent. The widespread occurrence of aeromonads establishes them in environments (e.g., wastewater treatment facilities, aquaculture, and mammalian gut microbiota) containing subinhibitory antibiotic concentrations, providing ideal conditions for horizontal gene transfer, including intergenus transfer. Prophylactic and clinical overuse of antibiotics in aquaculture, agriculture, and human medicine has led to *Aeromonas* strains becoming resistant to antibiotics of last resort, such as colistin (110).

For all of these reasons, more research is needed to further elucidate the pathogenicity and foodborne-illness potential of *Aeromonas* spp. More effective practices for treating food and water to reduce aeromonad contamination and proper identification from sources of infection and their corresponding clinical samples are examples of future research needs.

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16
Yersinia enterocolitica

Yersinia enterocolitica is a Gram-negative, non-sporeforming, non-toxin-producing, facultatively anaerobic, rod-shaped bacterium. Its growth temperature ranges from −2 to 42°C, and it is classified in the family *Enterobacteriaceae*. Only three *Yersinia* species (*Yersinia pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*) are pathogenic for humans, and the distribution of *Y. enterocolitica* in the environment is diverse (Fig 16.1). Foodborne *Yersinia* species are transmitted to humans by the oral route and can cause intestinal symptoms, such as abdominal pain, diarrhea, and fever. They are found worldwide, with a higher prevalence in countries with temperate and cold climates (1).

CELL METABOLISM

Y. enterocolitica is motile at 25°C but loses its motility at 37°C. It is a facultative anaerobe that anaerobically uses glucose as an energy source through fermentation:

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 + 2ATP$$

Most strains also reduce nitrates. When they are artificially cultured in laboratory settings, lactose fermentation is inhibited by the addition of bile salts in some growth media. When cultured in triple sugar iron medium, *Y. enterocolitica* produces urease but loses its ability to produce hydrogen sulfide (2). Coenzyme B12 is needed to break down 1,2-propanediol for carbon and energy utilization, and it is produced only anaerobically (3). *Y. enterocolitica* uses iron as a growth factor without producing a chelator, which is usually

necessary for the process. Most prokaryotic microorganisms acquire iron through an iron chelator known as a siderophore. *Y. enterocolitica*, however, uses siderophores from other bacteria for its iron uptake process. For example, it uses deferoxamine that is produced by *Streptomyces pilosus*. The pathogenicity of *Y. enterocolitica* is positively correlated with iron utilization. The need for iron varies among different strains of *Y. enterocolitica*, which affects their degree of virulence (2).

GENOME STRUCTURE

Y. enterocolitica strain 8081 has been sequenced and determined to have a circular DNA chromosome of 4,615,899 bp. The number of coding sequences is 4,037, with 7 rRNA operons and 81 tRNAs. The GC content of the chromosome is 47.27%. The average gene size is 968 bp, and the chromosome contains 60 insertion sequence elements and 4 prophage regions. In addition to the chromosome, *Y. enterocolitica* has four plasmids, i.e., pYVe8081, p29807, pYVa127/90, and pYVe227, which are 67,721, 2,682, 66,591, and 69,673 nucleotides, respectively, in length (3). *Y. enterocolitica* needs both the chromosome and a plasmid to be pathogenic, because the chromosome and the pYV pathogenic plasmid contain genes necessary for virulence. The virulent strains contain plasmids that carry genes for the low-Ca21 response, which is needed for pathogenicity (4). The unique features that distinguish the *Y. enterocolitica* genome from genomes of all other *Yersinia* species are the coenzyme B12 biosynthetic (*cbi*) and 1,2-propanediol degradation (*pdu*) genes. These gene clusters are located on a genomic island that is about 40 kb in size.

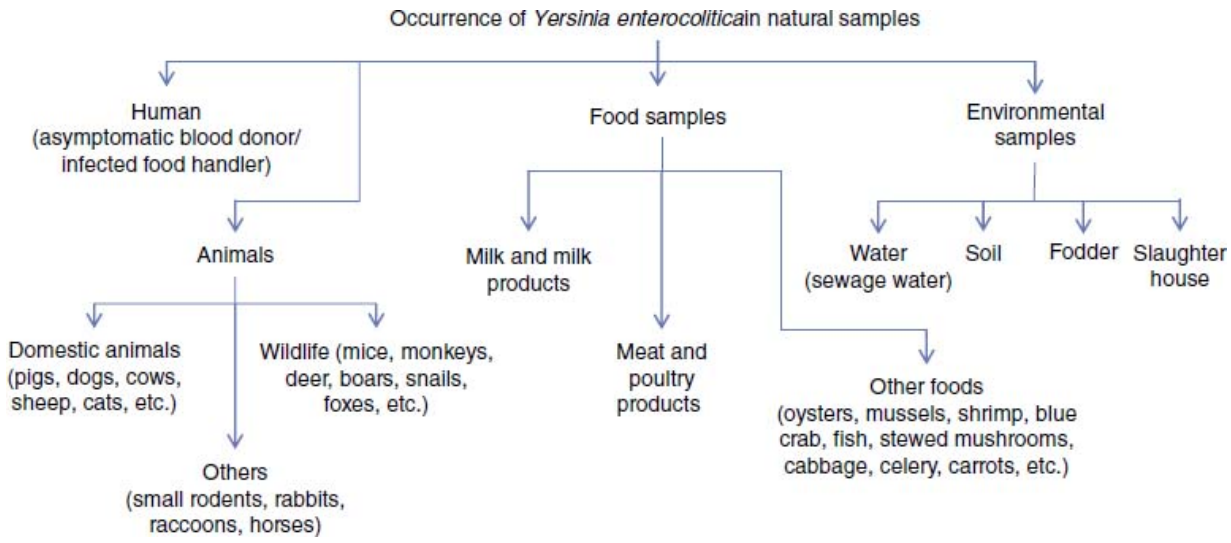


Figure 16.1 Sources of *Yersinia enterocolitica*. Adapted with permission from reference 23.

ASSOCIATION OF *Y. ENTEROCOLITICA* WITH FOODS

Food is a known source of *Y. enterocolitica* infection, although pathogenic isolates have seldom been obtained from food samples, with the exception of raw pork products. The low rates of isolation of pathogenic *Y. enterocolitica* from food samples are due to the limited sensitivity of culture methods, which have been largely used to obtain yersiniae from foods (5). PCR methods, which have not been used in many studies, have higher rates of *Y. enterocolitica* detection in raw pork products than culture methods (5). However, the isolation of pathogenic bioserotypes of *Y. enterocolitica* from raw pork is not common, except for edible pig offal, with the most common isolate being bioserotype 4/O:3 (Table 16.1.). Most studies addressing the detection and isolation of *Y. enterocolitica* in foods have focused on pig meat products (Table 16.1.). More studies are needed to determine the association of *Y. enterocolitica* with produce, because produce is grown in soil, and *Y. enterocolitica* can survive in soil (Fig 16.1). Soil, insects, animals, and humans can frequently come into contact with fruits and vegetables during their

growing, harvesting, and processing stages. Hence, their surfaces can be contaminated with soil-borne microbes such as *Y. enterocolitica*.

YadA is a collagen-binding outer membrane protein adhesin factor of *Y. enterocolitica*. Fredriksson-Ahomaa and Korkeala (6) determined the prevalence of *yadA*positive *Y. enterocolitica* in food in Finland, finding the highest prevalence in pig offal, including pig tongues (83%), livers (73%), hearts (71%), and kidneys (67%). Interestingly, the rate of detection in minced meat was greater with the PCR method than with the culture method (Table 16.1.). Ail is also an outer membrane protein adhesin factor of yersiniae. Thisted Lambertz and Danielsson-Tham (7) detected *ail*-positive *Y. enterocolitica* in 10% (9/91) of raw pork samples (loin, fillet, chop, ham, and minced meat) and in one of 27 ready-to-eat pork products. Vishnubhatla et al. (8) detected a high prevalence of *yst* (heat-stable enterotoxin)-positive *Y. enterocolitica* in ground beef. In the same study, *yst*-positive *Y. enterocolitica* was also detected in tofu by real-time PCR. These PCR results reveal that the rate of contamination of *Y. enterocolitica* in pork and other processed meats and foods is underestimated by culture methods. No pathogenic *Y. enterocolitica* was detected in fish or chicken samples in Finland but was detected in three (3%) lettuce samples. In Korea, Lee et al. (9) isolated one *ail*-positive *Y. enterocolitica* strain of bioserotype 3/O:3 from 673 ready-to-eat vegetables, revealing that vegetables may be a source of human infection. Sakai et al. (10) described a *Y. enterocolitica* O:8 outbreak in Japan in which the isolates from patients and salad had a similar pulsed-field gel electrophoresis pattern. Interestingly, Favier et al. (11) isolated *Y. enterocolitica* 2/O:9 from chicken eggshell surfaces in Argentina. The XbaI patterns from pulsed-field gel electrophoresis analysis of these isolates revealed genomic heterogeneity among the strains, suggesting that contamination came from different sources. Contamination of the egg surface might have occurred from direct or indirect contact with feces of other *Y. enterocolitica*-contaminated farm animals, such as pigs,

during collection of the eggs on farms, during transportation, or via handling in retail shops.

Table 16.1 Detection of pathogenic *Y. enterocolitica* in naturally contaminated samples using PCR and culture methods[a]

		No. (%) of samples		
Sample	Total	Culture positive[b]	PCR positive	Reference(s)
Animal				
Pig tonsils	185	48 (26)	58 (31)	80, 81
	252	0	90 (36)	82
	24	15 (63)	18 (75)	83
	829	411 (50)	0	19
	630	278 (44)	0	35
	212	72 (34)	186 (88)	84
Pig feces	255	0	80 (31)	82
	24	3 (13)	3 (13)	83
	2,793	114 (4)	345 (12)	85
	150	3 (2)	0	24
Mesenteric lymph nodes	257	0	103 (40)	82
	24	1 (4)	2 (8)	83
Submaxillary lymph nodes	24	1 (4)	3 (13)	86
Sheep feces	200	2 (1)	0	24
Dog feces	448	0	6 (1)	87
Food[c]				
Pig tongues	15	7 (47)	10 (67)	8
	99	79 (80)	82 (83)	6
Pig offal[d]	110	38 (35)	77 (70)	86

Chitterlings	350	8 (2)	278 (79)	82
Ground pork	350	0	133 (38)	86
	100	32 (32)	47 (47)	8
Ground beef	100	23 (23)	31 (31)	86
Minced pork	255	4 (2)	63 (25)	6
Pork[e]	300	6 (2)	50 (17)	88
	91	6 (7)	9 (10)	7
	62	0	20 (32)	89
Chicken	43	0	0	6
Fish	150	0	0	24
Heated soup	100	3 (3)		24
Cow milk	250	3 (1)		24
Lettuce	250	0	3 (3)	24
Tofu	50	0	6 (12)	8
Vegetables	27	1 (4)	4 (15)	90
Salad	42	16 (38)	16 (38)	10
Environment				
Water	105	1 (1)	11 (10)	91
Slaughterhouse/farm	89	5 (6)	12 (13)	66
	46	44 (96)	0	19
	45	31 (61)	0	35

[a] Adapted from reference 12.

[b] Pathogenicity of isolates confirmed.

[c] All meat samples were raw.

[d] Liver, heart, and kidney.

[e] Except pig offal and tongues.

FACTORS INFLUENCING SURVIVAL AND GROWTH

Y. enterocolitica is a facultatively anaerobic bacterium, having the ability to multiply under both aerobic and anaerobic conditions. The bacterium can survive in water at 4°C for up to 64 weeks and at freezing temperatures (12).

Temperature

The ability of *Y. enterocolitica* to multiply at low temperatures is a food safety concern. Its growth temperature range is from −2 to 42°C, with an optimum growth temperature of 28 ± 1°C (12). *Y. enterocolitica* can multiply at refrigeration temperature considerably more rapidly than *Listeria monocytogenes* (12). For example, in a food at neutral pH held at 5°C, *Y. enterocolitica* populations can increase from 10 CFU/ml to 2.8×10^7 CFU/ml in 5 days. Examples of thermal inactivation rates (D_T, where D is time and T is temperature) for *Y. enterocolitica* are as follows: $D_{55°C}$ = ~2 min, $D_{60°C}$ = ~0.5 min, and $D_{65°C}$ = ~2 s.

pH

The minimum pH for growth of *Y. enterocolitica* is 4.2 ± 0.2 at 22°C (12). The presence of organic acids reduces the ability of *Y. enterocolitica* to grow at low pH, and certain organic acids can completely inhibit growth at a low pH at which other less restrictive acids allow growth to occur. For example, acetic acid is more inhibitory than lactic and citric acids per gram-mole at a given pH, and the order of bactericidal activity of these acids is as follows: acetic acid > lactic acid > citric acid > sulfuric acid.

Water Activity

The minimum water activity (a_w) at which *Y. enterocolitica* can grow is 0.96. Salt (NaCl) can limit or prevent growth or inactivate

the bacterium. For example, *Y. enterocolitica* can grow in 5% salt but not in 7% salt, depending on the incubation temperature. At <3°C, 7% NaCl is bactericidal to the pathogen, and at 25°C it is bactericidal or bacteriostatic. The pathogen is inactivated in 9% NaCl at 25°C (12).

Preservatives and Growth Inhibitors

Potassium sorbate at pH 6.5 inhibits the growth of *Y. enterocolitica* in a dose-dependent manner up to 5,000 ppm, and concentrations of >1000 ppm at pH 5.5 completely inactivate yersiniae. Sodium nitrite at 150 ppm inhibits growth of *Y. enterocolitica* on bologna. Modifiedatmosphere packaging with 100% N_2 and a combination of gases (e.g., CO_2-N_2) inhibits yersiniae growth during storage at refrigeration temperature (12).

Y. enterocolitica can grow in vacuum-packed foods during refrigerated storage. However, it does not survive pasteurization or normal cooking, including boiling, baking, and frying temperatures. Several studies have revealed that *Y. enterocolitica* can multiply in foods held at refrigeration temperature and can compete well with the natural microflora of foods (1). For example, *Y. enterocolitica* at 10^4 CFU/g grew in the presence of 10^4 to 10^5 CFU/g of lactic acid bacteria at 8°C in vacuumpackaged cooked ham and sausage, whereas these lactic acid bacteria inhibited the growth of *L. monocytogenes* and *Escherichia coli* in the same experimental studies. Interestingly, growth of *Y. enterocolitica* O:9 was greater under anaerobic than aerobic conditions at 4°C in the presence of 1% lactic acid at pH 3.9 to 5.8 (12).

Pig carcasses are often chilled in rooms for 2 to 4 days after slaughter and prior to cutting. In addition, prepackaged raw meat products may remain in retail chilling cabinets for more than a week, depending on the product, packaging, package atmosphere, and rate of turnover. Pathogenic variants of *Y. enterocolitica* can propagate during this relatively long storage period. The growth of *Y. enterocolitica* is influenced by the package atmosphere. Under

anaerobic conditions, *Y. enterocolitica* is unable to grow in beef at pH 5.4 to 5.8, whereas growth can occur anaerobically at pH 6.0 (12). In 100% CO_2, *Y. enterocolitica* growth is inhibited. In vacuum-packed meat, *Y. enterocolitica* grew at all storage temperatures more rapidly than the spoilage microflora. In CO_2 packs, the bacteria could grow at both 5 and 10°C but not at lower temperatures. Growth of *Y. enterocolitica* was inhibited at both 4 and 10°C in a 60% CO_2–0.4% CO mixture, whereas the numbers of yersiniae in samples packed in a high-O_2 mixture (70% O_2–30% CO_2) increased from ca. 5×10^2 CFU/g at day 0 to 10^4 CFU/g at day 5 at 4°C and to 10^5 CFU/g during storage at 10°C. Growth in chub packs (stuffed in plastic castings) was even greater. Yersiniae inoculated into pasteurized milk at 10 or 1,000 CFU/ml grew to levels of 5.0 to 7.0 log CFU/ml after 7 days (12) at refrigeration temperature (13).

YERSINIOSIS

Yersiniosis is an infection largely associated with eating raw or undercooked pork contaminated with *Y. enterocolitica*. In the United States, *Y. enterocolitica* is estimated to cause approximately 117,000 illnesses, 640 hospitalizations, and 35 deaths annually (14). *Y. enterocolitica* infections have been observed on all continents, but they appear to be most prevalent in Europe (68). Foodborne outbreaks of yersiniosis are rare, and most occur sporadically withoutan apparent source (15). Cases of yersiniosis are sometimes severe, leading to prolonged acute infections, pseudoappendicitis, and long-term sequelae, such as reactive arthritis (ReA) and erythema nodosum. Secondary immunologically induced sequelae, such as ReA, are common, especially in HLA-B27-positive individuals. The prevalence of *Y. enterocolitica* O:3/O:9-specific antibodies in healthy blood donors is relatively high in Finland (31%) and Germany (43%), as determined by an immunoblotting technique (16, 17), suggesting a high rate of subclinical yersiniosis in healthy populations. Gastrointestinal symptoms of yersiniosis range from mild self-limiting diarrhea to acute mesenteric

lymphadenitis, which can lead to appendicitis (18, 19). Gastroenteritis caused by *Y. enterocolitica* is the most frequent form of yersiniosis, typically affecting infants and young children. The clinical manifestations of infection depend to some extent on the age and physical state of the patient, including the presence of underlying medical conditions and the biotype or pathotype of the organism. In older children, acute yersiniosis can manifest as a pseudoappendicular syndrome and is frequently confused with appendicitis. Sometimes it is followed by long-term sequelae, including ReA, erythema nodosum, uveitis, glomerulonephritis, and myocarditis. Sepsis is a rare complication of *Y. enterocolitica* infection and usually occurs during blood transfusions, especially in patients with predisposing underlying disease, as seen mostly in an iron-overloaded state (20).

Foodborne Yersiniosis

Foodborne yersiniosis is primarily a zoonotic gastrointestinal disease, like campylobacteriosis and salmonellosis, but is not as frequently reported (21). In Europe, outbreaks of yersiniosis are rare, but sporadic cases attributed to *Y. enterocolitica* are often reported (22). In England and Wales, the number of reported cases of yersiniosis in humans is lower than in other European countries (23). In 2005, fewer than 0.1 case of yersiniosis per 100,000 individuals was reported in the United Kingdom, compared to 12.2 cases in Finland and 6.8 cases in Germany (21). A higher prevalence of gastrointestinal illness, including fatal cases of yersiniosis, has been reported in developing countries, suggesting the need for further investigation of this bacterium as a food safety issue worldwide (24, 25).

Although pathogenic strains of *Y. enterocolitica* have infrequently been isolated from foods, foodborne outbreaks of yersiniosis have occurred (26). An important property of this bacterium is its ability to multiply at temperatures near 0°C, which allows it to survive and grow in many chilled foods. Sabina et al. (27) reported that different serotypes predominate in different countries. For example, in

China, serotype O:3 is primarily associated with infections, followed by O:9 and O:8, whereas the predominant serotype in Australia, Europe, and Canada is O:3, that in Japan is O:8, and that in Scandinavia and the Netherlands is O:9.

Epidemiologic Studies, Outbreaks, and Transmission

Indirect evidence suggests that swine are an important reservoir of *Y. enterocolitica* and the principal source of human infections. In case-control studies, a correlation between the consumption of raw or undercooked pork and the prevalence of yersiniosis has been reported (12, 15, 23, 28, 29). Serotyping methods have been used to subtype *Y. enterocolitica* strains to identify sources of infections and transmission vehicles (Table 16.2.).

In addition, several methods have been used to detect and obtain *Y. enterocolitica* from the environment (Fig 16.2). Conventional and molecular detection methods are the two most widely reported for epidemiologic studies. However, the most commonly used methods have been conventional methods, which are dependent on culture-based procedures and biochemical and serological identification. The efficacy of these methods depends on certain substrates in the assay media which can influence survival and growth, isolation, and identification (Fig 16.2). Thus, many factors related to the epidemiology of yersiniosis, such as sources and transmission routes, need further elucidation using more robust methods. Although animals, especially swine, have been identified as reservoirs for *Y. enterocolitica* and, hence, have been reported as principal sources of human infections (15, 23), most of the strains isolated from many animal sources differ both biochemically and serologically from strains isolated from humans with yersiniosis. *Y. enterocolitica* strains that belong to bioserotypes associated with human disease have frequently been isolated from the tonsils and fecal samples from slaughtered pigs. In several countries, *Y. enterocolitica* bioserotype 4/O:3, a pathogenic bioserotype, is the predominant bioserotype in asymptomatic pigs. Occasionally, bioserotype 4/O:3 has been isolated from dogs and cats (30), and

dogs harbor and excrete this *Y. enterocolitica* bioserotype in their feces for weeks after infection, suggesting that dogs and cats may be sources of human infections. *Y. enterocolitica* strains of biotypes 2 and 3 and serotypes O:5, 27, and O:9 have sporadically been isolated from slaughtered pigs, cows, sheep, and goats; however, the reservoir of these bioserotypes is not definitively established (31–33). In addition, wild rodents and pigs have been shown to be reservoirs for *Y. enterocolitica* O:8 strains in Japan (34).

Table 16.2 Epidemiologic studies of human infection with *Y. enterocolitica*[a]

Year(s)	Country	Description	Reference
1981–1990	Republic of Georgia	Report of 84 clinical isolates of *Y. enterocolitica*; the most frequently reported serotypes were O:5, O:10,46, and O:6,30.	92
1982–1991	The Netherlands	Analysis of clinical information from 261 Dutch patients with gastrointestinal infections caused by *Y. enterocolitica* serotypes O:3 and O:9	93
1982[b]	Canada	Outbreak of gastroenteritis among hospitalized patients associated with *Y. enterocolitica* serotype O:5	94
1982–1985	Canada	Examination of 125 isolates of *Y. enterocolitica*; serotypes O:7,8, O:5, and O:6,30 were frequently obtained from symptomatic patients.	95
1983	Finland	Report of 46 fecal isolates of *Y. enterocolitica*, including two serotypes (O:7 and O:6) associated with occurrence	77
1984[b]	Bangladesh	Case report of a fatal diarrheal illness associated with serotypes O:7 and O:8	96
1984[b]	Hong Kong	Report of *Y. enterocolitica*-associated septicemia in four patients regarding serotypes O:17	97
1984–1985	United Kingdom	Report of two nosocomial outbreaks of *Y. enterocolitica*	98

		serotype O:10 and O:6 infections in hospitalized children	
1986[b]	United Kingdom	Case report of nosocomial transmission of serotype O:6,30 associated with gastroenteritis	99
1986–1992	Canada	Report of 79 symptomatic children with culture-proven infection, including serotypes O:5, O:6,30, and O:7,8	100
1987	United Kingdom	Report of 77 *Y. enterocolitica* strains from patients, including serotypes O:6,30 and O:7	101
1987–1988	Australia	Report of 11 cases of *Y. enterocolitica* enteritis, including most frequently serotype O:6,30	102
1987–1989	Chile	Prospective case-control study of infants with diarrhea in Chile, showing a significant rate of reported serotypes O:6, O:7,8, O:7, and O:10	103
1988–1991	Nigeria	Of nine strains of *Y. enterocolitica* obtained from stool samples of children with diarrhea, six belonged to biotype four, serotype O:3.	104
1988–1993	New Zealand	Of 918 isolates of *Y. enterocolitica* from symptomatic patients, 98% were recognized human pathogens, with biotype 4, serotype O:3 being the most frequently recovered.	105
1968–2000	Brazil	Of 106 strains, 71 were bioserotype 4/O:3, isolated from human and animal clinical material, and 35	106

		were biotype 1A or 2, isolated from food.	
2002	Iran	Report of 8 cases of *Y. enterocolitica* infection out of 300 children with acute diarrhea aged 0–12 years who were attending a pediatric hospital in Tehran	107
2002–2004	Nigeria	Detection of *Y. enterocolitica* belonging to bioserotype 2/O:9 in investigating 500 human samples	24
2004	Japan	Report of food poisoning due to *Y. enterocolitica*	
2005–2006	Norway	Investigation of an outbreak involving 11 persons infected with *Y. enterocolitica* O:9	89
2001–2008	Germany	Almost 90% of *Y. enterocolitica* strains were diagnosed as serotype O:3.	108
2009[b]	Iraq	Identification of three children with diarrhea caused by *Y. enterocolitica* infection	25
2009	Australia	Report of 3 outbreaks due to consumption of roast pork contaminated with *Y. enterocolitica*	109

[a] Adapted from reference 23.

[b] Year(s) of publication.

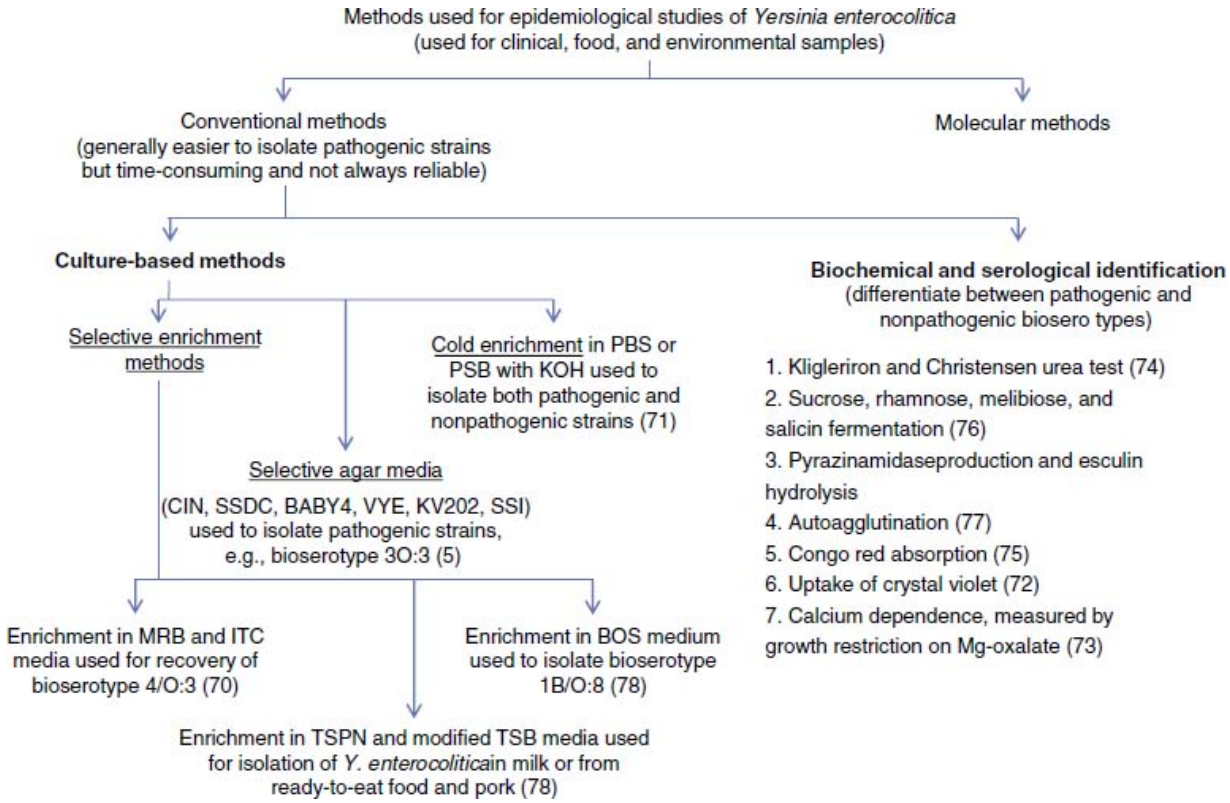


Figure 16.2 Methods used for epidemiological studies of *Y. enterocolitica* 1. Numbers in parentheses are reference numbers. Selective enrichment methods (70), selective agar media (5), a cold enrichment method (71), and biochemical and serological identification methods (71–78) are listed. BOS, bile-oxalate-sorbose medium; CIN, cefsulodin-irgasan-novobiocin; ITC, modified Rappaport base supplemented with irgasan, ticarcillin, and potassium chlorate; MRB, modified Rappaport broth containing magnesium chloride, malachite green, and carbenicillin; PBS, phosphate-buffered saline; PSB, phosphate-buffered saline with sorbitol and bile salts; SSDC, *Salmonella-Shigella* deoxycholate calcium chloride; SSI, Statens Serum Institut (Copenhagen, Denmark) enteric medium; TSB, tryptic soy broth; TSPN, TSB with polymyxin and novobiocin; VYE, virulent *Y. enterocolitica*. Adapted with permission from reference 23.

Strains of very rare bioserotypes, such as bioserotype 5/O:2,3, were isolated from sheep, hares, and goats, and bioserotype 3/O:1,2a,3 has been isolated from chinchillas (35). All of the environmental

isolates except one had a NotI profile identical to that of an isolate recovered from pig feces from the same farm. This suggests that the environment also represents a vehicle of *Y. enterocolitica* contamination from pigs. However, the prevalence of pathogenic *Y. enterocolitica* in the environment was lower than that in pigs. Several studies using different typing methods have been conducted to compare human strains with animal, mostly swine, isolates. Most of the results reveal that pigs are the main source of human *Y. enterocolitica* infections (31, 33).

VIRULENCE FACTORS

Y. pestis and *Y. pseudotuberculosis* have nearly identical chromosomal DNA sequences and are distantly related to pathogenic *Y. enterocolitica* (36, 37). Among the 11 species that make up the genus *Yersinia*, only *Y. pestis*, *Y. enterocolitica*, and *Y. pseudotuberculosis* are pathogenic for humans. These three species cause quite diverse diseases (37). *Y. enterocolitica* and *Y. pseudotuberculosis* induce gastroenteritis when consumed in contaminated food and have been isolated from patients with diarrhea. *Y. pestis* is the agent of bubonic plague and can cause oropharyngeal plague as a result of the consumption of inadequately cooked infected goat and camel meat or handling of meat from infected animals (15, 38, 39). The risk, morbidity, and mortality of contracting plague through consumption of food deliberately contaminated with *Y. pestis* are currently unknown but potentially real.

Y. pestis, *Y. enterocolitica*, and *Y. pseudotuberculosis* possess a range of virulence factors that aid attachment to host cells or tissues, causing dysfunction of host cells. Several researchers have reported that three plasmids are involved in the virulence of *Y. pestis*: pYV (virulence plasmid, 70 kb; Yops, or type III secretion system [T3SS]), pFra/pMT1 (96.2 kb; murine toxin, phospholipase, or F1 capsule-like antigen), and pCP1/pPst/pPla (9.6 kb; plasminogen activator) (40, 41). Among these plasmids' products,

the pYV-encoded T3SS (Yops) promotes cytotoxicity, leading to the common symptoms of plague, whereas the pYV plasmids of all three species are of the same size and genetically highly conserved (41). Once injected into the host cell cytoplasm, YopE, -H, -P, and -T cooperatively disrupt the cytoskeleton of epithelial cells, macrophages, and dendritic cells, thereby decreasing their capacity to engulf the invading bacteria. YopP/J can also facilitate evasion of adaptive immune responses by inhibiting the ability of dendritic cells to present antigens to $CD8^+$ T cells (42), either directly or possibly by decreasing the population of dendritic cells via induction of apoptosis (43–45). A similar strategy is employed by *Y. pseudotuberculosis* using the GTPase-activating protein YopE to circumvent phagocytosis by dendritic cells (42, 46). In addition to the *Yersinia* injectisome and effector proteins, at least three adaptor proteins—YopB, YopD, and VirF/LcrV (low-calcium-response V antigen)—are required for T3SS activity. VirF/LcrV (also called V antigen) is a multiple adaptational response family member that regulates the T3SS at the level of transcription and, when secreted into the extracellular host environment, contributes to virulence by downregulating inflammation (47).

Yersinia infections are biphasic and are initiated by a “quiet” 36-to 48-hour period of bacterial replication without a measurable host response. This initial quiet phase is followed by an influx of activated phagocytes into infected tissues and lymph nodes, which induces an acute inflammatory response characterized by cytokine production and tissue necrosis (48–51). The T3SS Yop effectors are likely responsible for the initial inhibition of phagocytic functions, but the mechanisms affecting such a sudden, bipolar “off-on” inflammatory response are presently not fully understood. The T3SS is absolutely required for effective colonization of systemic organs, and T3SS inactivation leads to rapid clearance of the bacteria by the host (52). As a result, yersiniae lacking a functional T3SS are avirulent and can function as live attenuated vaccine strains in mice (52–54).

T3SSs are not the sole secretion systems identified in the yersiniae that promote bacterial virulence. A type VI secretion system (T6SS) was recently identified in *Y. pseudotuberculosis*, which harbors four copies, one of which is regulated by temperature, growth phase, and the *N*-acyl homoserine lactone (AHL)-dependent quorum-sensing system, including the type IV pilus gene cluster, which contributes to pathogenicity (55). However, more studies are needed to elucidate the period of bacterial replication in a host cell leading to the disease state.

The Yops are *Yersinia* outer proteins that provide yersiniae the ability to target lymph tissues during infection and have genetic determinants responsible for infection and overcoming host defense mechanisms (36, 41). YopH, a protein tyrosine phosphatase, disrupts T-cell and Bcell activation by interfering with phosphorylation signaling events, resulting in decreased expression of the costimulatory molecules B7.2 and CD69, as well as the leukocyte mitogen interleukin 2 (IL-2) (56). Very little is known about YopM, but its deletion results in a dramatic decrease in virulence (57). YopM appears to be injected into host cells along with other T3SS effector proteins, but there is also evidence that YopM can bind to the extracellular acute phase protein α 1-antitrypsin (58).

All human-pathogenic *Yersinia* species harbor an approximately 70-kb virulence plasmid, which carries a set of genes whose transcription is activated at 37°C in the presence of millimolar concentrations of calcium (59). Virulence factors found on the surface of *Y. pseudotuberculosis*, *Y. enterocolitica*, and *Y. pestis* are shown in Fig 16.3. All three pathogens share Ail, YadB, and YadC, which are important to adhesion. However, only Pla is unique to *Y. pestis*, whereas YadA and invasin are important adhesins in *Y. pseudotuberculosis* and are also invariably expressed by *Y. pestis*.

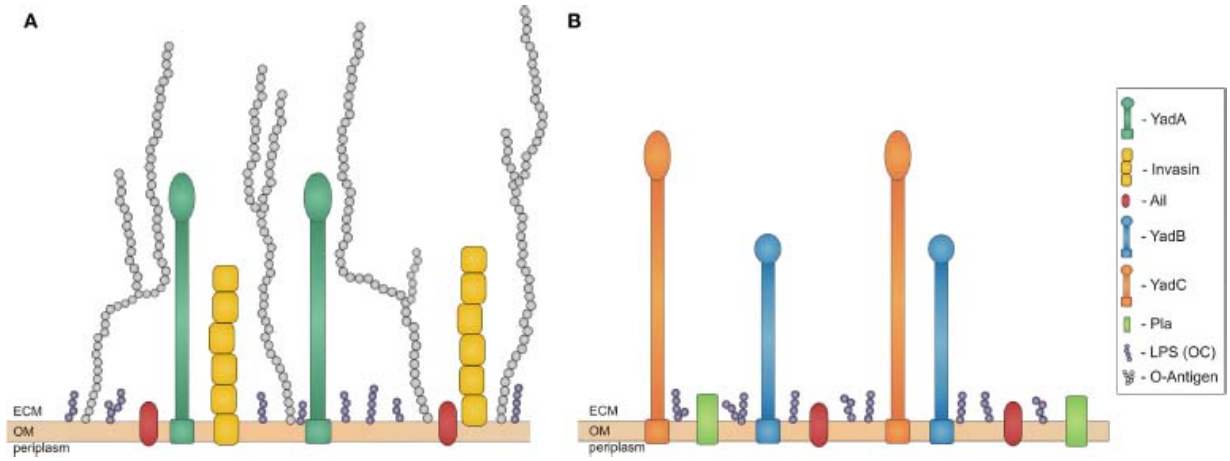


Figure 16.3 Virulence factors on the surface of *Y. pseudotuberculosis*, *Y. enterocolitica* (A), and *Y. pestis* (B). Invasin, YadA, Ail, and O antigen are shared by *Y. enterocolitica* and *Y. pseudotuberculosis* (YadB and YadC are absent from panel A for clarity), whereas Pla is unique to *Y. pestis*. YadA and invasin are important adhesins in *Y. pseudotuberculosis* and *Y. enterocolitica* but are not expressed by *Y. pestis*. Reprinted from reference 79.

Yersinia virulence determinants vary, but the most studied are those secreted via a T3SS. For yersiniae to efficiently deliver Yops into the host, it is essential that they adhere to the host cell surface and remain in close association during the delivery process. Apart from the T3SS virulence plasmid, two other plasmids that are unique to *Y. pestis*, pPCP and pMT (sometimes referred to as pFra), possess additional virulence factors. pPCP encodes the plasminogen activator Pla protease/adhesin (Fig 16.3). Pla converts plasminogen to plasmin (60), which then degrades extracellular matrices and confers on *Y. pestis* the ability to rapidly invade the host and migrate to lymphatic tissues (61). To ensure that this is possible, the yersiniae produce other virulence factors in addition to the T3SS. An active T3SS can deliver effector proteins into the host cell cytosol only if the bacterial cells make direct contact with, and bind tightly to, the host cell surface. Over the last 30 years, several chromosomally encoded or plasmid-encoded protein virulence factors have been identified that play a variety of roles in host cell attachment prior to effector protein injection. In each case,

attachment is not the exclusive function, and not all are present or active in all three human-pathogenic *Yersinia* species. However, a combination of these proteins confers the ability to adhere to and invade host cells or bind sufficiently to ensure successful T3SS delivery of Yops.

Invasin is a chromosomally encoded protein that mediates attachment to and entry into host cells by *Y. pseudotuberculosis* and *Y. enterocolitica*, although in *Y. pestis*, it is a pseudogene and therefore inactive (59). Invasin also promotes internalization in small intestine epithelial cells by binding to host cell target receptors known as β1-integrins that are present on the host cell surface. The integrins form clusters, resulting in the rearrangement of the host cell cytoskeleton, which promotes phagocytosis, ultimately leading to the internalization of the bacteria in the epithelial cells. Invasin has a greater (up to 100 times) affinity for some integrins than its natural ligand, fibronectin (59). The authors also reported that such strong associations are believed to be the major contributing factors influencing how internalization occurs and how Yop is delivered into host cells. Also, pYV is responsible for the calcium-dependent growth phenotype at 37°C. When pYV-bearing cells are cultivated in low-calcium or calcium-deficient media, they elicit a Mg^{2+}-dependent low-calcium response, resulting in the production of pYV-encoded virulence-associated antigens (LcrV and LcrW), including release of a series of proteins (Yops) (62). The low-calcium response is expressed phenotypically on solid media by the formation of pinpoint colonies (36, 41, 62). The pYVassociated virulence determinants include colony morphology and size, the low-calcium response, crystal violet binding, Congo red uptake, autoagglutination, hydrophobicity, mannose-resistant hemagglutination, expression of surface fibrils, and serum resistance (36, 41, 62). However, the expression of these physiological traits at 37°C also leads to the loss of pYV and the concomitant disappearance of the associated phenotypes.

MODE OF TRANSMISSION

The most common route for transmission of pathogenic *Y. enterocolitica* is fecal to oral via contaminated food. Direct person-to-person contact is not commonly recognized, but Lee et al. (63) reported *Y. enterocolitica* O:3 infections in infants who were likely exposed to infection by their caregivers. This may happen when basic hygiene and handwashing practices are inadequate. Indirect person-to-person transmission has been reported and occurs largely by transfusions of contaminated blood (20). However, transmission to a person having direct contact with pigs, a common risk factor for pig farmers and slaughterhouse workers, has also occurred. The main known sources of human infection are the consumption of contaminated pork and pork products. It has been established that pathogenic *Y. enterocolitica* can be transmitted from slaughterhouses to meat processing plants and then to the retail environment through contaminated pig carcasses and offal (64, 65). Cross-contamination of offal and pork can occur directly or indirectly via equipment and air and food handlers in slaughterhouses (66), retail shops (65), and residential kitchens.

The detection rate of pathogenic *Y. enterocolitica* in raw pork products is comparatively high, but because consumption of raw pork is not common in most developed countries, it is not likely a major direct source of yersiniosis. However, in Germany, raw minced pork with pepper and onion is considered a delicacy that can be purchased as a ready-to-eat product at butcher shops (86). This could be a source of infection if the product is contaminated. This is reinforced by a study in which the predominant genotype of *Y. enterocolitica* recovered from pig heart, liver, kidney, tongue, and ear samples was also found in a cat whose diet consisted mostly of raw pig hearts and kidneys (12). In addition, a dog which was fed raw minced pork excreted the genotype found in the minced meat (12). These findings further substantiate the idea that raw pork contaminated with *Y. enterocolitica* is a food safety concern.

CURRENT RESEARCH FINDINGS

Studies are under way to determine the effect of IL-12p40 on ReA, a chronic condition resulting from an infection with *Y. enterocolitica* (67, 68). IL-12 is a cytokine with two subunits, p40 being one of them. Mice with an IL-12p40 deficiency generally have defective immune systems. This research addressed the involvement of *Y. enterocolitica* antigens and Toll-like receptor (TLR) expression in causing ReA. TLR binds to portions of yersiniae that elicit arthritis. Understanding the mechanism of ReA development may enable the discovery of a treatment for this condition. It was determined that the Peyer's patches of IL-12p40-deficient mice were infected with substantially more yersiniae and contained higher levels of TLR than those of *Y. enterocolitica*-infected wild-type mice. These findings suggest that the absence of IL-12p40 contributes to ReA due to the inability of the host to remove *Y. enterocolitica* effectively (67, 68). The incomplete removal of bacteria triggers TLR expression, which leads to articular inflammatory response (69).

Another study attempted to determine if there was a relationship between the presence of food and the production of AHL, a quorum-sensing signal molecule, by *Y. enterocolitica*. Results revealed that AHLs are preferentially produced by *Y. enterocolitica* in fish and meat but inhibited in vegetables, suggesting that quorum sensing enables *Y. enterocolitica* to take up nutrients and to possibly reside in specific environments (69).

CONCLUSIONS

Y. enterocolitica is a zoonotic pathogen that can cause yersiniosis in humans and animals. Pigs are a main source of human yersiniosis. There have been challenges with associating *Y. enterocolitica* from clinical specimens with food and environmental samples. For example, because of the weak discriminating power of bioserotyping methods, which typically identify within a bioserotype a high degree of similarity between *Y. enterocolitica* isolates and the predominant

genotypes of *Y. enterocolitica*, such methods have limited usefulness in epidemiologic studies. Furthermore, conventional culture-dependent methods for isolating yersiniae from foods have several limitations, such as low sensitivity, long incubation times, and a lack of discrimination between pathogenic and nonpathogenic strains. With PCR-based assays, pathogenic *Y. enterocolitica* can be detected in natural samples relatively rapidly and with a high degree of specificity. Recently, several real-time PCR assays for qualitative detection of *Y. enterocolitica* in clinical, food, and environmental samples have been developed. However, to date, a PCR-based method has been applied in only a few reported epidemiologic studies.

Y. enterocolitica is the major cause of yersiniosis in humans, although *Y. pseudotuberculosis*-associated disease is likely underreported due to lack of surveillance and differences in the isolation strategies applied. Extreme heterogeneity among strains of *Y. enterocolitica* and *Y. pseudotuberculosis* further complicates efforts to link illnesses to the source and monitor human disease in a systematic manner comparable to that used for other, more thoroughly studied foodborne pathogens (e.g., *Salmonella*). In healthy individuals, the resulting illness can manifest as mild, self-limiting diarrhea, but in young children and immunocompromised individuals, yersiniosis can represent a significant source of morbidity and mortality. Additionally, chronic diseases, such as ReA, and secondary (or nosocomially derived) complications, such as sepsis, can develop in immunocompromised persons. Very little is known about yersinia-associated autoimmune disease and other chronic conditions. Two pathogenic strains (1B/O:8 and 3/O:9) of *Y. enterocolitica* have been sequenced, and their DNA sequences have been compared (3, 17) to gain insight into virulence mechanisms required to initiate infection and cause acute symptoms or chronic conditions in patients. Yersiniosis surveillance efforts concentrate almost exclusively on *Y. enterocolitica*, making attempts to accurately estimate the incidence of *Y. pseudotuberculosis*-associated gastroenteritis difficult. Presently, enteropathogenic

yersiniae cases are likely underestimated. Implementing a more robust global surveillance system would improve our understanding of yersiniosis trends and help identify which agricultural, hygienic, and clinical controls are most effective in reducing the incidence of yersiniosis.

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17
Listeria monocytogenes

Listeriosis has emerged as a major foodborne disease during the past 40 years, after a 1981 outbreak of listeriosis in Nova Scotia, Canada, was traced to contaminated coleslaw (1). However, the discovery of the causative agent of listeriosis, *Listeria monocytogenes*, dates back to the mid-1920s, when E. G. D. Murray and James Pirie independently reported detailed descriptions of listeriosis in small animals (2). A fascinating account of the discovery of *L. monocytogenes* has been compiled by Jim McLauchlin (3). The first documented retrospective case of human listeriosis involved a soldier who suffered from meningitis at the end of World War I, and there is a suggestion in the literature that listeriosis may have been the cause of Queen Anne's 17 unsuccessful pregnancies (4).

Between 1930 and 1950, only a few human listeriosis cases were reported. Today, the estimated incidence of listeriosis in most developed countries is 2 to 5 cases per 1,000,000 population, with nearly 1,600 cases estimated to occur annually in the United States (5). The emergence of listeriosis during the past 50 years is the result of complex interactions between various factors reflecting changes in social patterns. These factors include the following:

- improvements in medicine, public health, sanitation, and nutrition that have resulted in increased life expectancy, particularly in developed countries
- the greatly increased population of immunocompromised individuals at increased risk of listeriosis and other diseases as a result of the increased use of immunosuppressive medications for the treatment of malignancies, management of organ transplantations, and autoimmune diseases, as well as the ongoing epidemic of AIDS
- changes toward centralized and more consolidated food production and processing, the ever-expanding national and international distribution of foods, and increased use of refrigeration as a primary means of food preservation
- changes in food habits, including increased consumer demand for preservative-free, fresh-tasting convenience foods that can be purchased ready to eat (RTE), refrigerated, and rapidly prepared with minimal or no cooking before consumption
- expanded use of antacids and gastric-acid-suppressive medications
- improved diagnostic methods and enhanced public health surveillance

Listeriosis is an atypical foodborne illness of major public health concern because of the severity of the disease (meningitis, septicemia, and spontaneous abortion), the high case fatality rate (approximately 20 to 30%), the long incubation time (up to 70 days), and a predilection for individuals who have an underlying condition that leads to impairment of T-cell-mediated immunity. Control of *L. monocytogenes* in foods represents a significantly greater challenge than that of most foodborne pathogens in that it is widely distributed in the environment, is resistant to diverse environmental conditions, including low pH and high NaCl concentrations, and is facultatively anaerobic and psychrotrophic. The various ways in which *L. monocytogenes* can enter food processing plants, its capacity for prolonged survival in the environment (soil, plants, and water), on foods, and in food processing plants, and its ability to grow at low temperatures (2 to 4°C) and to survive in biofilms or on/in foods and food contact surfaces for prolonged periods under adverse conditions have made this bacterium a major concern of the agrifood industry for more than 35 years.

The significance of *L. monocytogenes* as a foodborne pathogen is complex. The severity and case fatality rate of the disease require appropriate preventive measures, but the characteristics of the microorganism are such that it is unrealistic to expect all food to be *Listeria* free. This dilemma has generated an ongoing debate concerning both the various strategies for prevention of listeriosis and the regulation of *L. monocytogenes* in foods. Since 1985, considerable research has been conducted to identify the routes of contamination, the behavior of this pathogen in a wide range of foods, and various microbial intervention strategies, including the use of *Listeria* growth inhibitors, thermal and nonthermal processing, and postpackaging pasteurization. Epidemiologic investigations of outbreaks have helped identify the vehicles of transmission and have led to an expanding list of RTE foods that have been associated with outbreaks. Basic research on the genetics, molecular biology, and immunologic responses of animals and humans to *L. monocytogenes* has provided detailed insights into the virulence characteristics of this fascinating pathogen.

CHARACTERISTICS OF THE ORGANISM

Classification—The Genus *Listeria*

Listeria is a member of the phylum *Firmicutes*, and while it was previously placed in the order *Bacillales*, genomic data support its placement in the *Lactobacillales* together with genera like *Staphylococcus*, *Streptococcus*, and *Lactobacillus* (6). Traditionally, *Listeria* is placed in the *Listeriaceae* together with the genus *Brochothrix*, although recent genomic analysis suggests that *Brochothrix* may be placed in a separate family in the future (6).

The genus *Listeria* currently contains 19 species. *L. monocytogenes* is one of six closely related species forming a group that also includes *Listeria ivanovii*, *L. innocua*, *L. seeligeri*, *L. welshimeri*, and *L. marthii* (7–9). This group is sometimes referred to as *Listeria sensu stricto* (9). The remaining 13 species are more phylogenetically distant from *Listeria sensu stricto* and can be subdivided into three distinct phylogenetic clades based on genomic data: (i) a clade containing *Listeria grayi*, (ii) a clade containing *Listeria fleischmannii*, *L. floridensis*, *L. aquatica*, *L. costaricensis*, *L. goaensis*, and *L. thailandensis*, and (iii) a clade containing *Listeria rocourtiae*, *L. weihenstephanensis*, *L. cornellensis*, *L. grandensis*, *L. newyorkensis*, *L. riparia*, and *L. booriae* (10). Within the genus *Listeria*, only *L. monocytogenes* and *L. ivanovii* are considered pathogenic, as evidenced by their 50% lethal doses (LD_{50}) in mice and their ability to grow in mouse spleen and liver. *L. monocytogenes* is the only human-pathogenic *Listeria* species of major public health concern, and *L. ivanovii* infections are primarily confined to animals.

The methods used for isolating *L. monocytogenes* from food and clinical samples have been reviewed and described elsewhere (11). Identification of *Listeria* to species level is based on a limited number of biochemical markers, among which hemolysin production has been traditionally used to differentiate between *L. monocytogenes* and frequently encountered nonpathogenic *Listeria* species, such as *L. innocua* (12). Other biochemical tests used to discriminate between species include acid production from various sugars. A wide range of miniaturized biochemical test kits are commercially available for species confirmation in addition to more rapid methods that utilize enzyme-linked immunosorbent assays and DNA probes for specific genes.

Further Characterization and Subtyping of *L. monocytogenes*

L. monocytogenes isolates are often characterized below the species level for the purposes of public health surveillance and to assist in outbreak investigations. Serotyping has proven its value over many years. There are 13 serotypes of *L. monocytogenes* that can cause disease, but more than 90% of human isolates belong to three serotypes: 1/2a, 1/2b, and 4b (13). Because of the low discriminatory power of this method, phage-typing systems were developed, and prior to the introduction of molecular typing methods, they were a primary means of distinguishing between strains of the same serotype.

Since 1989, various molecular typing methods have been applied to *L. monocytogenes*, including multilocus enzyme electrophoresis, ribotyping, DNA microrestriction (DNA fragments generated with

high-frequency-cutting enzymes and separated by conventional agarose gel electrophoresis) and macrorestriction (large DNA fragments generated with infrequently cutting enzymes and separated by pulsed-field gel electrophoresis [PFGE]), and random amplification of polymorphic DNA (14). Due to their ability to type all strains and the high discriminatory power of some of them, these methods have become standard tools for epidemiologic investigations. In addition, unlike serotyping and phage typing, which require specialized reagents (typing sera and bacteriophages) available to only a few reference laboratories, these molecular methods can be performed in any reasonably well-equipped laboratory. This includes the routine subtyping and reporting of *Listeria* isolates by public health and regulatory laboratories in the United States and other countries via the Centers for Disease Control and Prevention's (CDC) PulseNet. This international network of laboratories uses highly standardized subtyping protocols (i.e., PFGE) to detect foodborne disease clusters that may have a common source. *L. monocytogenes* was added to PulseNet in 1999, and a standardized PFGE protocol is available (15). Whole-genome sequencing (WGS) technologies are now largely replacing PFGE for *L. monocytogenes* surveillance both in the United States and in parts of the European Union (16, 17). Laboratories using WGS surveillance use a combination of core genome multilocus sequence typing and single-nucleotide-variant methods to detect outbreak clusters (18).

Both early Sanger sequencing-based research and WGS data have revealed that most *Listeria* isolates are part of groups of genetically highly similar strains, called clonal complexes (CCs). While genetic variability within these CCs is limited, genomic divergence between closely related CCs is usually high (19). Some of these CCs were recognized before the introduction of sequence-based methods and were found to be often involved in outbreaks and are called epidemic clones (ECs). Evans et al. (20) examined several outbreak strains of *L. monocytogenes* 4b and classified them as ECI and ECII. The ECI isolates were involved in outbreaks prior to 1998, whereas ECII isolates were involved in multistate outbreaks in the United States in 1998 and 2002. ECII isolates have diverged in the serotype-specific region of the genome compared with other serotype 4b strains. Maury et al. (21) found that some CCs are more likely to be associated with human central nervous system or maternal-neonatal listeriosis than with foodborne illness and that they were hypervirulent in a humanized mouse model of listeriosis. Some of these hypervirulent CCs overlap with previously recognized ECs, such as ECI, while others have not been recognized as ECs previously (e.g., CC6).

Susceptibility to Physical and Chemical Agents

L. monocytogenes can grow at 0 to 45°C, with growth occurring more slowly at lower temperatures. The average generation times for 39 *L. monocytogenes* strains were 43, 6.6, and 1.1 h at 4, 10, and 37°C, respectively, and the associated lag times were 151, 48, and 7.3 h (22). Temperatures below 0°C preserve or moderately inactivate the bacterium. Survival and injury during frozen storage depend on the substrate and the rate of freezing. Among foodborne pathogens, *L. monocytogenes* is considered one of the most resistant to freeze injury. *L. monocytogenes* is inactivated by exposure to temperatures above 50°C.

Zheng and Kathariou (23) identified three genes (*ltrA*, *ltrB*, and *ltrC*) in *L. monocytogenes* that are essential for low-temperature growth. When *ltrB* was used as a probe, it hybridized only with outbreak-associated ECI serotype 4b strains of *L. monocytogenes*.

The pH range for the growth of *L. monocytogenes* was thought to be 5.6 to 9.6, although the bacterium can initiate growth in laboratory media at pH values as low as 4.4. Growth at low pH values is influenced by the incubation temperature and the type of acid. At pH values below 4.3, listeriae may survive but do not multiply. Experimentally, the presence of up to 0.1% acetic, citric, and lactic acid in tryptose broth inhibits the growth of *L. monocytogenes*, with inhibition increasing as the incubation temperature decreases. The antilisterial activity of these acids is related to their degree of dissociation, with citric and lactic acids being less detrimental than acetic acid at an equivalent pH.

L. monocytogenes grows optimally at a water activity (a_w) of 0.97. For most strains, the minimum a_w for growth is 0.93, but some strains may grow at a_w values as low as 0.90. Further, the bacterium may survive for long periods at a_w values as low as 0.83 (24). An inverse relationship exists between the

thermal resistance of *L. monocytogenes* and the a_w of the medium in which it is suspended (25), which must be addressed by manufacturers who rely on low a_w and thermal treatment for food preservation.

L. monocytogenes can grow in the presence of 10 to 12% sodium chloride and can grow to high populations at moderate salt concentrations (6.5%). The bacterium survives for long periods at high salt concentrations, with survival in such environments being significantly increased at lower temperatures.

The inoculum level of *L. monocytogenes* affects its ability to grow under adverse environmental conditions (temperature, pH, and a_w). For example, at 25°C and an a_w of 0.997, growth can be initiated at pH 4.45 by fewer than 10 CFU/ml, whereas growth can be observed at pH 3.9 when the cell concentration exceeds 6×10^6 CFU/ml (26). Multiple predictive microbiology models based on data obtained from studies using microbiological media and various foods describe the effects and interactions of temperature, pH, a_w, sodium chloride content, organic acid concentration, and sodium nitrite concentration on both growth and survival of *L. monocytogenes*.

LISTERIOSIS AND RTE FOODS

Certain RTE processed foods that support the growth of *L. monocytogenes* are high-risk vehicles for transmitting listeriosis to susceptible populations, as determined by active surveillance for sporadic listeriosis and epidemiologic investigation of listeriosis outbreaks. These foods are usually preserved by refrigeration and offer an appropriate environment for the multiplication of *L. monocytogenes* during manufacture, aging, transportation, and storage. The risk is likely to be increased if the RTE foods are exposed to marginal abuse temperatures (8 to 15°C) or have extended refrigerated shelf lives. Foods in this category include unpasteurized milk and dairy products prepared from unpasteurized milk, such as soft unripened and surface-ripened cheeses, frankfurters that were not reheated before consumption, delicatessen meats, and smoked fish, as well as some seafood and certain fresh-cut fruits and vegetables.

In Canada, the regulatory policy directs inspection and compliance action on RTE foods that can support the growth of *L. monocytogenes*. The highest priority is given to foods that have previously caused listeriosis and those that have more than 10 days of shelf life (27). The need to establish effective, risk-based food safety controls has resulted in *L. monocytogenes* being the focus of a large number of qualitative and quantitative microbiological risk assessments in various RTE foods (28–35). The most comprehensive of these assessments was conducted by the U.S. Food and Drug Administration (FDA), in collaboration with the U.S. Department of Agriculture (USDA) Food Safety and Inspection Service and the CDC. The assessment was undertaken to predict the potential relative risk of listeriosis from eating certain RTE foods among three age-based groups of people: perinatal (16 weeks after fertilization to 30 days after birth), elderly (60 years of age and older), and intermediate age (general population, less than 60 years of age). This assessment evaluated foods within 23 categories considered to be principal potential sources of *Listeria* (Table 17.1.) and found that RTE meats, including deli meats and frankfurters, posed the greatest risk. Subsequent risk assessments have examined in detail a variety of RTE foods and various sectors of the food chain (36).

From the exposure models and “what-if” scenarios used in the risk assessment, it was determined that the following five factors affected consumer exposure to *L. monocytogenes* at the time of food consumption: (i) amount and frequency of the food consumed, (ii) prevalence and levels of *L. monocytogenes* in the food, (iii) likelihood of *L. monocytogenes* growth in the food during refrigerated storage, (iv) refrigerated-storage temperature, and (v) duration of refrigerated storage of the food before consumption. The risk assessment model was used to estimate the likely impact of control strategies by changing one or two input parameters and measuring the change in the model outputs. For example, one “what-if” scenario determined that the predicted number of listeriosis cases would be reduced by 69% if all home refrigerators were consistently operating at or below 7.2°C. Another scenario determined that reducing the maximum storage time of deli meats from 28 to 14 days would reduce the median number of cases in the elderly population by 13.6%.

Fluid Milk Products

Raw milk is a well-documented source of *L. monocytogenes*. The first major outbreak of foodborne listeriosis was traced to California-produced Mexican-style cheese in 1985 (37), followed by an outbreak in North Carolina in 2000, both of which were likely caused by the use or partial use of unpasteurized milk for cheese making (38). The prevalence of *L. monocytogenes* in bulk tank raw milk varies from 1 to 13%, while its prevalence in milk processing plants ranges from 7 to 28% (39). Therefore, raw milk and food products made from raw milk are potential sources of *L. monocytogenes*. Based on numerous surveys, about 2.5, 3.6, and 5.2% of the raw milk samples tested in North America, Europe, and elsewhere, respectively, were positive for *L. monocytogenes* (40). However, the ability of *L. monocytogenes* to survive and proliferate in raw dairy products stored at refrigeration temperatures makes this bacterium a particular concern for the dairy industry. Contamination levels that are initially low (e.g., <1 CFU/25 g) may increase to high cell numbers that could pose a human health hazard if milk and certain cheeses are subjected to long-term refrigerated storage (40).

Most thermal-inactivation studies have shown that cells of *L. monocytogenes* suspended in milk were effectively inactivated by high-temperature short-time (HTST) pasteurization (71°C for 15 s or equivalent) (40, 41). One multistate survey showed a very low frequency of isolation (1 of 5,519 samples) for *L. monocytogenes* in commercial pasteurized fluid milk products sold in the United States (42), with the same being true for dairy products prepared from pasteurized milk (40). Differences were observed when thermal inactivation of freely suspended *L. monocytogenes* cells was compared with that of cells that were internalized within phagocytic leukocytes (43). The physiological state (actively growing cells versus cells in stationary phase) and growth of *Listeria* at elevated temperatures (e.g., in infected cows that may have developed fever) before pasteurization are some of the potential factors that may have caused these cells to become more heat resistant. Lado and Yousef (44) state that the following factors should be considered in evaluating the effect of pasteurization on *L. monocytogenes*: (i) the safety margin of pasteurization for inactivation of the bacterium may be lower than previously thought; (ii) the level of contamination in pooled milk is lower than that used in most inoculation studies; (iii) homogenization of milk destroys the integrity of phagocytic cells in milk, thus removing any protection offered to bacterial cells; and (iv) thermoduric spoilage microorganisms surviving HTST pasteurization are likely to outcompete *L. monocytogenes*. Hence, there is general agreement with the observations of the WHO informal study group, which concluded that "pasteurization is a safe process which reduces the number of *L. monocytogenes* in raw milk to levels that do not pose an appreciable risk to human health" (276). Recognizing the small margin of safety offered by HTST pasteurization, most milk processors have adopted processes that employ temperatures well above the minimum legal requirements for pasteurized milk. *L. monocytogenes* grows in pasteurized milk, with the numbers increasing 10-fold in 7 days at 4°C. Further, listeriae grow more rapidly in pasteurized milk than in raw milk when incubated at 7°C. Therefore, fluid milk that is contaminated after pasteurization and stored under refrigeration may attain very high populations of *L. monocytogenes* after 1 week. Temperature abuse may further enhance growth rates, as was evidenced in pasteurized-milk-associated outbreaks in Illinois (45) and Massachusetts (46).

Table 17.1 Relative risk ranking and predicted number of cases of listeriosis for the total U.S. population on a per-serving and per-annum basis[a]

		Predicted median no. of cases of listeriosis for 23 food categories				
		Per-serving basis[b]			Per-annum basis[c]	
Relative risk ranking	Risk category	Food	No. of cases	Risk category	Food	No. of cases
1	High	Deli meats	7.7×10^{-8}	Very high	Deli meats	1,598.7
2		Frankfurters, not reheated	6.5×10^{-8}	High	Pasteurized fluid milk	90.8
3		Pâté and meat spreads	3.2×10^{-8}		High-fat and other dairy products	56.4
4		Unpasteurized fluid milk	7.1×10^{-9}		Frankfurters, not reheated	30.5
5		Smoked seafood	6.2×10^{-9}	Moderate	Soft unripened cheese	7.7
6		Cooked RTE crustaceans	5.1×10^{-9}		Pâté and meat spreads	3.8
7	Moderate	High-fat and other dairy products	2.7×10^{-9}		Unpasteurized fluid milk	3.1
8		Soft unripened cheese	1.8×10^{-9}		Cooked RTE crustaceans	2.8
9		Pasteurized fluid milk	1.0×10^{-9}		Smoked seafood	1.3
10	Low	Fresh soft cheese	1.7×10^{-10}	Low	Fruits	0.9
11		Frankfurters, reheated	6.3×10^{-11}		Frankfurters, reheated	0.4
12		Preserved fish	2.3×10^{-11}		Vegetables	0.2
13		Raw seafood	2.0×10^{-11}		Dry/semidry fermented sausages	<0.1
14		Fruits	1.9×10^{-11}		Fresh soft cheese	<0.1
15		Dry/semidry fermented sausages	1.7×10^{-11}		Semisoft cheese	<0.1
16		Semisoft cheese	6.5×10^{-12}		Soft ripened cheese	<0.1
17		Soft ripened cheese	5.1×10^{-12}		Deli-type salads	<0.1
18		Vegetables	2.8×10^{-12}		Raw seafood	<0.1
19		Deli-type salads	5.6×10^{-13}		Preserved fish	<0.1

20		Ice cream and other frozen dairy products	4.9×10^{-14}		Ice cream and other frozen dairy products	<0.1
21		Processed cheese	4.2×10^{-14}		Processed cheese	<0.1
22		Cultured milk products	3.2×10^{-14}		Cultured milk products	<0.1
23		Hard cheese	4.5×10^{-15}		Hard cheese	<0.1

[a] Adapted from reference 6.

[b] Foods were classified as high risk (>5 cases per billion servings), moderate risk (≤5 but ≥1 case per billion servings), and low risk (<1 case per billion servings).

[c] Foods were classified as very high risk (>100 cases per annum), high risk (>10 to 100 cases per annum), moderate risk (>1 to 10 cases per annum), and low risk (<1 case per annum).

Cheeses

L. monocytogenes can survive the cheese manufacturing and ripening process because of its relative hardiness to temperature and pH fluctuations, its ability to multiply at refrigeration temperature, and its salt tolerance. Growth in cheese milk is retarded but not completely inhibited by lactic starter cultures. During cheese making, *L. monocytogenes* is concentrated about 10-fold in the curd, with the remaining cells appearing in the whey. The behavior of listeriae in the curd is influenced by the type of cheese, ranging from growth in feta cheese to significant inactivation during cottage cheese manufacture. During cheese ripening, the *L. monocytogenes* population may increase (Camembert), decrease gradually (Cheddar or Colby), or decrease rapidly during early ripening and then stabilize (blue cheese). Consumption of soft Mexican-style cheeses (e.g., queso fresco or queso blanco) and surface-ripened cheeses (e.g., Brie or Camembert) by susceptible persons is an important risk factor for sporadic and epidemic listeriosis in North America and Europe. Over 3 decades ago, 49% of the sporadic listeriosis cases in France were attributed to consumption of soft cheese. Prevention efforts have reduced this incidence by 68% between 1987 and 1997 (47, 48). The first documented foodborne listeriosis outbreak in Japan resulted in 86 cases of infection (mainly gastroenteritis) and was traced to *L. monocytogenes* serotype 1/2a contamination in washed-type cheese (49).

Meat and Poultry Products

The growth potential for *L. monocytogenes* in meat and poultry products depends on the meat variety, pH, and type and number of competing microorganisms. A 2006 survey in the United States revealed that 3.5% of commercial ground beef samples were positive for *L. monocytogenes* (50). Poultry supports the growth of *L. monocytogenes* better than other meats, whereas properly fermented sausage does not permit *Listeria* growth. Contamination typically occurs via symptomatic or asymptomatic carriage of *L. monocytogenes* by the animal before slaughter, followed by contamination of the carcass during or after slaughter. Because *L. monocytogenes* tends to concentrate and multiply in the kidneys, mesenteric and mammary lymph nodes, livers, and spleens of infected animals, organ meats may be more hazardous to consume than muscle tissue (51). Regardless of the contamination route, *L. monocytogenes* strongly attaches to the surface of raw meats and is difficult to remove or inactivate. *L. monocytogenes* multiplies readily in meat products, including vacuumpackaged beef, at pH values near 6.0, whereas little or no multiplication occurs at pH 5.0 (51). RTE meat products that have received a heat treatment followed by cooling in brine before packaging may provide an environment that is particularly conducive to multiplication of *L. monocytogenes* because of the reduction in competitive microbiota and the high salt tolerance of the bacterium.

Based on the USDA monitoring program for *L. monocytogenes*, the prevalence of this pathogen in cooked RTE meat and poultry products decreased from 4.61% in 1990 to 0.19% in 2017 (52), with much of this decrease being due to the adoption of mandatory hazard analysis and critical control point

programs for the industry beginning in 1998. Since 2004, producers of such products in the United States must also use one of the three USDA alternatives for control of *Listeria*, namely, (i) use of a postlethality treatment (e.g., postpackaging pasteurization) and use of an antimicrobial agent (e.g., lactate or diacetate) or process (e.g., freezing) that suppresses or limits the growth of the organism, (ii) use of either a postlethality treatment or an antimicrobial agent or process that suppresses or limits growth of the organism, or (iii) use of only sanitation to control the organism (53). Surveys of both meat and poultry processing facilities have identified both transient and persistent strains of *L. monocytogenes*, with the pathogen in some cases persisting at the same location for months or years (54–57).

Cooked, RTE meat and poultry products have been frequent sources of sporadic and outbreak-associated listeriosis in both North America and Europe. Consumption of unreheated frankfurters and undercooked chicken was first identified as a risk factor for sporadic cases of listeriosis in the United States (58) after a contaminated turkey frankfurter product was linked to a sporadic *L. monocytogenes* infection in a cancer patient in 1989 (59). Nationwide outbreaks of listeriosis were traced to contaminated frankfurters or deli meat in the United States in 1998, 2000, and 2002 (54–56) and in Canada in 2008 (60). In 2017 and 2018, the largest outbreak of listeriosis recorded to date affected over 1,000 people and caused 204 deaths in South Africa. The source was polony, a processed deli meat, contaminated with *L. monocytogenes* sequence type 6. The total cost of the outbreak has been estimated at $250 million (61).

Cross-contamination of RTE meat and poultry products with *L. monocytogenes* can occur during both production and retail sale (62). In a large U.S. survey of RTE foods obtained from different retail markets, in-storepackaged deli meats were about seven times more likely to harbor *L. monocytogenes* (2.7% positive) than manufacturer-packaged products (0.4% positive) (63). Using more recent retail contamination rates (34) and consumer purchasing habits (64), approximately 80% of all listeriosis cases and deaths involving deli meat can be attributed to products sliced at retail with mechanical delicatessen slicers now recognized as the major source of *Listeria* cross-contamination (34, 65, 66).

Seafood

The role of seafood in human listeriosis was reviewed in 2000 (67). The same year, Huss et al. (68) classified the following seafood as potential high-risk foods for listeriosis: (i) mollusks, including fresh and frozen mussels, clams, and oysters (in shell or shucked); (ii) raw fish; (iii) lightly preserved fish products, including salted, marinated, fermented, cold-smoked, and gravad fish; and (iv) mildly heat-processed fish products and crustaceans. Smoked seafood and cooked RTE crustaceans were identified as moderate-to high-risk foods in the FDA 2003 risk assessment. Shrimp, smoked mussels, and imitation crabmeat have been implicated as sources of human listeriosis. Gravad rainbow trout and cold-smoked rainbow trout were responsible for two outbreaks in Sweden and Finland, respectively (69, 70).

In the United States, *L. monocytogenes* has been isolated from both domestic and imported fresh, frozen, and processed seafood products, including crustaceans, molluscan shellfish, and finfish (71). An FDA survey of domestic and imported refrigerated or frozen cooked crab meat in 1987 and 1988 determined contamination levels of 4.1% for domestic products and 8.3% for imported products (72). Crab and smoked fish samples analyzed by the FDA between 1991 and 1996 yielded *L. monocytogenes* contamination rates of 7.5% and 13.6%, respectively. From 1999 to 2003, 2.9% and 14.9% of the hot-and cold-smoked, respectively, seafood samples (primarily fish) tested positive for *L. monocytogenes* (71) with 3.6% of RTE crustaceans (primarily crab and shrimp) also being positive during the same period. However, consumption of smoked seafood in the United States is still much less than that of meats and cheeses, and the much smaller scale of production of these products helps to explain the general lack of large-scale outbreaks in the United States. Cold-smoked fish continues to be the focus of efforts to develop new control measures and systems for *L. monocytogenes* (73).

As was true for meat processing plants, *L. monocytogenes* is also frequently found in facilities producing raw and RTE seafood. When Hoffman et al. (74) compared the ribotypes of *L.*

monocytogenes strains isolated from smoked fish processing environments and raw fish, various unique ribotypes were recovered from raw fish samples, indicating that the raw fish was possibly contaminated from a source other than the processing environment.

Products of Plant Origin

Compared to dairy, meat, poultry, fish, and seafood products, *L. monocytogenes* has until recently received only limited attention regarding its prevalence and growth in products of plant origin. At the time when the previously discussed *Listeria* risk assessment was published, in 2003, *L. monocytogenes* was recognized as an infrequent contaminant of vegetables (e.g., broccoli, cabbage, celery, cucumbers, green beans, lettuce, mushrooms, onions, potatoes, radishes, spinach, sprouts, tomatoes, and yams) and fruits (e.g., apples, blueberries, cantaloupe, melons, pears, pineapple, and watermelon) (75). More recent surveys, including many conducted outside North America, have yielded similar findings (76). Vegetables and fruits are prone to both pre-and postharvest contamination. Strawn et al. (77) recovered *L. monocytogenes* from 17.5% of 263 field and 30% of 74 water samples collected over a 5-week period from 21 produce farms in New York State.

Risk factors associated with increased isolation included manure application, presence of wildlife, soil cultivation, and field worker activity. During subsequent postharvest processing, fresh produce is prone to contamination during slicing, dicing, and washing (78). While chemical sanitizers are typically added to flume water to minimize cross-contamination during washing, their effectiveness typically decreases over time as the levels of soil and organic material increase in the water during continued processing. While the prevalence of *L. monocytogenes* in fresh produce is presumed to be quite low (<5%), reliable data for specific vegetables and fruits are still lacking due to limited collection and sampling.

Fruits and vegetables differ in their ability to support the growth of *L. monocytogenes* (79), with the manner of product handling during postharvest processing having an impact on maximum attainable populations. Many types of fresh produce to be further processed undergo one or more washing steps in which a chemical sanitizer is added to the water to minimize cross-contamination through the wash water. However, given the increasing number of *Listeria* recalls involving fresh-cut produce, washing alone cannot completely ensure end product safety. Further processing steps, such as slicing, dicing, and shredding, can spread *Listeria* to previously uncontaminated product, as has been shown for celery (80) and onions (81). Listeriae can reportedly grow in such products, including diced celery (82), diced onions (82), and shredded lettuce (83), with the extent of growth being dependent on the degree of temperature abuse and the type of packaging. Caramel apples are also capable of supporting growth if *Listeria* enters the center of the apple via the stick (84). However, recent studies indicate that *L. monocytogenes* is far less thermally resistant than *Salmonella* in low-moisture foods (e.g., nuts, spices, and powders), with the latter organism being the primary pathogen of concern (85).

Involvement of fruits and vegetables in human listeriosis dates back at least to a 1981 Canadian outbreak in which coleslaw prepared from *Listeria*-contaminated cabbage was responsible for 7 adult and 34 perinatal cases of listeriosis (1). Fertilization of the cabbage with manure from *Listeria*-infected sheep was cited as the most probable route of contamination. More than 25 years then passed without any major reports of systemic listeriosis from consumption of produce, which supported the low ranking of fresh fruits and vegetables in the 2003 *Listeria* risk assessment. However, in 2008, fresh produce once again emerged as a vehicle for listeriosis, with a multistate outbreak in the United States involving sprouts being linked to 20 cases of illness during 2008 and 2009. Subsequent outbreaks in the United States have been traced to consumption of diced celery in 2010 (10 cases, 5 deaths) (86), cantaloupe in 2011 (147 cases, 33 deaths) (87), nectarines and peaches in 2014 (2 cases, 1 death) (88), mung bean sprouts (5 cases, 2 deaths) (89), and commercially prepared caramel apples (35 cases, 7 deaths) (90), all of which have been reviewed by Garner and Kathariou (91). Leafy greens also have been implicated in two multistate outbreaks, one originating in Colorado in 2011 (99 cases, 515 deaths) (92) and the other in Ohio in 2016 (19 cases, 1 death) (93), in addition to frozen vegetables from Washington State in 2016 (9 cases, 3 deaths) (94).

EFFECTS OF NEWER METHODS OF FOOD PRESERVATION

New trends in food preservation have recently been developed, including the use of bacteriocins as biopreservatives, as well as vacuum and modified-atmosphere packaging, all of which have been reviewed by Lado and Yousef (44). Nisin, produced by certain strains of *Lactococcus lactis* subsp. *lactis*, is by far the most widely recognized bacteriocin. The antagonistic effect of nisin on *L. monocytogenes*, which is well documented, is strongly dependent on the pH and chemical composition of the food to which it is added. Pediocins (from *Pediococcus pentosaceus* and *Pediococcus acidilactici*) and bavaracin A (95) (from *Lactobacillus bavaricus*) transiently affect *L. monocytogenes* growth and survival in various beef systems, especially at low temperature. Meat-borne lactic acid bacteria can effectively inhibit the growth of *L. monocytogenes* by rapid production of lactic acid and bacteriocins and can be used as potential biopreservatives in cooked meat products (96). A similar effect was observed with the bacteriocin carnocin from *Carnobacterium piscicola* in broth and skimmed milk (97). However, the emergence of resistant mutants can be a drawback to the use of bacteriocins, as has been observed with bavaracin A and nisin (95, 98).

In vitro studies have also demonstrated antilisterial activity of essential oils that function by partitioning the lipids of the cell membrane and mitochondria, resulting in increased membrane permeability and cell leakage. Examples of essential oils include carvacrol, thymol, eugenol, perillaldehyde, cinnamaldehyde, and cinnamic acid, which have MICs of 0.05 to 5 μl/ml *in vitro* (54). However, these essential oils must be carefully paired with particular food so as to not impart undesirable flavor characteristics.

A survey of vacuum-packaged processed meat in retail stores in the early 1990s revealed that 53% of the samples tested were contaminated with *L. monocytogenes* and that 4% contained more than 1,000 CFU/g (99). This observation corroborates experimental evidence that the growth of *L. monocytogenes* (a facultative anaerobe) is not significantly affected by vacuum packaging. There has been considerable interest in modified-atmosphere packaging of meat products (low oxygen and high carbon dioxide concentrations) in recent years because of the increasing demand for refrigerated convenience foods with extended shelf lives. Studies with meat juice, raw chicken, and precooked chicken nuggets revealed that such atmospheres do not significantly affect the growth of *L. monocytogenes*.

L. monocytogenes is sensitive to low doses of irradiation. Sublethal heating, freezing, sanitizer exposure, and exposure to various nonthermal processing methods, including high-intensity pulsed light, high pressure, irradiation, and pulsed electric fields, can injure a substantial portion of surviving cells (100). Heat-stressed *L. monocyte genes* may be considerably less pathogenic than nonstressed cells. While injured cells can repair the damage induced by stress and grow on nonselective agar, the additional stress encountered when cells are grown on selective agar leads to variable recovery. Hence, when present in foods, sublethally damaged cells may go undetected.

RESERVOIRS

The role of improperly fermented silage in the transmission of animal listeriosis, first referred to as “circling disease” in 1929, to ruminants is now well known. This pathogen is geographically widespread in both rural and urban environments (101) and can survive and/or grow in improperly fermented silage, pasture grass, decaying vegetation, soil, sewage, and various aquatic environments, including surface water of canals, lakes, ditches, and freshwater tributaries (102). Alfalfa and other crops grown on soil treated with sewage sludge can be contaminated with *Listeria*. In one study, half of the radish samples grown in *L. monocytogenes*-inoculated soil were confirmed positive 3 months later (103). The wide spread presence of *L. monocytogenes* in soil is likely due to contamination by decaying plant and fecal material, with the soil providing a cool, moist environment and the decaying material providing the nutrients (104).

L. monocytogenes has been isolated from the feces of many healthy animals and birds; listeriosis in many animal species has been recorded. Humans exhibiting symptoms of listeriosis and asymptomatic carriers shed the organism in their feces. Figure 17.1 illustrates the many ways in which *L.*

monocytogenes can be spread from the environment to animals and humans and back to the environment.

Food Processing Facilities

Entry of *L. monocytogenes* into food processing facilities can occur through soil on workers' shoes and clothing, transport equipment, animals that excrete the bacterium or have contaminated hides or surfaces, raw plant tissue, raw food of animal origin, and possibly healthy human carriers. Growth of *Listeria* is favored by high humidity and the presence of nutrients. *L. monocytogenes* is most often recovered from moist areas such as floor drains, condensate, stagnant water, floors, and residues on processing equipment (105). *L. monocytogenes* can attach to various types of surfaces, including stainless steel, glass, plastic, and rubber, and form biofilms, as has been described for meat and dairy processing environments (106). *Listeria* spp. can survive on fingers after hand washing and in aerosols. The presence of *L. monocytogenes* in the food chain is evidenced by the widespread distribution of the listeriae in processed products. Contaminated effluents from food processing plants increase the spread of *L. monocytogenes* in the environment. Sources of *L. monocytogenes* in dairy processing plants include the environment (floors and floor drains, especially in areas in and around coolers or places subject to outside contamination) and raw milk. Efforts to ensure that milk is safe from *L. monocytogenes* contamination should focus on promoting appropriate methods of pasteurization and on identifying and eliminating sources of postpasteurization contamination (40). Strict adherence to the guidance in the Pasteurized Milk Ordinance (http://ncims.org/wp-content/uploads/2018/06/2017-PMO-FINAL.pdf) helps minimize *Listeria* contamination in dairy processing environments and better ensure the safety and quality of the final product.

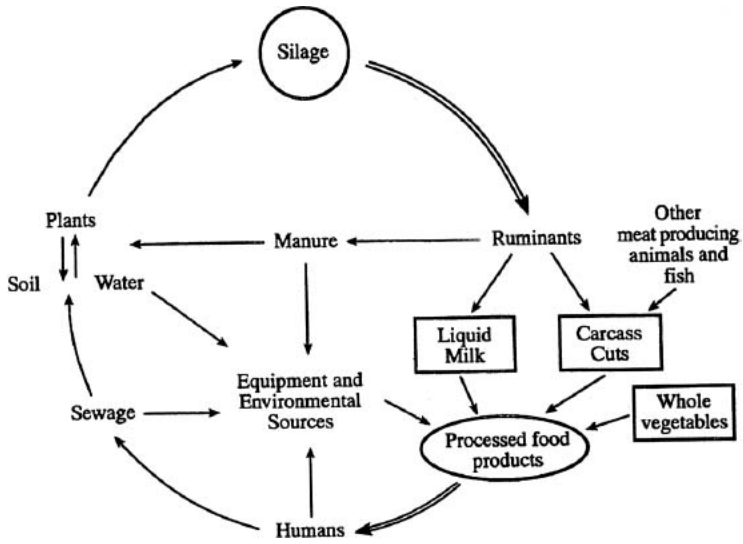


Figure 17.1 Potential routes of transmission of *L. monocytogenes*. Circles or ovals indicate areas of greatest risk of *L. monocytogenes* multiplication. Boxes indicate where direct consumption of minimally processed products (whole fresh vegetables, cooked carcass cuts of meat and fish, and effectively pasteurized milk) presents a low risk. Double arrows indicate consumer risk. Adapted from reference 117.

The presence of *L. monocytogenes* on carcasses is usually attributed to contamination by fecal matter during slaughter, evisceration, and fabrication. A high percentage (11 to 52%) of healthy animals are fecal carriers. Up to 45% of pigs can harbor *L. monocytogenes* in their tonsils, and 24% of cattle have contaminated internal retropharyngeal nodes (107, 108). *L. monocytogenes* has been recovered from both unclean and clean zones (especially workers' hands) in slaughterhouses, with the most heavily contaminated work areas involving cow dehiding and pig stunning and hoisting. Surveys conducted at both turkey and broiler slaughterhouses failed to recover *L. monocytogenes* from feathers, scald tank water overflow, neck skin, livers, hearts, ceca, or large intestines. In contrast, *L. monocytogenes* was

isolated from feather plucker drip water, chilling water overflow, recycling water for cleaning gutters, and mechanically deboned meat. These findings demonstrate the importance of the defeathering machine, chillers, and recycled water in product cross-contamination (109).

Contamination of processed RTE foods with *L. monocytogenes* occurs primarily after processing. To date, there is no evidence to indicate that *L. monocytogenes* can survive validated heat processing protocols used to render foods safe. Having the propensity to adhere to food contact surfaces and form biofilms, *L. monocytogenes* is particularly difficult to eliminate from the food processing environment even when well-designed sanitation programs are used (110). For example, *L. monocytogenes* can persistently survive on stainless steel food contact surfaces, such as those found on dicing machines, and repeatedly contaminate RTE meats (54).

Because *L. monocytogenes* is a frequent contaminant of raw foods and ingredients, ample opportunity exists for the reintroduction of *Listeria* into food processing facilities. Extensive information on the problem of *L. monocytogenes* in various food processing environments and approaches for control has been provided by Kornacki and Gurtler (110). Tompkin (111) outlined a six-step *Listeria* control program for food processing environments that includes (i) prevention of the establishment and growth of *Listeria* species in niches or other sites that can lead to contamination of RTE foods, (ii) implementation of a sampling program to assess how well the control program is working, (iii) rapid and effective response when the sampling program yields positive results for *Listeria* species, (iv) verification by follow-up sampling to ensure that the source of contamination has been identified and corrected, (v) short-term assessment of the last four to eight samplings to facilitate early detection of problems and trends, and (vi) long-term assessment at appropriate intervals (quarterly, annually, etc.) to identify widely scattered contamination events and to measure overall progress towards continuous improvement. Longitudinal studies based on various molecular subtyping analyses can assist in identifying *L. monocytogenes* contamination patterns in food processing plants and validating intervention strategies (54, 55, 74, 112, 113). The design of the environmental sampling program and response to positive findings will determine the overall effectiveness of the *Listeria* control program in food processing environments (111).

Surveys of Foods for Prevalence of *L. monocytogenes* and Regulatory Status in Different Countries

Numerous surveys that have been reviewed and summarized elsewhere confirm that *L. monocytogenes* contamination of fermented (114) and unfermented (40) dairy products, meat (51), poultry and eggs (115), fish and seafood (71), and fresh produce (116) is widespread in many parts of the world. One large-scale qualitative/quantitative survey of retail RTE foods was conducted by investigators at the National Food Processors Association in the United States (117). Product categories examined included luncheon meats, deli salads, Mexican-style cheeses, packaged salads, blue-veined and soft-ripened cheeses, smoked seafood, and seafood salads. Of 31,705 samples examined, 1.82% (the range by sample category varied from 0.17% to 4.7%) were positive for *L. monocytogenes*. Pathogen levels in positive samples ranged from <0.3 CFU/g to 1.5×10^5 CFU/g. A dose-response model was developed by combining the food survey data with concurrent data on illness in the population that consumed the foods surveyed. Based on this model, control strategies focused on foods that yielded higher cell numbers of *L. monocytogenes* are likely to have greater positive public health impact than the current zero-tolerance policy (28, 118).

In a survey conducted in Denmark during 1994 to 1995, *L. monocytogenes* was isolated from 14.2% and 30.9% of raw fish and raw meats, respectively (71). Preserved, non-heat-treated fish and meat products were more frequently contaminated (10.8% and 23.5%, respectively) than heat-treated meat products (5%). *L. monocytogenes* was present at levels exceeding 100 CFU/g in 1.3% of the preserved (non-heat-treated and heat-treated) fish and meat products that were packed under vacuum or modified atmosphere for extended shelf life. In contrast, another survey covering the same product types that were not packed under vacuum or modified atmospheres revealed significantly lower contamination levels (0.3 to 0.6%) (119). In a Japanese survey of retail foods, *L. monocytogenes* was isolated from 12,

20, 37, and 25% of minced beef, pork, chicken, and pork-beef mixture, respectively, with only five chicken samples having populations greater than 100 CFU/g. *L. monocytogenes* was isolated from 5.4% of smoked salmon samples and 3.3% of RTE uncooked seafood (120).

A survey conducted in Spain revealed *L. monocytogenes* contamination in 9.3% of RTE foods and 2.9% of foods intended to be cooked before consumption (121). In another investigation, *L. monocytogenes* was isolated from 22% of retail smoked fish samples (122). *L. monocytogenes* has been isolated from many vegetables, including bean sprouts, cabbage, cucumbers, leafy vegetables, potatoes, prepackaged salads, radishes, salad vegetables, and tomatoes in North America, Europe, and Asia, with coleslaw, chopped celery, cantaloupe, apples, and most recently lettuce having been linked to major outbreaks of listeriosis.

Because of the frequent occurrence of *L. monocytogenes* in foods and the persistence of this pathogen in food processing environments, food regulatory agencies in many countries have established tolerance levels other than "zero" (i.e., absence in the sample tested) for *L. monocytogenes*. Following a nationwide outbreak of listeriosis in Canada in 2008 that was traced to RTE deli meats, the Canadian government revised its policy on *L. monocytogenes* in RTE foods to include new end product compliance criteria in addition to environmental monitoring and the use of postlethality treatments and/or *Listeria* growth inhibitors for RTE meat products. Foods are divided into two risk categories, with category 1 foods (those that support *L. monocytogenes* growth to 100 or more CFU/g) being regulated more stringently than foods in categories 2a (those that support *L. monocytogenes* growth to 100 CFU/g or less throughout shelf life) and 2b (those that do not support *L. monocytogenes* growth during the expected product shelf life) (123). In the European Union, according to European Food Safety Authority regulation 2073/2005, RTE foods are divided into three groups as follows: (i) RTE food for populations "at risk," where absence in 25 g is required; (ii) RTE foods able to support the growth of *L. monocytogenes*, where absence in 25 g is also required while the food is under the control of the food producer; and (iii) RTE foods which cannot support the growth of *L. monocytogenes*, where up to 100 CFU/g is permitted.

In contrast, the United States, while acknowledging the widespread distribution of *L. monocytogenes* in the food supply and the difficulties in producing *L. monocytogenes*-free foods, has decided not to adopt tolerance levels for *L. monocytogenes* in RTE foods. It is argued that establishing "acceptable" levels for *L. monocytogenes* would require knowledge of the number of listeriae unlikely to cause human infection. While progress is continually being made to establish such tolerance levels through various risk assessments, the United States has thus far maintained its "zero-tolerance" policy for *L. monocytogenes* in RTE foods (43), particularly in view of a recent outbreak that involved extremely low levels of *L. monocytogenes* in ice cream (124, 125).

Human Carriage

Asymptomatic fecal carriage of *L. monocytogenes* has been studied in various human populations, including healthy people, pregnant women, outpatients with gastroenteritis, slaughterhouse workers, laboratory workers handling *Listeria*, food handlers, and patients undergoing renal transplantation or hemodialysis (126). *L. monocytogenes* can be found in 2 to 6% of fecal samples from healthy people, and listeriosis patients often excrete high numbers. In one study, stool specimens from 21% of patients contained 10^4 or more *L. monocytogenes* CFU/g, and 18% of household contacts of patients with listeriosis fecally shed the same serotype and multilocus enzyme type of *L. monocytogenes* as the index case patient (127, 128). Among household contacts of 18 pregnant women with listeriosis, 8.3% asymptomatically shed *L. monocytogenes*, whereas no listeriae were isolated from 30 household contacts of age-, sex-, and hospital-matched controls (63). Results from a 1985 outbreak investigation in California showed that community-acquired outbreaks might be amplified through secondary transmission by fecal carriers. The very low prevalence of *L. monocytogenes* in human stools and the short duration of fecal shedding argue against routine stool screening of persons with possible work-related exposure to the pathogen (e.g., dairy workers) as a tool for prevention of listeriosis (129). *L. monocytogenes* isolated from asymptomatic carriers may carry the full complement of virulence genes or may be attenuated by truncated virulence-associated genes such as *inlA* and *actA* (130).

L. monocytogenes has not been isolated from oropharyngeal samples of healthy people, and the presence of listeriae in cervicovaginal specimens may be associated with pregnancy-related listeriosis. The role of healthy carriers in the epidemiology of listeriosis is unclear.

FOODBORNE OUTBREAKS

Foodborne transmission of listeriosis, although suggested in the early medical literature, was not definitively documented until 1981, when a case-control study was simultaneously used with strain typing investigation in an outbreak in Canada (1). Since 1981, epidemiologic investigations have repeatedly revealed that consumption of contaminated food is the primary mode of transmission of listeriosis. Since 1981, more than 10 major (>30 cases) outbreaks of listeriosis have been traced to various foods, including soft cheeses, delicatessen meats, cantaloupe, caramel apples, frozen corn, and the deli meat polony.

An outbreak of listeriosis in Massachusetts in 1979 may have been caused by raw produce, but the food source was not positively identified. Twenty patients with *L. monocytogenes* serotype 4b infection were hospitalized during a 2-month period; only nine cases had been detected in the previous 26 months. Ten of the patients were immunosuppressed adults, and 5 out of the total 20 patients died. Fifteen patients are thought to have acquired the infection in the hospital. Consumption of tuna fish, chicken salad, or cheese was associated with illness, but no specific brand was implicated. It was postulated that the raw celery and lettuce, served as a garnish with the three foods, may have been contaminated with *L. monocytogenes* (131). Although the source of infection was not definitively identified in this outbreak, consumption of cimetidine or antacids was implicated as a risk factor for listeriosis, a factor that has been identified in several case-control studies. Decreased gastric acidity might have increased the survival of *L. monocytogenes* cells as they passed through the stomach.

The first confirmed foodborne outbreak of listeriosis occurred in 1981 in Nova Scotia, Canada. Thirty-four pregnancy-associated cases and seven cases in nonpregnant adults were reported during a 6-month period. A case-control study implicated locally prepared coleslaw as the vehicle, and the epidemic strain was subsequently isolated from an unopened package of the product. Cabbage fertilized with manure from sheep suspected of having *Listeria* meningitis was the probable source. Harvested cabbage was stored over the winter and spring in an unheated shed, which provided a definite growth advantage for this psychrotrophic pathogen (1).

Pasteurized milk was identified as the most likely source of infection in another large outbreak of listeriosis, in Boston, MA, in 1983 (132). Forty-nine cases occurred during a 2-month period, 42 in immunosuppressed adults and 7 in pregnant women; the overall case fatality rate was 29% (119). A case-control study implicated pasteurized milk with 2% fat as the vehicle. Multiple serotypes of *L. monocytogenes* were isolated from raw milk at the implicated dairy, but none belonged to the outbreak strain. No deviations from approved pasteurization processes were noted at the dairy, suggesting that the contamination occurred after pasteurization.

In 1985, the first outbreak of listeriosis to attract widespread attention occurred in Los Angeles County, CA, when contaminated soft Mexican-style cheese was linked to 142 cases over an 8-month period (37). Pregnant women accounted for 93 cases. The remaining 49 cases were in nonpregnant adults; 48 of 49 nonpregnant adults had a predisposing condition for listeriosis. Among pregnancy-associated cases, 87% occurred in Hispanic women. The case fatality rates were 32% for perinatal cases and 32% for nonpregnant adults. Inadequate pasteurization and mixing of raw milk with pasteurized milk likely resulted in the contaminated cheese.

L. monocytogenes-contaminated Vacherin Mont d'Or cheese (a soft surface-ripened variety) was responsible for a 4-year-long (1983 to 1987) outbreak in Switzerland involving 122 cases (J. Bille, presented at the Foodborne Listeriosis Symposium, Wiesbaden, Germany, 7 September 1988). Contaminated pâté was epidemiologically linked to 300 cases of listeriosis in the United Kingdom during 1989 to 1990 (133). Contaminated pork tongue in aspic marketed in France was the principal vehicle for 279 cases of listeriosis reported during a 10-month period in 1992 (134). Potted pork

("rillettes") was associated with 39 cases in 1993, and soft cheese was the vehicle for 33 cases in 1995 (135). Recalling the implicated food, advising the general population through the mass media to avoid consuming the implicated product, and taking appropriate action to prevent further *L. monocytogenes* contamination during manufacture and handling terminated these large outbreaks.

A large multistate outbreak of listeriosis occurred in the United States between August 1998 and March 1999. A total of 101 outbreak-associated cases (including 15 perinatal cases) were identified in 22 states. Fifteen adult deaths and six miscarriages or stillbirths were associated with this outbreak. A case-control study implicated turkey franks from a major manufacturer produced at one facility. *L. monocytogenes* serotype 4b of the epidemic PFGE subtype was isolated from opened and unopened packages of frankfurters from the implicated factory (136, 137).

Between December 1998 and February 1999, an increase in listeriosis cases due to *L. monocytogenes* serotype 3a was recognized in Finland. A total of 25 cases, most of which occurred in hematological or organ transplant patients, were identified as part of the outbreak; six patients died from *Listeria* infection. Butter served at a tertiary-care hospital was implicated as the source of infection. The epidemic strain was isolated from all 13 butter samples obtained from the hospital kitchen and from several lots from the dairy and wholesale store. One sample contained 11,000 *L. monocytogenes* CFU/g, with the others having lower counts (5 to 60 CFU/g) (27). Another outbreak of listeriosis associated with butter occurred in 2003 in the United Kingdom.

Between May and November of 2000, a listeriosis outbreak in the United States impacted 11 states. When subtyped, the *L. monocytogenes* isolates from these cases were all serotype 1/2a and were indistinguishable from each other by PFGE. Eight perinatal and 21 nonperinatal cases were reported. Among the 21 nonperinatal case patients, the median age was 65 years (range, 29 to 92 years); 13 (62%) were female. This outbreak resulted in four deaths and three miscarriages or stillbirths. A casecontrol study implicated sliced processed turkey meat from a delicatessen. A traceback investigation identified a single food processing plant as the source of this outbreak and led to the recall of 16 million pounds of processed turkey meat. The same plant had been identified in a *Listeria* contamination event that had occurred a decade previously (138).

An outbreak of listeriosis, identified in North Carolina in 2000, affected 13 people, of whom 12 were 18-to 34-year-old Hispanic females. This outbreak, which resulted in five stillbirths, was traced to Mexican-style cheese produced from contaminated raw milk at a local dairy (38). Another listeriosis outbreak in Texas was associated with legally imported but illegally distributed cheese prepared in Mexico. Five of six case patients reported eating the implicated cheese. These outbreaks underscore the need for educating Hispanic women about food safety considerations during pregnancy and enforcing existing laws that regulate the illegal sale and importation of certain cheeses that have been prepared from raw milk.

During October 2002, another multistate outbreak of listeriosis was recognized in Pennsylvania and eight other states in the United States (139). This outbreak of 54 cases included eight deaths and three fetal deaths. Case-control studies implicated delicatessen turkey meat as the source of the outbreak. In traceback studies, the outbreak strain, which was related to the 1998-1999 outbreak strain according to PFGE, was found in the environment of one turkey processing plant and in turkey products produced by another plant. Together, the two plants recalled more than 30 million pounds of turkey meat products (Table 17.2.). This outbreak, together with the outbreak in 2000 that was traced to delicatessen turkey, led to the three previously discussed alternatives for control of *Listeria* in RTE meats, with no additional large-scale RTE meat-related outbreaks having been identified in the United States since 2002. However, in 2008, a nationwide outbreak in Canada that included 57 cases of illness and 23 fatalities was traced to various delicatessen meats that became contaminated during packaging at a larger manufacturing facility in the province of Ontario. The *L. monocytogenes* isolates from this outbreak belonged to serotype 1/2a but were of two distinct PFGE patterns (140). This outbreak led to the adoption of additional preventive measures in Canada, as described above.

Since the initial link between consumption of coleslaw and listeriosis in 1981 that confirmed *L. monocytogenes* as a foodborne pathogen, fresh produce was thought to play a minor role in invasive

illness. However, in 2010 consumption of commercially diced celery was linked to 10 cases of listeriosis, including 5 fatalities in the state of Texas (141). In 2011, a much larger outbreak of listeriosis in the United States was traced to cantaloupes that were grown and packed in Colorado, with a total of 146 cases of illness, including 30 deaths, being reported across 28 states from July 31 to October 27. The victims ranged in age from <1 to 96 years with a median age of 77 years (87). An unusual feature of this outbreak was the involvement of four different *L. monocytogenes* strains, belonging to serotypes 1/2a and 1/2b, that exhibited widely divergent PFGE patterns and genome sequences (142). Three of these four strains were recovered from the processing line or packing area, suggesting multiple incoming sources of contamination. In subsequent years, several other listeriosis outbreaks in the United States have been linked to produce, including outbreaks linked to mung bean sprouts in 2014 (5 cases, including 2 deaths) (89), prepackaged caramel apples in 2014 to 2015 (35 cases, including 7 deaths) (90), packaged salads in 2016 (19 cases, including 1 death) (93), and frozen vegetables in 2016 (9 cases, including 3 deaths) (94).

Table 17.2 Selected invasive listeriosis outbreaks, 1990 to 2018

Yr(s)	Geographic location	No. of persons affected	No. of deaths or fetal deaths	Vehicle	Serotype[a]	Reference(s)
1989–90	Denmark	26	6	Blue mold cheese or hard cheese	4b	266
1992	France	279	85	Pork tongue in jelly	4b	135
1993	France	38	11	Pork rillettes	4b	267
1994	Illinois	3	0	Chocolate milk	1/2b	45, 268
1994–95	Sweden	6	1	Gravad rainbow trout and cold-smoked rainbow trout	4b	69, 134
1995	France	33	4	Raw-milk soft ripened cheese	4b	267
1998–99	United States (multiple states)	108	14/4	Frankfurters	4b	136
1999–2000	France	10	3	Rillettes	4b	269
1999–2000	France	32	10	Pork tongue in jelly	4b	269
2000	United States (multiple states)	30	4/3	Turkey deli meat	1/2a	138
2000	North Carolina	13	0/5	Homemade Mexican-style cheese	4b	38
2002	United States (multiple states)	54	8/3	Delicatessen turkey meat	4b	139
2007	United States	5	3	Pasteurized flavored and unflavored milk	NA	46
2008	Canada	57	23	RTE deli meats		60
2009–10	Austria, Germany, Czech Republic	34	8	Acid curd cheese (Quargel)	1/2a	270
2010	Louisiana	8	2	Hog head cheese	1/2a	271
2010	United States	10	5	Diced celery	1/2a	86
2011	United States	146	30	Cantaloupe	1/2a and 1/2b	64, 65, 87
2014	United States	5	2	Mung bean sprouts	NA	89
2014	United States	2	1	Stone fruit	4b	88
2014-15	United States	35	7	Prepackaged caramel apples	4b	90
2016	United States	9	3	Frozen vegetables	NA	94
2017–18	South Africa	1,060	216	Polony (RTE meat product)	ST6, 4b[b]	272

[a] NA, not available.

[b] Inferred from multilocus sequence typing (19).

The *L. monocytogenes* strains responsible for the major outbreaks from 1981 to 1992 (Canada, 1981; California, 1985; Switzerland, 1983 to 1987; France, 1992) were all serotype 4b and belonged to a small number of closely related clones, as evidenced by ribotyping, multilocus enzyme electrophoresis, and DNA macrorestriction pattern analysis (143). This group of closely related, epidemic-associated strains was designated ECI. The 1998–1999 and 2002 multistate outbreaks in the United States were caused by strains of a novel epidemicassociated lineage, ECII (20).

During the past decades, several outbreaks of febrile gastroenteritis caused by *L. monocytogenes* have been documented (Table 17.3.). These gastroenteritis outbreaks differ in several respects from the invasive outbreaks just described. They affect persons with no known predisposing risk factors for listeriosis. The infectious dose appears to be much higher (1.9×10^5 to 1.6×10^9 CFU/g or CFU/ml) than that for typical invasive listeriosis in the susceptible population. Finally, the symptoms of gastrointestinal listeriosis appear within a day or less (typically in 18 to 27 h) of exposure (similar to other bacterial enteric infections), in contrast to the several weeks or more of incubation observed for invasive listeriosis.

Table 17.3 Outbreaks of gastrointestinal manifestation of listeriosis in humans with no known predisposing condition[a]

Yr(s)	Geographic location	Incubation time (h)	No. of persons affected	Symptoms	Vehicle Vehicle	Serotype	Contamination level (CFU/g or ml)	Ref
1986–87	Pennsylvania	Unknown	Unknown	Fever, vomiting, and diarrhea in the week before positive culture	Unknown	Multiple serotypes	NA	273
1989	United States	NA	9	Fever, muscle pain, gastroenteritis	Shrimp	4b	NA	273
1991	Tasmania, Australia	NA	4	Gastrointestinal	Smoked mussels	1/2a	NA	274
1993	Italy	18–43	18	Gastrointestinal, flu-like	Rice salad	1/2b	NA	275
1996	Illinois	20	80	Fever, diarrhea	Chocolate milk, temperature abused	1/2b	10^9	102
1998?	Finland	<27	5	Nausea, abdominal cramps, diarrhea, fever	Cold-smoked rainbow trout	1/2a	1.9×10^5	70
1997	Northern Italy	24	1,566	Headache, abdominal pain, diarrhea, fever	Cold salad of corn and tuna	4b	10^6	144
2000	Australia		31	Fever, muscle pain, headache, diarrhea	RTE corned beef and ham	1/2	1.8×10^7	277
2001	Los Angeles, CA	NA	16	Body aches, fever, headache, diarrhea, vomiting	Precooked, sliced turkey	1/2a	1.6×10^9	278
2001	Sweden	31	120	Diarrhea, fever, stomach cramps, vomiting	Cheese from fresh, raw milk	1	6.3×10^7	279
2001	Japan	24–144	38	Gastrointestinal, flu-like symptoms	Locally made cheese	1/2b	$<0.3 \times 10^7$ to 4.6×10^7	49
2002	British Columbia, Canada	NA	86	Febrile gastroenteritis	Cheese made from pasteurized milk/water	NA	10^2 to 10^9	280

[a] NA, not available.

CHARACTERISTICS OF DISEASE

Human disease caused by *L. monocytogenes* predominantly occurs in certain well-defined high-risk groups that include pregnant women, neonates, immunocompromised adults, and the elderly but has also been documented in individuals with no predisposing underlying conditions. In nonpregnant adults, *L. monocytogenes* primarily causes septicemia, meningitis, and meningoencephalitis, with a mortality rate of 20 to 30%. Other infrequent manifestations of listeriosis in this population include endocarditis in persons with underlying cardiac lesions (including prosthetic or porcine valves) and various types of focal infections, including endo-ophthalmitis, septic arthritis, osteomyelitis, pleural infection, and peritonitis (102). Clinical conditions known to predispose persons to the serious manifestations of listeriosis include malignancy, organ transplants, immunosuppressive therapy, HIV infection, and advanced age. Although pregnant women, particularly in the third trimester of pregnancy, may experience only mild flu-like symptoms (fever and myalgias with or without diarrhea) from exposure to *L. monocytogenes*, the infection has serious consequences for the fetus, leading to stillbirth or abortion. In neonates who are less than 7 days old, sepsis and pneumonia are the predominant syndromes, whereas in older neonates, the infection manifests as meningitis and sepsis.

Several investigations of listeriosis outbreaks show that *L. monocytogenes* can also cause febrile gastroenteritis in otherwise healthy hosts (Table 17.3.). Interestingly, Murray and Pirie clearly stated in their original descriptions of cases from the 1920s that diarrhea was a common feature of listeriosis in small animals (3). The most compelling evidence for the gastrointestinal manifestation of *L. monocytogenes* infections comes from outbreak investigations by Dalton et al. (45) and Aureli et al. (144). Fever and diarrhea are the most consistent symptoms in gastrointestinal listeriosis. Also, the incubation time for the enteric form is rather short, usually in the range of 18 to 27 hours. However, the incubation time varied between 24 and 144 hours in a 2001 Japanese outbreak involving cheese, possibly because of wide-ranging contamination levels (Table 17.3.). In the five investigations in which attempts were made to quantify the numbers of *L. monocytogenes* cells in the implicated foods, contamination levels were very high (range, 1.9×10^5 to 1.6×10^9 CFU). From the data available thus far for gastrointestinal listeriosis, it appears that infection requires a high dose of *L. monocytogenes*. It is not known whether the strains involved in the gastrointestinal forms of listeriosis possess additional virulence factors similar to those of common enteric pathogens.

While listeriosis outbreaks attract the most attention, most cases of human listeriosis occur sporadically. However, some of these sporadic cases may be unrecognized common-source clusters. The source and route of infection of most of these cases remain unknown, although foodborne transmission has been either demonstrated or suspected in most instances. Many cases have not been associated with food because of the difficulties associated with prospective investigations. Long incubation times (up to 10 weeks) make accurate food histories difficult if not impossible to obtain, and incriminated foodstuffs almost always have been consumed or discarded.

Understanding the epidemiology of sporadic cases is critical to the development of effective control strategies. An active surveillance program for listeriosis in the United States from 1989 to 1991 based on a population of 19 million people yielded an estimated incidence of 7.9 cases per million. A case-control study of dietary risk factors conducted during the same period indicated that foodborne transmission was responsible for about one-third of cases and that patients with listeriosis were more likely than controls to have eaten soft cheeses or foods purchased from delicatessen counters (145, 146). By 1993, the incidence of sporadic cases of listeriosis in the active surveillance sites had declined to 4.4 cases per million (147), with this decrease being attributed to enhanced efforts by the food industry and regulatory agencies to prevent contamination of processed foods. The 2010 data from CDC's foodborne diseases active surveillance program (FoodNet) indicate that the incidence of listeriosis in the United States had declined to 2.4 cases per million population (148), with an estimated 1,600 cases occurring annually, including 255 fatalities (5).

Although exposure to *L. monocytogenes* is common, invasive listeriosis is rare. It is unclear whether this is due to early acquired protection, to intrinsic resistance of the average healthy host, or to most strains being only weakly virulent. Despite improvements in the emerging picture concerning the key

events affecting the relative susceptibility of humans to this pathogen (149), there remain gaps in our understanding of the onset of disease (see below). Between 2 and 6% of healthy individuals are asymptomatic fecal carriers of *L. monocytogenes*. The risk of clinical disease in intestinal carriers of *L. monocytogenes* is unknown but must be very low, given the rarity of diagnosis. Endogenous infections by *L. monocytogenes* in the gut are plausible, especially in patients receiving immunosuppressive therapy, which can impair resistance to infection and alter the intestinal defense mechanisms to favor listerial invasion. Nevertheless, asymptomatic fecal carriage has been observed in pregnant women who proceed to normal birth at term, and women who have given birth to infected infants do not necessarily suffer the same problem in later pregnancies. Similarly, recent transplant recipients may harbor *L. monocytogenes* in the gut without developing the disease.

Epidemiologic studies since 1981 have focused on the role of contaminated food in transmission of listeriosis. However, two unusual transmission routes have been described. Hospital-acquired listeriosis is sporadically observed, mainly in nurseries, with equipment serving as the vehicle. During intrauterine infections, amniotic fluid can contain up to 10^8 *L. monocytogenes* CFU/ml (150). Mineral oil was also implicated in one outbreak of neonatal listeriosis (151). Primary cutaneous infections without systemic involvement have been observed as an occupational disease in veterinarians and farmers, with most cases being caused by manipulation of presumably infected bovine fetuses or cows.

INFECTIOUS DOSE AND SUSCEPTIBLE POPULATIONS

Infectious Dose

The infectious dose of *L. monocytogenes* depends on many factors, including the immunological status of the host, the type of food consumed, the degree of virulence of the particular strain, and the numbers ingested, as seen from the previously discussed outbreaks (152). The severity of the disease is such that tests with human volunteers are impossible. Studies involving monkeys and other animals suggest that reducing levels of exposure will reduce clinical disease (153). However, these experiments do not help to determine the minimal infective dose for humans. Further, the current assumption is that for infectious and toxicoinfectious agents, a single cell has a small but finite probability of causing disease. In the case of *L. monocytogenes*, a risk assessment by the WHO and the Food and Agriculture Organization of the United Nations estimated that the median probability for a single cell causing listeriosis is in the range of 10^{-12} to 10^{-14} (154) based on exposure data and annual disease statistics. When exposed to *L. monocytogenes* at the beginning of the third trimester, pregnant rhesus monkeys showed an increased risk of delivering a stillborn infant with pathology similar to that of humans, including acute inflammation, placentitis, fetal liver necrosis, and isolation of *Listeria* from the placental and fetal tissues (155).

The populations of *L. monocytogenes* in contaminated food responsible for epidemic and sporadic foodborne cases usually contained more than 100 CFU/g. The frankfurters implicated in the 1998 listeriosis outbreak in the United States contained less than 0.3 CFU/g (124), while ice cream implicated in a 2014–2015 outbreak yielded *L. monocytogenes* at an average most probable number of 8/g (125). However, these results may not accurately reflect the numbers consumed, since *Listeria* populations in the food would be expected to change between the time of consumption and analysis. It is clear that the risk of foodborne listeriosis is overwhelmingly associated with RTE foods that support the growth of *L. monocytogenes* at refrigeration or marginal abuse temperatures. Hence, additional epidemiologic information is clearly needed to more accurately assess the infectious dose.

Susceptible Populations

Most human cases of listeriosis occur in individuals who have a predisposing disease that leads to impairment of their T-cell-mediated immunity (152). The percentage of patients suffering from a known underlying condition varies from 70 to 85% in some surveys to nearly 100% in others (156, 157). The most commonly affected populations include those at either age extreme (neonates and the elderly), pregnant women, and individuals who are immunosuppressed by medication (corticosteroids

or cytotoxic drugs), especially after organ transplantation or illness (hematologic malignancies, such as leukemia, lymphoma, and myeloma, as well as solid malignancies). Listeriosis is 300 times more frequent in people with AIDS than in the general population (158). In addition to impaired T-cell immunity, a small percentage of listeriosis patients suffer from chronic diseases not usually associated with immunosuppression, such as congestive heart failure, diabetes, cirrhosis, alcoholism, and systemic lupus erythematosus, alone or in association with known predisposing diseases. Mortality in listeriosis cases is almost exclusively associated with predisposing diseases and conditions (159), with recent transplant patients being the subpopulation at greatest risk, as they are ~2,500 times more susceptible than healthy adults under 65 years of age (154).

Concurrent infection can also influence susceptibility to listeriosis. This was exemplified by a cluster of cases in 1987 in Philadelphia that were caused by a number of different strains. Clinical and epidemiologic investigations suggested that individuals who were previously asymptomatic for listerial infection but whose gastrointestinal tract harbored *L. monocytogenes* became symptomatic, possibly because of a coinfecting agent (158). However, a single food vehicle could not be identified due to the diversity of strains.

VIRULENCE FACTORS AND MECHANISMS OF PATHOGENICITY

In the early 1980s, *L. monocytogenes* was an attractive model system to study both intracellular parasitism and the immunological response of humans and animals to infection. This was due to five main factors: (i) *L. monocytogenes* grows well in culture media, (ii) it can be genetically manipulated, (iii) it belongs to a genus that contains pathogenic and nonpathogenic species, (iv) it infects mammalian cells in tissue culture, and (v) several laboratory animals are susceptible to *Listeria* (160). The *L. monocytogenes* genome was first sequenced in 2001, paving the way for the discovery of a series of new virulence factors (102, 161, 162). Since that initial study, genome sequencing has become the tool of choice for outbreak investigations of *L. monocytogenes*. In addition, as genomic data can be used for taxonomic purposes, genome sequences for representatives of all *Listeria* species are available (10, 163).

Pathogenicity of *L. monocytogenes*

Many tests for addressing *L. monocytogenes* pathogenicity have been developed, including tissue culture assays and tests using laboratory animals, in particular immunocompetent and immunocompromised mice (164, 165). Animal models routinely involve intraperitoneal or intragastric infection, and virulence is evaluated either by comparing the LD_{50}s or by enumerating bacteria in infected target organs, in particular the spleen and liver. Heterogeneity in the virulence of *L. monocytogenes* has been observed in several *in vitro* and *in vivo* studies (LD_{50}s of *L. monocytogenes* strains range from 10^3 to 10^9 CFU) (153, 166, 167), depending on the animal or *in vitro* system being used and whether the immune system of the animal was suppressed prior to exposure. No clear correlation between the level of virulence and the origin (human, animal, category of food, or environment) or the strain characteristics (serotype, phage type, multilocus enzyme type, or DNA micro-or macrorestriction patterns) could be established. Diet may also play a role, as a recent study showed that even short-term exposure to a high-fat diet increased susceptibility of mice to *Listeria* infection (168).

However, only 3 of the 13 known serovars of *L. monocytogenes*, 1/2a, 1/2b, and 4b, account for more than 90% of human cases of listeriosis (158, 169). Among the listeriosis-associated serotypes, 4b strains cause a large proportion of listeriosis outbreak cases worldwide, serotypes 1/2a and 1/2b account for a significant portion of sporadic cases, and serotypes 1/2a, 1/2b, and 1/2c predominate in food isolates (67, 170–172). As discussed earlier, specific ECs of serotype 4b and 1/2a have been linked to specific single nucleotide polymorphisms. The relatively low percentage of cases caused by serotypes 1/2a, 1/2b, and 1/2c from foods appears to be strongly influenced by mutations leading to premature stop codons in *inlA*, which encodes internalin A. Although rare nonpathogenic or weakly pathogenic *L.*

monocytogenes isolates have been reported, all strains of *L. monocytogenes* are considered potentially capable of causing human disease.

There has been substantial debate over the appropriateness of various routes of administration and animal models for studying *L. monocytogenes* pathogenicity, particularly in regard to fetal infections. Since most listeriosis cases are associated with foodborne transmission, it is generally accepted that an oral route of administration is most suitable, particularly since there can be substantial differences in the ability of *L. monocytogenes* strains to cross the intestinal barrier. Crossing both the intestinal and placental barriers involves two proteins, internalin A (InlA) and internalin B (InlB), which mediate high-specificity attachment to specific epithelial cells and low-specificity attachment to a wide variety of cell types, respectively (173). The former attaches to the Ecadherin receptor, while the latter attaches to the hepatocyte growth factor (Met) receptor. E-cadherin receptors differ among mammalian species, with InlA from *L. monocytogenes* binding to the receptors on the intestinal epithelial cells of humans, guinea pigs, rabbits, and gerbils but not those of mice or rats, bringing into question the appropriateness of using mice to study orally transmitted listeriosis. Similarly, there are differences in host specificity related to the ability of *L. monocytogenes* to cross the placental barrier, with the pathogen invading the barrier in humans, rats, rabbits, and gerbils but not in mice or guinea pigs. High-efficiency invasion of the placental barrier requires receptors for both InlA and InlB (173, 174). There have been an increasing number of dose-response trials and related research that have focused on the use of rhesus monkeys, guinea pigs, gerbils, or "humanized" mice (155, 175–178).

Experimental Infection and Cell Biology of the Infectious Process

Much of the original work on the disease course of *L. monocytogenes* was performed with mice injected intravenously, with subsequent monitoring of bacterial growth kinetics in the spleen and liver. Within 10 minutes after intravenous injection, 90% of the inoculum is taken up by the liver and 5 to 10% by the spleen. During the first 6 hours, the number of viable *Listeria* cells in the liver decreases 10-fold, indicating rapid destruction of most of the bacteria. Surviving *Listeria* cells then multiply within susceptible macrophages and grow exponentially in the spleen and liver for the next 48 hours, peaking at day 2 or 3 postinfection (179). Rapid inactivation ensues during the next 3 to 4 days, indicating recovery of the host. Convalescent mice are resistant to challenge and have a delayed-type hypersensitivity characterized by swelling of the footpads injected with crude cell preparations of *L. monocytogenes*.

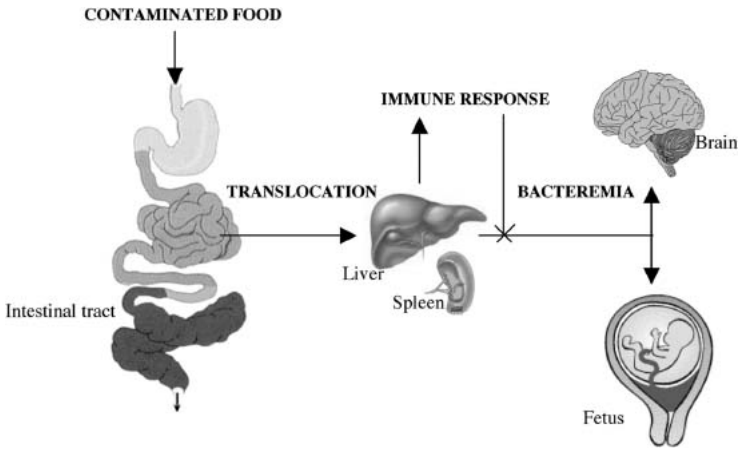


Figure 17.2 Schematic representation of the pathophysiology of *Listeria* infection.

However, as described above, the natural route of foodborne infections with *L. monocytogenes* in humans is via the gastrointestinal tract. *L. monocytogenes* infects intestinal epithelial cells in a process that requires the interaction of InlA, expressed at the bacterial surface, with epithelial E-cadherin, expressed at the surface of epithelial cells (180). Although *L. monocytogenes* has a relatively broad host range, the relative susceptibility of mammalian species to ingested *L. monocytogenes* varies based

in large part on the attachment to and invasion of the intestinal epithelium. As indicated above, this reflects differences in the epithelial receptors. For example, mice are relatively resistant to intestinal infection with *L. monocytogenes* because of a single amino acid difference between human and mouse E-cadherin (181). Recent studies using different *L. monocytogenes* strains report that, in contrast to strains from other serovars, serotype 4b epidemic strains appear to be able to cause systemic infection in mice infected orally. This suggests that there might be serovar-specific virulence factors playing a role in mouse susceptibility to orally acquired listeriosis (182). The probability that an oral infection will lead to perinatal infections has been related to the probability of specific "key events": (i) survival of the pathogen in the upper gastrointestinal tract, (ii) attachment to and uptake into the intestinal epithelial cells, (iii) survival and escape from phagosomes and transfer to phagocytes, (iv) transfer across the placenta, and (v) pathogen growth leading to fetal morbidity and mortality (149).

From the intestinal lumen, bacteria traverse the epithelial-cell layer and disseminate via the bloodstream to other organs, such as the spleen and liver, where they are internalized by splenic and hepatic macrophages in which they can survive and replicate (Fig 17.2). When the *Listeria* cells reach the liver and the spleen, most are rapidly killed. In the initial phase of infection, infected hepatocytes are the targets for neutrophils and later for mononuclear phagocytes that are responsible for control and resolution of infection. The bacterial cells are subsequently transported via the blood to regional lymph nodes. Depending on the level of T-cell response induced in the first days following initial infection, further dissemination may subsequently occur via the blood to the brain or, in the pregnant animal, the placenta.

L. monocytogenes possesses the ability to cross the maternofetal barrier and leads to placental abscesses, chorioamnionitis, and finally infection of the fetus. As the highest concentrations of *L. monocytogenes* are encountered in the gut and in the lung, it is thought that infection might be amplified through ingestion of contaminated amniotic fluid rather than solely as a consequence of the hematogenous transplacental route (183). Several animal models have been developed to study pregnancy-associated (15, 184–186) and pulmonary (186) *L. monocytogenes* infections.

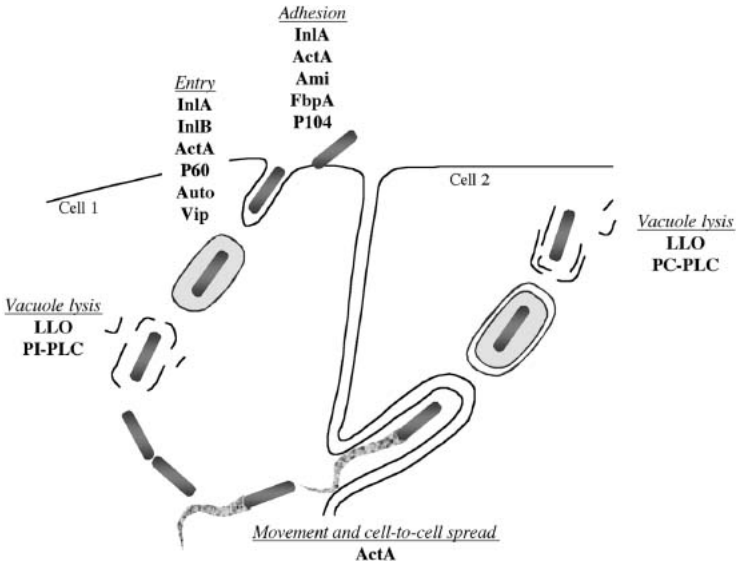


Figure 17.3 Schematic representation of the successive steps of the cell infection process. Factors implicated in the different steps are indicated.

L. monocytogenes has, in addition to its ability to cross initially the intestinal barrier and the maternofetal barrier, the capacity to cross the blood-brain barrier and reach the central nervous system (CNS) and cause meningitis, encephalitis, and brain abscesses. Invasion of brain cell endothelial cells seems a prerequisite for meningeal pathogens that penetrate the CNS. It has been proposed that *L. monocytogenes* utilizes penetration of human brain microvascular endothelial cells as a means of

crossing the blood-brain barrier. *L. monocytogenes* adheres to human brain microvascular endothelial cells through the microvilli and then infects them by an InlBdependent mechanism (187).

Once inside a susceptible host, *L. monocytogenes* has the potential to disseminate and multiply in a wide vari ety of cell types and tissues. *Listeria* is primarily found intracellularly due to its ability to induce its own phagocytosis into many cell lines (e.g., macrophages, fibroblasts, hepatocytes, and epithelial cells) that are normally nonphagocytic (188). Detailed analysis of infected-tissuecultured cells reveals a complex series of interactions between the bacteria and the cell (Fig 17.3). Host cell infection begins with the adhesion and internalization of the bacteria either by phagocytosis in the case of macrophages or by induced phagocytosis (invasion) in the case of normally nonphagocytic cells. It has been suggested that at the intestinal epithelium, InlA provides high-specificity adhesion while InlB activates the c-MET receptor, which in turn accelerates junctional endocytosis into the enterocytes (189), with goblet cells being the specific location of attachment (190). Bacterial invasion starts by a close contact with the plasma membrane that progressively enwraps the bacterium. This process is usually referred to as the "zipper" mechanism, in contrast to the "trigger" mechanism used by *Salmonella* or *Shigella* (191).

Following internalization, bacteria reside within membrane-bound vacuoles (phagosomes) for ~30 min. *L. monocytogenes* escapes the vacuoles by perforating the membrane by the combined action of listeriolysin O (LLO) and two phospholipases. Neumann et al. (192) concluded that *L. monocytogenes* could escape phagosomes independent of listeriolysin in interleukin-4-deactivated human macrophages. Once free in the cytosol, *L. monocytogenes* replicates and becomes covered with actin filaments. These filaments rearrange within 2 hours into long comet tails (up to 40 μm in length) left behind in the cytosol while the bacteria move ahead at a speed of ~0.3 μm/s (193, 194). When moving bacteria contact the plasma membrane, they induce the formation of bacterium-containing protrusions. Contact between these protrusions and neighboring cells results in the internalization of the protrusion (195). In the newly infected cell, the bacterium is surrounded by two plasma membranes that must be lysed to initiate a new cycle of multiplication and movement. Lysis of the two membrane vacuoles requires at least four virulence factors: LLO, the metalloprotease Mpl, and two phospholipases (see below). The entire cycle is completed in about 5 hours. This process allows *L. monocytogenes* to disseminate by direct cell-to-cell transfer, thereby avoiding humoral defenses such as antibodies. This accounts for the early observations that antibodies are not protective and that immunity to *Listeria* is T cell mediated (160).

Virulence Factors

The overall pathogenesis of *L. monocytogenes* and identification of individual virulence determinants have been studied extensively using a variety of genetic techniques. Conjugative transposons from the Tn*1545*-Tn*916* family or Tn*917* transposons were initially exploited to generate mutant libraries, allowing the identification of several *L. monocytogenes* virulence factors (196, 197). Additionally, Tn*1545*-and Tn*917*-derived tagged transposons were also used for signature-tagged mutagenesis (198, 199). As previously discussed, genome sequencing of multiple strains has allowed the direct comparison of pathogenic and nonpathogenic isolates for the identification of specific virulence determinants. Plasmid vectors originating from *Bacillus subtilis* and *Escherichia coli* are used for genetic studies in *L. monocytogenes*, including allelic exchange of chromosomal DNA, cloning, gene expression, or reporter gene fusion (200). For allelic exchange, inframe deletion, or site-specific mutagenesis, thermosensitive vectors such as pKSV7 (201) and pAUL-A (202) are used. A novel thermosensitive plasmid, pMAD, harboring the β -galactosidase gene, facilitates the screening and the generation of allelic exchanges (203). For complementation and gene expression, shuttle plasmids replicating in *E. coli* and *L. monocytogenes* are mostly used, such as pAT18, pMK4, and pAM401 (204–206).

Complementation of deletion mutants can now be achieved using a new integrative phage-derived plasmid, pPL2, that allows a single-copy integration at a specific location in the *L. monocytogenes* chromosome (207). Listeriae do not seem naturally competent; however, transformation with plasmid DNA can be obtained on protoplasts or by electroporation (208, 209). In addition to traditional

molecular methods, WGS and single-nucleotide-polymorphism analyses have been used extensively to study the evolution of *Listeria* virulence, *L. monocytogenes* population genetics, and genomic evolution.

These tools have led to the successive identification of multiple virulence determinants that are necessary to modulate the ability of *L. monocytogenes* to cause disease and determine the relative virulence ability of individual isolates. Gene expression analysis is providing an appreciation of the large number of genes (>70) that may be involved in *in vivo* infections and the complexity associated with the disease course and the ability of the microorganism to evade cellular defenses (210). Some of the well-defined virulence determinants are described in more detail below in relation to their role in *L. monocytogenes* pathogenesis.

Adhesion to Mammalian Cells

The first step in the disease course of foodborne listeriosis is the attachment of *L. monocytogenes* to the intestinal epithelium. In addition to its role as the first step in the internalization and passage through the intestinal epithelium, adhesion may promote colonization of the gastrointestinal tract, may direct the bacteria to appropriate target cells or tissues such as the CNS or the placenta, and may even facilitate cell invasion by activating cell signal transduction pathways or triggering the synthesis of a target cell receptor required for invasion.

Adherence involves a number of surface proteins, including InlA, ActA, Ami, p104, and FbpA. InlA is the first member of the internalin multigene family, a family characterized by the presence of a leucine-rich repeat (LRR) region (211). InlA promotes adherence and entry into cells expressing its receptor, the adhesion protein Ecadherin (180) (see above and below). ActA, the surface protein required for actin-based motility, may also promote attachment via host cell proteoglycans (212, 213). An ActA-deficient mutant is significantly impaired in attachment to and entry into IC-21 murine macrophages and Chinese hamster ovary epithelial cells. Ami is a bacterial surface amidase that presents an N-terminal domain similar to the amidase domain of the Atl autolysin of *Staphylococcus aureus* and a C-terminal, cell wallanchoring domain made up of eight GW dipeptide modules (214). Besides its lytic activity on *Listeria* cell walls (215), Ami is implicated in bacterial adhesion to target cells (216). Ami mutants are attenuated, indicating that Ami plays a role in the virulence of *L. monocytogenes*. Finally, FbpA is a fibronectin-binding protein shown to be required for intestinal and liver colonization after oral infection of transgenic mice expressing human Ecadherin. FbpA binds to immobilized human fibronectin and increases adherence of wild-type *L. monocytogenes* to HEp-2 cells. Despite the lack of conventional secretion and anchoring signals, FbpA is detected on the bacterial surface and was shown to be a substrate of the SecA2 pathway (199). In addition, FbpA behaves as a chaperone for two important virulence factors, LLO and InlB, probably preventing their degradation. The roles of surface proteins, including adhesion, have been reviewed by Bierne and Cossart (217).

Entry into Mammalian Cells

InlA and InlB were the first listerial factors identified as mediating bacterial invasion into different target cell types. As discussed above, their cellular receptors have also been identified. The molecular signaling cascades triggered during *Listeria* entry into host cells are being characterized in detail. InlA and InlB are just two of a family of internalins (e.g., InlC, InlD, InlH, and InlJ) that are characterized by the presence of LRRs (211). Some internalins are present in most lineages, whereas others are associated with specific lineages or serotypes (8). Some are associated with adhesion to enterocytes (InlA), whereas others may play a role in stimulating junction endocytosis (see above). Recent findings suggest that other molecules are also necessary for internalization (P60, ActA, Auto, and Vip), revealing a complex dialogue between *Listeria* and eukaryotic cells during the early phases of the infection cycle.

Internalins

InlA (800 amino acids) displays a classical signal sequence followed by an LRR region comprising 15 repeats of 22 amino acids. An interrepeat (IR) region separates the LRR region from a second repeat

region (called the B repeat region). The carboxy terminus displays a cell wall-sorting motif, LPTTG, which is the substrate for the enzyme sortase A and allows covalent linkage of the protein to the peptidoglycan. The LRR and IR regions are necessary and sufficient to promote *Listeria* entry into human epithelial cells (181). E-cadherin, the InlA receptor (180), belongs to the cadherin superfamily of cell adhesion molecules, which are transmembrane glycoproteins located at adherens junctions and allow cell-cell adhesion. The species-specific nature of the interaction between *L. monocytogenes* and animal hosts (see above) involves recogni tion of a proline at position 16 (Pro16) of the E-cadherin molecule (218). Also, as mentioned above, the truncation of InlA that occurs naturally in some strains associated with foods helps to explain the decreased incidence of listeriosis cases associated with 1/2a, 1/2b, and 1/2c from food sources, and a recent epidemiologic survey indicates that clinical strains express a full-length InlA far more frequently than do strains recovered from food products (219, 220), revealing that InlA is critical for the pathogenesis of human listeriosis.

InlB (630 amino acids) is another member of the internalin family involved in entry of *Listeria* into a broad range of cell lines, including hepatocytes and nonepithelial cells (221) (see above). It contains a signal peptide, seven LRR repeats, one IR region, and one B repeat. The C-terminal domain presents three tandem repeats (GW modules) that mediate attachment of InlB to the bacterial cell wall through noncovalent interaction with lipoteichoic acids (211). The LRR region of InlB is sufficient to confer invasiveness to noninvasive *L. innocua* or to latex beads (222). InlB directly binds to cellular glycosaminoglycans (GAGs) through its GW modules. InlB-dependent entry into epithelial cells is strongly affected by depletion of the cellular plasma membrane GAGs. It was proposed that interaction of InlB with GAGs through its GW modules leads to its detachment from the bacterial surface, allowing its clustering at the cellular surface through binding to Met by its LRR domain and favoring the local activation of the signaling pathway downstream of Met (223).

P60 Protein

For a long time, Δ*iap* (encoding invasion-associated protein) mutants could not be obtained, suggesting that the protein was essential for bacterial viability. Therefore, the role of P60 (encoded by *iap*) was first evaluated in spontaneous rough mutants expressing lower levels of P60 and forming long filamentous structures composed of bacterial chains (224). The rough mutants are less virulent and enter certain eukaryotic cells less efficiently, suggesting a role for P60 in bacterial invasion (225).

Ultimately, a viable Δ*iap* mutant was obtained, allowing more precise studies of the role of P60. Like rough mutants, these also had a defect in septum formation and in virulence after intravenous infection of mice. In addition, the Δ*iap* mutant is impaired in bacterial movement and spreading from cell to cell due to an improper localization of ActA at the surface of *L. monocytogenes* (226, 227). However, in contrast to studies using rough mutants, the invasiveness of the Δ*iap* mutant in mouse fibroblasts and human epithelial cells is only slightly diminished compared with that of wild-type bacteria (226, 227). P60 is an autolysin both secreted by and associated with the bacterial cell wall (224, 228). Secretion is mediated as for FbpA by the recently identified auxiliary secretion system SecA2, which mediates the secretion of at least 17 secreted and surface proteins of *L. monocytogenes* (226, 227). In addition, P60 plays an important role in the immune response against *L. monocytogenes* infection.

ActA Protein

ActA is a surface protein implicated in the attachment to cells (see above) and responsible for the actin-based motility of *Listeria* (see below). Moreover, expression of ActA in *L. innocua* is sufficient to promote bacterial entry in some epithelial cell lines (212).

Auto Protein

A novel autolysin-encoding gene, *aut*, has been identified (229). It is the only autolysin gene that is absent from the nonpathogenic species *L. innocua*. The *aut* gene encodes a surface protein, Auto, with an autolytic activity, as expected from the presence of a domain harboring homologies with autolysin-

encoding genes, especially *N*-acetylglucosaminidases. The protein Auto possesses a C-terminal cell wall-anchoring domain made up of four GW modules, similar to those observed in the other autolysin, Ami, and in InlB (230). The morphology of an *aut* deletion mutant is similar to that of the wild type, with no defect in septation and cell division, suggesting no role for Auto in these functions (229). Auto is required for entry of *L. monocytogenes* into different nonphagocytic eukaryotic cell lines. An *aut* deletion mutant has reduced virulence following intravenous inoculation of mice and oral infection of guinea pigs, which correlates with its low invasiveness. However, the autolytic activity of Auto by itself, rather than an invasive ability, might be critical for virulence. Indeed, Auto may control the general surface architecture exposed to the host by *L. monocytogenes* and/or the composition of the surface products released by the bacteria, hence affecting the host response to infection (229).

Vip Protein

The *vip* gene encodes an LPXTG surface protein absent from nonpathogenic *Listeria* species (231). *vip* is positively regulated by PrfA, the transcriptional activator of the major *Listeria* virulence factors. Vip is anchored to the *Listeria* cell wall by sortase A and is required for entry into some mammalian cells. Using a ligand overlay approach, a Vip cellular receptor was identified. It is the endoplasmic reticulum-resident protein Gp96, reported to also interact with Toll-like receptors (232). The Vip-Gp96 interaction is critical for bacterial entry into some cells. Comparative infection studies using oral and intravenous inoculation of nontransgenic and transgenic mice expressing human Ecadherin demonstrated a role for Vip in *Listeria* virulence not only at the intestine level but also in late stages of the infectious process. Vip thus appears to be a new virulence factor exploiting Gp96 as a receptor for cell invasion and/or signaling events that may interfere with the host immune response in the course of the infection (231).

Sortases

The genome of *L. monocytogenes* EGD-e contains 41 genes that encode LPXTG proteins, including InlA and Vip (230). LPXTG proteins are anchored to the peptidoglycan by transpeptidases named sortases (230). *srtA* encodes a sortase responsible for the anchoring of InlA, Vip, and at least 13 other proteins to the peptidoglycan (217). *L. monocytogenes* mutants lacking *srtA* are defective in internalizing into human enterocyte-like Caco-2 cells and hepatocytic HepG2 cells, as well as in colonizing the livers and spleens of mice infected orally. The *Listeria* genome encodes another sortase, SrtB, which has a limited number of substrates containing a C-terminal NXXTN sorting motif. SrtB is not required for the infectious process (233).

Escape from the Phagosome and Intracellular Growth

Once *L. monocytogenes* is internalized, the next key step in the disease course is to escape the vacuole or phagosome via the concerted action of LLO and two phospholipases C, each of which is considered a virulence determinant. Once in the target cell cytoplasm, *Listeria* then multiplies, acquiring nutrients from the cytosol.

Listeriolysin O

Nonhemolytic *L. monocytogenes* strains can be found in the environment but are avirulent in experimentally infected mice. Nonhemolytic mutants generated by conjugative transposons were found to be similarly avirulent in mice (234). Both wild-type and nonhemolytic strains entered mammalian cells but were unable to grow intracellularly due to an inability to escape from the phagocytic vacuole (235). Subsequent analysis indicated that the mutations resulted in disruption of *hly*, which encodes LLO. LLO is a pore-forming toxin that enables the bacterium to escape into the cytosol, where it replicates. LLO accomplishes this by binding to cholesterol and then oligomerizing into large complex pores. LLO is a 60-kDa secreted protein involved in *Listeria*'s escape from both primary and secondary vacuoles (236). LLO is a member of the pore-forming, cholesterol-dependent cytolysin family. These thiol-activated toxins are produced by several Gram-positive bacteria, including *Streptococcus*, *Clostridium*, and *Bacillus* species (236). They are active only on cholesterol-containing

membranes, with cholesterol likely acting as the receptor in the membrane. Unlike all other thiol-activated toxins, LLO has a maximal activity at pH 5 and is inactive at pH 7, and thus, its deleterious effect on cellular membranes is impaired when the bacterium is free in the cytosol. In addition, optimal pore formation by LLO occurs between pH 5.5 and 6.0 (the pH of the early phagosome). A second hemolysin, listeriolysin S, has been isolated from specific strains of lineage I *L. monocytogenes*, leading to the suggestion that this second hemolysin may account for the increased pathogenicity of this lineage (188).

Phospholipases

Two phospholipases are produced and secreted by *L. monocytogenes*: phosphoinositide-phospholipase C, encoded by the gene *plcA*, and a broad-range phospholipase, phosphatidylinositol-specific phospholipase C, encoded by *plcB* (237). The two phospholipases have membranedamaging activity and are involved in bacterial escape from primary and/or secondary phagosomes (237).

Once free in the cytosol, *L. monocytogenes* starts multiplying, with an approximate doubling time of 1 hour (238). A number of genes are upregulated as the microorganism grows within the host tissue (210). This includes several genes encoding virulence or metabolic determinants induced during the intracellular life of *L. monocytogenes*, including those involved in phagosomal lysis, actin-based motility, and cell-to-cell spreading. In contrast to most bacteria, *L. monocytogenes* replicates in the cytosol when it is directly microinjected into cells (239). The cytosol permissiveness for *L. monocytogenes* growth is likely due to the bacterium's ability to use a variety of cytosolic nutrients, as suggested by the fact that intracellular multiplication of several auxotrophic mutants is not affected (240). Intracytosolic growth of *L. monocytogenes* is highly dependent on the product of the *hpt* gene, which encodes a sugar uptake system (239), and is tightly regulated by PrfA (241).

Intracytoplasmic Movement and Cell-to-Cell Spreading

Induced phagocytosis, escape from the phagosome, and intracellular multiplication are essential steps for infection of individual cells but are not sufficient to achieve infection. *L. monocytogenes* must also efficiently infect tissues by direct cell-to-cell spreading. As described above, direct cell-to-cell spreading is the result of intracellular movement, protrusion formation, phagocytosis of the protrusion by a neighboring cell, formation of a doublemembrane-bound vacuole, and lysis of the two-membrane vacuole. Intracytoplasmic movement is strictly coupled to continuous actin assembly that provides the force for bacterial propulsion (Fig 17.4). Actin polymerization requires expression of the *actA* gene. *actA* mutants are invasive, escape from the phagosome, replicate, and form intracellular microcolonies but are not covered with actin, do not move intracellularly, and do not spread from cell to cell. ActA is a 639-amino-acid protein that presents a signal sequence at its amino-terminal domain and a transmembrane motif at its carboxyl-terminal domain that anchors the molecule to the bacterial surface (242). Immunolocalization of the ActA protein in infected cells has revealed that ActA is asymmetrically distributed on the bacterial surface, with an increasing concentration towards the opposite pole of the bacterial cell, which is the site of comet tail formation. The amino-terminal region of ActA alone can induce bacterial movement (243). Actin filaments in *Listeria* tails, as in *Shigella* tails, are branched, in contrast to those in *Rickettsia conorii* tails, which display longer and unbranched actin filaments (244).

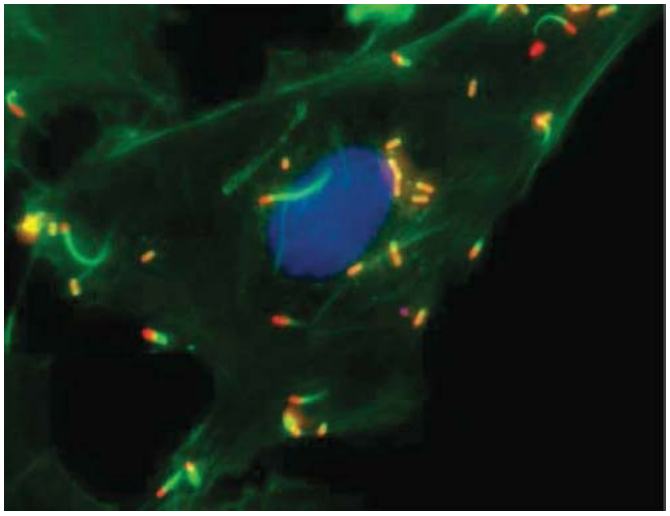


Figure 17.4 Directional actin polymerization by *L. monocytogenes*. *L. monocytogenes* isolates were processed for triple-labeling fluorescence microscopy, 5 h after the infection of Vero cells was started. Bacteria (red) were visualized with a polyclonal anti-*Listeria* antibody, actin (green) with phalloidin, and cell nuclei (blue) with DAPI (4′,6′-diamidino-2-phenylindole). Magnification, ×100.

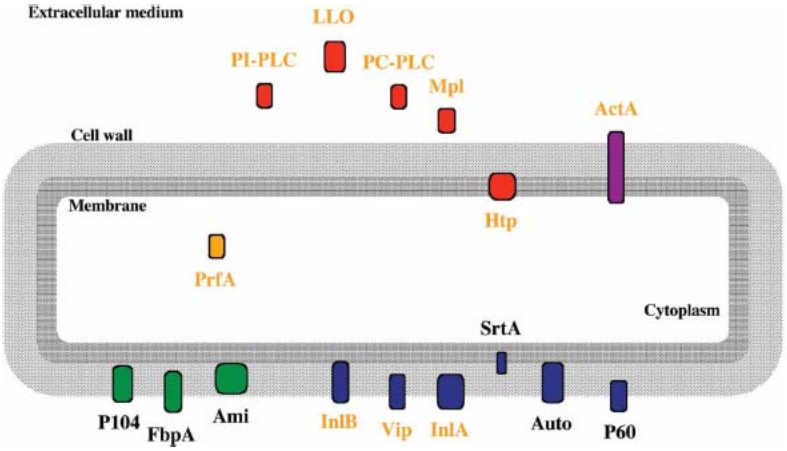


Figure 17.5 Schematic representation of *L. monocytogenes* virulence factors. The localization of factors implicated in adhesion (green), entry (blue), escape from the phagosome and intracellular growth (red), and intracytoplasmic movement and cell-to-cell spreading (purple) is indicated. The names of factors whose expression is regulated by PrfA are in orange. PI-PLC, phosphoinositide-phospholipase C; PC-PLC, phosphatidylinositol-specific phospholipase C.

Coregulation of Virulence Factors

Most *Listeria* virulence genes involved in the steps of the infection cycle (i.e., *prfA*, *plcA*, *hly*, *mpl*, *actA*, *plcB*, *inlA*, *inlB*, *inlC*, *hpt*, *bsh*, and *vip*) are regulated by the transcriptional activator PrfA (Fig 17.5) (245). PrfA is a protein comprising 233 amino acids that belongs to the Crp/Fnr family. Genes regulated by PrfA contain a binding site in the –41 region, the PrfA box, consisting of a 14-bp palindromic sequence to which the putative helix-turnhelix motif of PrfA binds *in vitro* (246). Expression of *prfA* is a complex phenomenon. *prfA* is the second gene of the *plcA-prfA* operon, whose primary promoter is regulated by PrfA. Hence, PrfA autoregulates (activates) its own synthesis. *prfA* can also be transcribed from its own promoter region. It has been determined that in a *prfA* mutant transcription at the *prfA*-specific promoter is upregulated. PrfA-regulated virulence factors such as LLO, PlcA, PlcB, and Vip are produced at higher levels as temperature increases to 37°C, the temperature of the host (237). At low temperatures, the untranslated leader region of the *prfA* mRNA

forms a stable secondary structure masking the Shine-Dalgarno sequence (247). Consequently, PrfA is not translated. At high temperatures, this structure, named the thermosensor, melts. Mutations destabilizing the thermosensor at 30°C mimic the effect of an increase in temperature in that they unmask the ribosome-binding site and lead to virulence gene expression. Knowledge of the *L. monocytogenes* genome sequence (161) allowed analysis of the complete PrfA regulon. A whole-genome array based on the sequence of the *L. monocytogenes* EGDe strain was constructed to study the PrfA regulon (241). This transcriptomic analysis revealed that PrfA could act both as an activator and as a repressor. It led to the identification of many new PrfA-regulated genes in addition to genes previously known to be part of the PrfA regulon. However, in many cases, the effect of PrfA is probably indirect. This analysis has been expanded upon in a second transcriptional analysis to identify the genes that are positively controlled by PrfA and upregulated in the host (210).

Stress Proteins of *L. monocytogenes*

L. monocytogenes has evolved a series of adaptive responses in order to cope with a large variety of stresses to survive and/or multiply under harsh environmental conditions, outside as well as inside the host. The LisRK two-component signal transduction system is implicated in virulence, acid and ethanol tolerance, and oxidative stress (248). *virR*, a gene encoding the response regulator of another two-component system, was shown to regulate a key regulon in *L. monocytogenes*, controlling virulence by a global regulation of surface component modifications (198). The DnaK heat shock chaperone protein is required for survival of *L. monocytogenes* at high temperatures and under acidic conditions as well as for efficient phagocytosis with macrophages (249). The major heat shock chaperones, GroES and GroEL, are induced at high temperature, at low pH, and during cell infection (250). The ClpC stress protein is required for survival under iron deprivation or high temperature or osmolarity in bone marrow macrophages and in organs of mice (251). Two other proteases have been reported to be involved in stress response and virulence, namely, ClpE and ClpP (252, 253).

The *fri* gene, which encodes ferritin, is necessary for optimal growth in minimal medium in both the presence and absence of iron, as well as after cold and heat shock. Ferritin also provides protection against reactive oxygen species and is essential for full virulence of *L. monocytogenes*. A comparative proteomic analysis revealed an effect of *fri* deletion on the levels of LLO and several stress proteins (254, 255). Adaptation to osmotic stress depends on the intracellular accumulation of osmolytes and thus on uptake systems that include BetL, GbuABC, OpuC, OpuB, and OppA (256, 257). Bile tolerance of *L. monocytogenes* involves the bile salt hydrolase Bsh, an enzyme that deconjugates bile salts and that is required for both intestinal and hepatic phases of listeriosis (258). The transporter BtlA (259) and other systems, such as the putative transporter of the glutamate decarboxylase GadE and the zinc uptake regulator ZurR, contribute to tolerance to bile or various other stresses, e.g., low pH, salt, ethanol, detergent, and antibiotic (260). The alternative sigma factor B contributes to the ability of *L. monocytogenes* not only to survive and/or multiply under stressful conditions outside the host (acid, osmotic, or oxidative stresses, low temperature, or carbon starvation) (261) but also to persist within the host during the infectious process.

It has been demonstrated that σ^B contributes to survival following exposure to bile salts (262) and also to transcription of the virulence gene activator PrfA (1). Characterization of the σ^B-dependent general stress regulon confirmed the broad role of this sigma factor (263). The lessons learned in relation to *L. monocytogenes* responses to stressors within the host are beginning to be used to study the impact of the pathogen's expression of stress responses and virulence determinants in foods and food processing environments (264, 265).

CONCLUDING REMARKS

Public health surveillance, outbreak investigations, and applied and basic research conducted during the past 40 years have helped to characterize the disease listeriosis, define the magnitude of its public health problem and its impact on the food industry, identify the risk factors associated with disease, and develop appropriate and targeted control strategies. The food processing industry has made

progress towards reducing the prevalence of *L. monocytogenes* in processing plant environments and in high-risk foods, and preventive measures have been developed and implemented for persons at increased risk of infection. These gains were reflected in a substantial decline in the incidence of listeriosis during the late 1990s and early 2000s. For example, in the United States, the incidence of listeriosis in 2005 was only 0.3 case per 100,000 population. However, since that time, the number of listeriosis cases has plateaued, with no substantial declines in the United States, and several European countries and South Africa have experienced increased incidences of listeriosis during the past 10 years. Furthermore, we have seen the association of listeriosis outbreaks with new foods such as fresh-cut produce. Despite ever-increasing knowledge concerning this pathogen, translation of that knowledge into effective control programs has lagged. It also implies that "doing the same old thing" is not likely to further reduce the public health burden of *L. monocytogenes*. Hopefully, the same progress in understanding the basic biology of this important pathogen during the past decade will translate in the next decade into effective risk-based food safety systems for preventing this rare but potentially lifethreatening foodborne disease.

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18
Clostridium botulinum

Botulism is a neuromuscular paralytic disease in humans and animals resulting from the actions of botulinum neurotoxins (BoNTs), which are produced by *Clostridium botulinum* and rare strains of *Clostridium butyricum* and *Clostridium baratii* (1–4). BoNTs are the most poisonous toxins known for humans and vertebrate animals (5–7) and are unique among most foodborne toxins in being highly toxic by the oral route (2, 8, 9). *C. botulinum* produces seven serotypes of BoNTs (A, B, C1, D, E, F, and G), which are distinguished by neutralization of toxicity in mice using homologous antisera prepared against the purified toxins (10). In the United States, *C. botulinum* and BoNTs are categorized as tier 1 select agents (11), the most dangerous group of biological agents (5, 7, 12, 13; www.selectagents.gov), and work with *C. botulinum* and SA quantities (>1 mg) of BoNTs requires high-security laboratory facilities and rigorously trained personnel (5, 12, 14, 15). Botulism is a serious neurological disease and can be life threatening, generally due to respiratory paralysis and suffocation and occasionally due to secondary disease sequelae (2, 7). After being absorbed into the circulation, BoNT binds to and is internalized in nerve terminals. The pri mary target of BoNTs is neuronal end plates, particularly the neuromuscular junction of the motor nerve, which has been termed “the most vulnerable synapse” (16). Although botulism is an acute and not a permanent intoxication, the syndrome can paralyze all muscles in the body, and the paralysis can last for days to several months, with severe morbidity and approximately 5% fatality (2, 17–19).

Human botulism has been an important public health issue ever since its recognition in the 1800s, when it was contracted from consumption of contaminated blood sausages in Germany (20–23). The importance of botulism to the food industry stems from the wide distribution of *C. botulinum* spores in the environment, the high resistance of endospores to food processing technologies, and the ability of *C. botulinum* to grow and form BoNTs in foods, even under conditions that are inhibitory to most other foodborne bacterial pathogens (24–26). Spores can form BoNTs in foods, in the intestines of infants, in wounds, and by inappropriate injection of BoNTs in therapeutic and cosmesis applications (5, 27, 28). The control of *C. botulinum* and formation of BoNTs in foods are a continuing challenge, particularly with increasing consumer demands for fresh and organic commodities, since these foods undergo minimal processing and are not treated with preservatives inhibitory to *C. botulinum*. Botulism is considered a public health emergency in the United States and many countries worldwide, and its occurrence triggers intensive regulatory and medical responses (5, 29, 30).

Consequently, a perennial goal of the food industry and of regulatory agencies is to limit the occurrence of viable *C. botulinum* spores in raw foods and to prevent their germination and vegetative-cell growth with formation of BoNTs in foods for consumption. Certain regulations, laws, and preservation practices have been designed and enacted in the food industry primarily to prevent growth and BoNT formation by *C. botulinum*, specifically, the 12D “bot cook,” low-acid-food regulations, and inhibitory water activity (a_w) values (see chapter 2 of this volume). Botulism has the highest costs per hospitalized human patient of all foodborne diseases (31–33). Due to the importance of *C. botulinum* and BoNTs in food safety, *C. botulinum* and BoNTs have served as archetypes for food safety systems and have stimulated global innovation in research strategies and advances in food microbiology and processing technologies (34, 35).

The structure, pathophysiology, and molecular mechanisms of BoNTs causing neuromuscular paralysis in animals and humans have been expertly and extensively reviewed (36–40). The remarkable development of BoNT for medical treatment of nearly 200 neuronal diseases in humans has also been described (41–44), and clinical and basic studies of structure-function and clinical uses of BoNTs in medicine currently constitute the most active area of BoNT research, providing the majority of publications in the *C. botulinum* literature. Although botulism is rare in humans, its importance in food safety is highlighted by the severity of the illness with an approximately 5% fatality rate, the requirement of intensive critical care for hospitalized patients, and the necessity of weeks to months for recovery of normal functions. Botulism outbreaks can have a dramatic impact on the human and animal populations in which they occur (2, 45–49). Animal botulism is the most prevalent type of botulism worldwide, and annual outbreaks cause devastating losses of thousands of domestic and wild animals, particularly of wild waterfowl and fish, as well as domestic animals such as cattle, mink, and some other farmed animals (20, 47).

This chapter focuses primarily on areas of neurotoxigenic clostridia and BoNTs that impact food safety and emphasizes the microbiology and control of neurotoxigenic clostridia in foods and properties of BoNTs related to food safety.

DISCOVERY OF *C. BOTULINUM*, BoNT, AND THE PATHOPHYSIOLOGY OF BOTULISM

Botulism was suspected as a dreaded food poisoning over 1,000 years ago during the reign of Emperor Leo VI of Byzantium (886 to 911 CE) (20, 21, 23). The first definitive studies of human botulism were conducted in Germany in the early 1800s by Georg Steinbuch and Justinus Kerner, who reported that consumption of raw blood and liver sausages was associated with a disease characterized by muscle paralysis, decreased secretions such as salivation and sweating, and ensuing suffocation in the patient (21–23). Kerner showed that a poison developed within the sausages and extracts of the sausages, which caused severe paralytic symptoms and death in animal models. Kerner even experimented on himself: he administered a “few drops” of diluted poisoned-sausage extract on his tongue and reported symptoms including drying of his tongue and throat, difficulty swallowing, altered tension of eye muscles, and reduced secretions including salivation and sweating, which are today recognized as anticholinergic symptoms (21).

Following the observations in Germany, the etiologic cause of botulism remained obscure for more than 50 years until a series of remarkable experiments were conducted by the Belgian microbiologist Emile Pierre van Ermengem (50). His background included studies under Robert Koch and Claude Bernard (5, 23), and he was well prepared to investigate the etiology of botulism. In 1897, van Ermengem published a classic comprehensive treatise describing isolation of an anaerobic bacillus from a raw salted ham implicated in a botulism outbreak that affected 34 individuals at a funeral wake in Belgium (50). van Ermengem (50) established the etiology of botulism by isolation of “*Bacillus botulinus*” from the ham and from the large intestines of persons who had died from consumption of the ham. He determined that the bacillus produced a very potent toxin that was released into the food. His success in isolating the anaerobe and demonstrating the extracellular nature of the toxin was a triumph in food and medical microbiology. In 1897, Kempner (51) subsequently determined that van Ermengem’s cultures produced a protein-like substance that on injection in an inactive form gave rise to antitoxin in the blood of goats, which subsequently prevented death when administered to animals prior to toxin challenge. These classic experiments provided the first evidence that antibodies to BoNT could neutralize toxicity in the circulation and prevent illness and death using serum therapy (52).

van Ermengem's and Kempner's landmark investigations established several principles of botulism that remain valid today and form the cornerstone for understandingand control of the disease: (i) foodborne botulism is a true toxemia caused by BoNTs and is not an infection; (ii) the toxin is produced by a bacterium, "*Bacillus botulinus*," currently designated *C. botulinum*; (iii) the toxin causes a paralytic disease and death in vertebrate animals when administered by the intravenous, intramuscular, and oral routes; (iv) the toxin is inactivated by moderate heat and alkali and is stable under acidic conditions; (v) *C. botulinum* does not grow and form BoNT in foods containing sufficient salt or acid; (vi) *C. botulinum* endospores are highly resistant to heat and certain chemicals; (vii) animals can develop immunity to BoNTs by exposure to inactive toxin (toxoid); (ix) antibodies in antitoxins can neutralize BoNT when they are present in the circulation but not after it has entered nerves in animal models.

Following van Ermengem's findings in Belgium, a similar paralytic disease was described in several countries worldwide from consumption of various foods, especially home-canned vegetables and meats in terrestrial environments and home-prepared fish and marine mammals in coastal regions of many countries (45, 53, 54). Foodborne botulism occurs worldwide from consumption of a variety of foods, yet the occurrence and kind of poisonous food vary depending on the geographic region, country, and food processing and handling practices. Most foodborne-botulism cases have resulted from home-prepared foods, while large outbreaks involving several persons have also resulted from food obtained from food service operations and restaurant-prepared foods (2, 19, 55). Foodborne botulism in a given class of foods is generally associated with a specific serotype of *C. botulinum* and its BoNT (53, 54, 56). Recent botulism outbreaks are listed in Table 18.1..

Following the discovery of foodborne botulism, different mechanisms of BoNT intoxication in humans and animals were discovered. In the 1940s, botulism from a wound infection was reported, and currently, wound botulism occurs mainly in intravenous-drug users (56–59). In 1976, botulism resulting from the colonization of the intestinal tracts of infants (<12 months of age) by *C. botulinum* and production of BoNT were discovered by Pickett et al. (60) and confirmed by Midura and Arnon (61). In the 1980s, strains of *C. butyricum* and *C. baratii* that produce serotypes E and F, respectively, were isolated in Italy and in New Mexico from infant botulism cases (62–64). Rare forms of botulism, including colonization of the intestinal tracts of adolescents and adults by *C. botulinum*, have also been observed, generally following surgery and antibiotic treatment of patients (18, 65–68). While foodborne botulism is considered the most common type of botulism in most countries world-wide, infant botulism is the most prevalent type of botulism in the United States (69, 70). Since its discovery in the United States in the 1970s, infant botulism has been recognized globally on all continents except Antarctica (70).

The worldwide occurrence of human botulism from commercial and home-prepared foods has been promulgated by globalization of the food supply, whereby ingredients in a food may be sourced from diverse geographic regions and most of the seven continents (see chapter 2).

In recent years, there has been an increase in incidence of iatrogenic botulism, which results from inappropriate injection of BoNTs for medical treatments for cosmesis and therapeutic disorders (71–73). There is also a growing problem of injection of counterfeit preparations of BoNTs and "BoNT-like substances" for cosmetic purposes, whereby the toxins are prepared without proper quality control and governmental approval (17, 71, 74).

In all categories of botulism, BoNT is solely responsible for morbidity and mortality, and thus, botulism is a true toxemia (2, 36). The fact that BoNT is the sole entity causing disease was proven in laboratory animal studies of intoxication using highly purified BoNTs and absence of *C. botulinum* organisms. For laboratory production of BoNTs, *C. botulinum* strains are grown in

complex culture media under anaerobic conditions. The titers of BoNTs produced depend on the strain, the medium, and the culture conditions (42, 75–77).

The beginnings of purification of BoNTs and the understanding of their pathophysiology occurred early in the 1900s. In 1916, Sommer determined that addition of a mineral acid to type A broth cultures to achieve a pH of ca. 3.5 resulted in flocculation of more than 90% of the active toxic activity which settled from the culture (42). Acid precipitation is the first step in most purifications of nearly all serotypes of BoNTs. In the 1940s at Fort Detrick, Maryland, the purification of the botulinum toxin type A complex from precipitated BoNT was accomplished (42, 78, 79), and this process was further perfected by Edward J. Schantz at the University of Wisconsin (42, 80). The purification in the 1940s entailed repeated acid precipitations, washing of the precipitates with water and sodium phosphate buffer, an ethanol precipitation of the toxin at −5°C, and formation of toxin crystals by slow and tedious addition of ammonium sulfate over several days (42). During this early period in protein purification and characterization, protein chromatography was not available for protein purification, and therefore, these differential precipitation techniques were exclusively used for toxin isolation. Ultracentrifugation and diffusion experiments revealed that botulinum toxin type A consisted of a large protein of approximately 900 kDa (42, 81). It was subsequently determined that this large molecule comprised a protein complex that contained the active neurotoxin of ca. 150 kDa noncovalently linked with nontoxic proteins, including hemagglutinins that coagulated red blood cells and a nontoxic nonhemagglutinin protein (42, 82). Depending on the serotype and production methods, these complexes vary in content and ratios of BoNTs and the nontoxic proteins. These large protein complexes were termed progenitor toxins by Japanese researchers (83–85).

Table 18.1 Examples of recent botulism outbreaks (adapted from Parkinson et al. [203])

Year	Product	Country	No. of cases	Probable cause
2017	Nacho cheese sauce	U.S.	10	Improper handling/temperature abuse
2016	Pruno/hooch	U.S.	16	Illicit potato fermentation
2016	Fish heads/seal oil	U.S.	2	Improper preparation and fermentation
2014	Pesto	U.S.	2	Inadequate heat treatment
2012	Adzuki bean soup	Japan	2	Temperature abuse
2011	Korma sauce	Scotland	3	Unknown
	Olive paste	France	9	Incorrect thermal processing
	Potato soup	U.S.	1	Temperature abuse
2010	Olives stuffed with almonds	Finland	2	Defective container
2009	Smoked fish	France	3	Unknown
2008	Chicken enchilada	France	2	Temperature abuse
2007	Hot dog chili sauce	U.S.	8	Incorrect thermal process
2006	Carrot juice	U.S./Canada	6	Temperature abuse
	Smoked fish	Finland	2	Unknown
	Bamboo shoots	Thailand	209	Improper preparation

The association of the neurotoxin components with nontoxic proteins was subsequently shown to confer stability on the BoNT in the intestinal tracts of animals and humans and probably to facilitate absorption of BoNTs from the intestinal tract (8, 83). Once absorbed into the lymph and then into the blood circulation, they are trafficked to peripheral nerves, which they enter and where they cause intoxication. The intoxication process results in reduced secretion of acetylcholine and likely other signaling molecules, including hormones (5, 36, 37). The inhibition of acetylcholine secretion at neuromuscular junctions prevents muscle activation and causes muscle atrophy and shrinkage. The early studies of the pathophysiology of BoNTs were recently reviewed (5).

In the 1960s, ion exchange and gel filtration column chromatography became available, which facilitated isolation of the 150-kDa neurotoxin component from the complexing proteins (82, 86). The isolation of the purified BoNT component enabled definitive studies on the mode of action of BoNTs (36, 37, 87). It was determined that BoNTs specifically bind to gangliosides and then to receptor proteins on the terminal surfaces of nerves, are internalized in them, and enter the nerve cytosol, where they cleave SNARE (soluble *N*-ethylmaleimide-sensitive factor protein receptor) proteins. SNARE proteins make up a superfamily of small proteins that constitute the machinery for transmission of vesicles to the cell surface and enable exocytosis. In 2013, the Nobel Prize in Physiology or Medicine was awarded for the elucidation of the mechanism of SNARE-mediated protein and metabolite trafficking. BoNTs played a role in understanding this intricate cellular process by cleaving the SNARE proteins as specific targets and preventing release of acetylcholine at the neuromuscular junction or hormones in nonneuronal cell types. The inhibition of acetylcholine prevents muscle contraction and is responsible for the symptoms of muscle paralysis, atrophy, and severe muscle weakness characteristic of botulism (38, 40, 88). When BoNT accesses the respiratory neuromuscular junctions, it causes suffocation and ensuing death through inhibition of acetylcholine release from nerves to muscle.

A triumph in botulism research was the determination of the three-dimensional structure of BoNT A by Stevens and his lab in the 1990s, which provided valuable insights into the mechanisms of intoxication (89, 90). The first structure determination of BoNT A was followed by structures for BoNT B, BoNT E, and the individual domains (40, 91). The availability of the structures of BoNT revealed that BoNTs have three major protein domains, including a receptor-binding domain, a translocation domain for passage of BoNTs into the nerve cytosol, and a domain with catalytic activity that specifically cleaves SNARE proteins and prevents neurotransmitter exocytosis (40, 88). Elucidation of the structure and demonstration of specific proteolytic activity on the SNARE apparatus provided a definitive understanding at the molecular level of the intoxication process of BoNTs. The intoxication process has been studied by several accomplished laboratories in molecular biology and has been definitively reviewed (36, 37, 40, 87).

TAXONOMY OF NEUROTOXIGENIC *CLOSTRIDIUM* SPECIES

The genus *Clostridium* is classified in the kingdom *Bacteria*, phylum *Firmicutes*, order *Clostridiales*, family *Clostridiaceae* (92, 93). Over the past 3 decades, the genus *Clostridium* became a collection (“dump”) of more than 200 heterogeneous bacteria, including nonsporeformers. To more concisely define the taxonomy of *Clostridium* species, the taxonomic classification of neurotoxigenic and related species of *Clostridium* has recently been reexamined by advanced taxonomic methods and described according to the *Code of Bacterial Nomenclature* (93). These studies have placed neurotoxigenic clostridia, including *C. botulinum* and BoNT-producing strains of *C. baratii*, *C. butyricum*, and *C. tetani*, in *Clostridium* taxonomic cluster I, as initially defined by the pioneering studies of John Johnson, David Collins, and colleagues (94,

95). Cluster I is designated *Clostridium sensu stricto*, and the type species is *C. butyricum* Prazmowski. *C. butyricum* is likely the species that Louis Pasteur isolated in his famous studies of spoilage of wines (5, 92). Nontoxigenic *Clostridium* spp. taxonomically similar to *C. botulinum* in group I include *C. sporogenes* (type A), *C. novyi* (type C), and *C. subterminale* (type G). *Eubacterium*, *Sarcina*, and many other "*Clostridium*" species need further study for clear delineation of the genus and species.

For many decades, neurotoxigenic clostridia have been classified as the single species *C. botulinum* based on BoNT formation, since this designation was convenient and easily recognized by the medical professions as well as by the food industry. Currently, clostridial isolates that produce BoNTs with the characteristic high potency of approximately 10^8 50% lethal doses (LD_{50}s) for mice per mg belong to the species *C. botulinum*, as well as BoNT-producing strains of *Clostridium argentinense*, *C. baratii*, and *C. butyricum*.

Recently, whole-genome sequencing, genome mining, and bioinformatics have identified gene clusters in various organisms that possess BoNT gene clusters analogous to those in *C. botulinum*. These strains include *C. botulinum* strain 111, an *Enterococcus faecium* strain, *Chryseobacterium piperi*, *Weissella oryzae*, and certain other bacterial groups (96–99). Although catalytic activity on SNARE proteins has been shown for the light chains of these organisms, the putative holotoxins in cell extracts have no activity in neuronal cell models and *in vivo* in mice. These species have not been shown to produce BoNTs with the neural specificity and high toxicity of BoNTs from *C. botulinum*, and therefore, at this time these organisms cannot be considered neurotoxigenic clostridia that produce authentic BoNTs. Efforts are in progress to carefully evaluate the ability of these distantly related bacteria to produce true BoNTs. Certain isolates produce toxins that are active on SNARE substrates, but their toxicity is not in the range of the select agent designations for BoNTs (≤100 ng/kg in a mouse bioassay).

ISOLATION AND CHARACTERIZATION OF BoNT-PRODUCING CLOSTRIDIA

An important aspect of investigations of neurotoxigenic clostridia is their isolation and culture. The isolation, identification, and maintenance of pure cultures of botulinogenic clostridia present certain practical difficulties. Clostridia tend to grow as consortia, and pure cultures are sometimes difficult to achieve and maintain. The purity of neurotoxic clostridia is ascertained by microscopy, by plating on nonselective media to ascertain uniform morphology colonies, and by phenotypic and molecular methods described below. Since botulinogenic clostridia have complex nutrient requirements, rich media are commonly used for their cultivation (4, 10, 100). Enrichment of spore-forming neurotoxic clostridia is often carried out by heating food, environmental, or clinical samples (e.g., 60 to 80°C for 10 min) or by treating the samples with 50% ethanol to kill vegetative bacteria and enrich for sporeformers (14, 101). For isolation of *C. botulinum*, a small quantity of food or clinical specimen extracts is added to 10 ml of cooked meat medium with 0.5% glucose or Trypticase-peptone-glucose-yeast extract broth in anaerobic tubes (14, 101). These are incubated for 1 to 3 days for vegetative growth and up to 4 weeks to promote endospore formation.

Once neurotoxic *Clostridium* cultures are isolated and maintained in pure culture, they are characterized phenotypically and genetically. Neurotoxic clostridia are strict anaerobes that obtain energy by fermentation and substrate-level phosphorylation and do not reduce sulfate to sulfide (92). Microbiological phenotypic characterization may include the lipase reaction, Gram and spore stains, proteolysis of meat particles and casein, and substrate utilization patterns (92, 102). Formerly, the carbon and nitrogen compound assimilation in cultures was determined by

growth tests, and formation of fatty acids and alcohol end products was routinely determined by gas-liquid chromatography (102), but these labor-intensive tests are not currently routinely performed with *Clostridium* species. Such phenotypic characterizations are still required for characterization of commercial strains of *C. botulinum* used for BoNT production as a pharmaceutical (Kevin Munson, personal communication). All manipulations of neurotoxigenic clostridia should be performed in an anaerobic chamber, since these organisms are strictly anaerobic and colonies are killed on exposure to air. For example, plating of a culture in an aerobic environment followed by placing it in an anaerobic jar or chamber will generally kill about 90 to 99% of the clostridial cells (my unpublished results).

Currently, cultures of *C. botulinum* are mainly characterized by cell morphology, genetic analyses, and partial-or whole-genome sequencing. In *C. botulinum* and *C. tetani*, the spores are generally wider than the vegetative organisms in which they are formed, imparting a spindle or "tennis racket" shape to the cells, the characteristic toxigenic clostridial form (4, 10, 22, 92) (Fig 18.1). In early stages of culture, neurotoxigenic clostridia typically grow as rod-shaped bacteria and often form filaments or chains. The vegetative cells are often curved, their sides parallel, and their ends rounded (4). Neurotoxigenic clostridia are generally referred to as Gram-positive spore-forming rods (92), but the Gram reaction may be weak or appear negative in 24-h or older cultures (4). Spores are generally formed when cells are grown in rich media, but their microscopic presence may require several days to weeks of incubation to reach a significant spore titer in the primary culture of vegetative cells. In media favoring BoNT formation, spore formation is usually limited and the cells in the culture undergo mass lysis following early stationary phase, which usually occurs at 12 to 24 h in the growth cycle (75). Autolysis has long been postulated to release BoNT from the cells, but it is likely there is also an active secretion system for BoNTs. Furthermore, in some serotypes, such as type E, the culture does not generally undergo mass lysis, and BoNT remains adhered to the surface of the cells.



Figure 18.1 Characteristic morphology of *C. botulinum* with an endospore.

In addition to morphology and growth tests, molecular techniques are primarily used for species and strain characterization. These include the determination of the nucleotide sequences of genes encoding rRNA, multilocus sequence typing and other DNA typing methods such as restriction fragment length polymorphism analysis, PCR analyses for BoNT and nontoxic nonhemagglutinin protein genes, and pulsed-field gel electrophoresis (PFGE) of isolated DNA digested with restriction enzymes (103–108). Whole-genome sequencing has become the preferred method of characterization of BoNT-producing clostridia and is rapidly being adopted in academic, industry, and government laboratories. A critical test for neurotoxigenicity is the demonstration of BoNT. The traditional method of testing for BoNT is by the mouse bioassay, which is currently the only method approved by the FDA for food and clinical detection of BoNT and is considered the gold standard (10, 109, 110). It involves injection of diluted extracts of foods or clinical samples intraperitoneally into mice and observation of the mice for symptoms of botulism and death. It is important to also inject a set of tubes containing neutralizing antibodies to specifically prevent toxicity, particularly since mice often die from toxic non-BoNT compounds in foods and clinical samples, referred to as nonspecific death. The lack of antibody neutralization indicates false-positive toxicity results, which can lead to hugely impactful

regulatory and industry responses, such as recalls of massive quantities of products (111). Several ancillary methods, including PCR and whole-genome sequencing, are used by some laboratories to determine the presence of the *bont* and *ntnh* genes in cultures (103, 107, 112). A method based on antibody binding and mass spectroscopy detection of SNARE protein cleavage is being evaluated as a more rapid test for BoNT that does not involve mice and can be performed more rapidly than the mouse bioassay (113, 114). Cleavage of SNARE proteins in cultured human neuronal induced pluripotent stem cells in culture is also being used for detection of BoNTs (115), and two tests have been approved by the FDA for quantitation of BoNT in pharmaceutical preparations. It would be valuable to determine if neuronal cells could be used to detect BoNT in foods, since such an assay would not use animals and would shorten the time required to obtain results for the presence of active BoNTs in foods.

A few other species of *Clostridium* also produce neurotoxins, most famously tetanus neurotoxin, produced by *C. tetani*, which causes a drastic spastic paralysis (116), and epsilon toxin from *C. perfringens*, which causes morphological changes and death of neurons in culture (117). Although *C. tetani* produces a neurotoxin with high homology to BoNTs, it does not form complexes with nontoxic proteins in the progenitor form and is not toxic by the oral route, and therefore, it does not cause foodborne disease.

METABOLIC AND GENOTYPIC CHARACTERISTICS OF NEUROTOXIGENIC CLOSTRIDIA

Neurotoxigenic clostridia are heterogeneous in their physiology and metabolism, and many of these differences are important in food safety, including conditions for spore germination, resistance properties of spores, temperature range for growth of vegetative cells, acid and salt tolerance, resistance to antimicrobials, proteolytic activity, substrate utilization, and BoNT expression (Tables 18.2 and 18.3; see also chapter 2) (102). In 1970, the observations of differences in metabolic properties of *C. botulinum* led to the designation of three metabolic groups (groups I to III) (Table 18.2.). When *C. botulinum* type G was isolated and characterized in 1970, it was placed in group IV (118, 119). *C. botulinum* type G (group IV) was taxonomically classified as *C. argentinense* (118). The classification of *C. botulinum* in physiological groups I to IV has been widely accepted (1–3, 23). Following this early metabolic classification, groups I to IV were determined to be genetically distinct and to comprise four species lineages (120–122). Groups I and II are responsible for human botulism, while group III mainly causes animal botulism, and group IV is not known to cause disease in humans or animals. Rare BoNT-producing strains of *C. baratii* and *C. butyricum* were initially recovered from infant botulism cases and more recently from foodborne-botulism cases as well as the environment (23,123–125). It has been suggested that neurotoxigenic *C. baratii* and *C. butyricum* make up metabolic groups V and VI of neurotoxigenic clostridia (126, 127), but most researchers in the botulinum field prefer to maintain the species names instead of adding more groups. Furthermore, the metabolism of *C. baratii* and *C. butyricum* has not been studied in sufficient detail to validate two additional metabolic groups (128). Extensive studies of phylogenomics by wholegenome sequencing have substantiated the six genomic lineages of neurotoxigenic clostridia. For many years, and recently, it has been proposed that the groups be designated with different species names (127), but such nomenclature would be confusing to the medical profession and the food industry, and *C. botulinum* should be currently retained as the sole species in groups I to IV.

METABOLIC PROPERTIES OF *C. BOTULINUM* GROUPS I TO IV, *C. BUTYRICUM*, AND *C. BARATII*

C. botulinum group I comprises the proteolytic strains of serotypes A, B, and F (Table 18.2.). The organisms in this group digest gelatin, milk, and meat particles in culture. The gene clusters encoding BoNTs reside on chromosomes and/or on plasmids (129–133) (Table 18.4.). Like all fermentative clostridia, neurotoxic *Clostridium* spp. produce short-chain organic acids and alcohols from anaerobic metabolism (92, 102). Group I *C. botulinum* also produces ammonia and putrefactive amines. Arginine and phenylalanine are the nutrients most strictly required for growth by group I type A and B strains (100). The nutritional requirements for amino acids and glucose in proteolytic type F strains have not been studied. Glucose, fructose, and maltose are utilized for growth and toxin production, while mannose, lactose, sucrose, and trehalose are not utilized (92). The optimum temperature for growth of group I is 35 to 40°C. The minimum temperature for growth of group I *C. botulinum* is 10°C, and the maximum growth temperature is 45 to 50°C. Growth is inhibited by an aw of 0.94 (with NaCl as the humectant in solution). The minimum pH for growth is designated as 4.6, but many strains do not grow below pH 5, especially with other inhibitory factors in the medium or food. The strains are sensitive to several antibiotics (134, 135), and although these are not used for control of foodborne or infant BoNT botulism, they may be used in wound botulism, especially since wound infections are polymicrobial. Group I has the highest heat resistance of organisms in groups I to IV and *C. baratii* and *C. butyricum* (see chapter 2). The *D* value for spore inactivation in group I varies, yet the food industry generally defines 0.21 min at 121°C as the standard value. The spores of group I *C. botulinum* have remarkable heat resistance, with a *D* value (100°C) of approximately 25 min (53) (also see chapter 2). Thus, from a practical perspective, the boiling of foods does not readily inactivate group I spores, and caution is needed in canning and production of foods in the home and in food service settings and restaurants. *C. botulinum* group I is closely related to *C. sporogenes*, but certain strains of this species, including *C. sporogenes* PA3679, have temperature resistance approximately 10 times greater than that of *C. botulinum* group I (53,136–138). *C. sporogenes* is often used as a surrogate for group I *C. botulinum* in processing and in food challenge studies (137). Due to its higher heat resistance, it is useful as an indicator of food processing efficacy, as it is a “worst case” surrogate and provides a conservative safety margin in processing and food challenge studies. *C. sporogenes* cannot be considered a true surrogate, as it is not isogenic to *C. botulinum*, except by only having a deleted *bont* gene, and *C. sporogenes* also has higher resistance properties than group I *C. botulinum*. Therefore, *C. sporogenes* has a genome distinct from that of group I strains of *C. botulinum* (138) (see chapter 2).

Table 18.2 Groups and relevant growth and resistance properties of BoNT-producing clostridia[a]

				Group		
		C. botulinum				
Property	I	II	III	IV (*C. argentinense*)	*C. butyricum*	*C. baratii*
Neurotoxin type(s)	A, B, F	B, E, F	C, D	G	E	F
Growth temp (°C)						
Minimum	10	3	15	12	10	20
Optimum	35–40	18–25	35–40	35–40	30–37	30–40
Maximum	48	45	NA	45	~40	NA
Minimum pH	4.6	5.0	NA	NA	~3.6	NA
Inhibitory a_w	0.94	0.97	NA	NA	0.97	NA
Inhibitory NaCl concn (%)	10	5	3	>3	~5	5
D value of spores (min) at:						
100°C	~25	<0.1	NA	NA	NA	NA
121°C	0.21	<0.005				

[a] Data are for a limited number of strains. Inhibitory factors interact, and hence, the inhibitory values may be affected by sublethal combinations of factors. *C. sporogenes*, often considered a surrogate for group I *C. botulinum*, has *D*121°C values ranging from 0.5 to 6 min. NA, insufficient data.

Group II includes nonproteolytic strains of types B and F, as well as type E. The BoNT genes are also located on chromosomes and/or on plasmids as described for group I. The organisms in this group are deemed nonproteolytic and they do not digest casein or meat particles, but gelatin is usually degraded. Glucose, fructose, maltose, and sucrose are fermented and support growth and toxin formation, but lactose is not utilized. This group is commonly referred to as saccharolytic due to their need for sugars to grow, which is in contrast to group I organisms, which primarily require amino acids as carbon and energy sources. The optimum temperature for growth is 25 to 30°C depending on the serotype. The minimum growth temperature is 3.3°C, and the maximum growth temperature is 40 to 45°C. At 3.3°C, and depending on the food system and the number of inoculated spores or cells, growth and BoNT formation occur very slowly, requiring weeks to months to form sufficient BoNT to cause mouse lethality (139). Growth is inhibited by an a_w of 0.97 (5% NaCl as the humectant in solution). The minimum pH for growth is 5.0. The spore heat resistance is much lower than that of group I, and strains typically have *D* values of <0.1 min at 100°C. Therefore, spores are readily killed at 80 to 100°C, and industry typically targets a 5-to 6-log thermal inactivation in thermally processed foods for group II nonproteolytic strains. Surrogate organisms for group II *C. botulinum* are not well defined, and there is a need to construct isogenic mutants of members of this *C. botulinum* group that have only a deletion of the gene encoding BoNT. Newer genetic methods such as the CRISPR (clustered regularly interspaced short palindromic repeats)-Cas method are being used to isolate mutants of other clostridial species (140–142), and this method should be valuable in creating defined true *bont* deletion surrogates for processing and food challenge studies in groups I and II.

Table 18.3 Nutritional substrates metabolized by botulinogenic clostridia[a]

Clostridium species and type(s) (group)	Glucose	Fructose	Sucrose	Maltose	Lactose	Gelatin	Milk	Meat	Lipase	Lecithinase
C. botulinum										
Types A, B, F (I)	+	+/-	+/-	+/-	-	+	+	+	+	-
Types B, E, F (II)	+	+	+/-	+/-	-	+	-	-	+	-
Types C, D (III)	+	+/-	+/-	+/-	-	+	+/-	-	+	-
C. argentinense (IV)	-	-	-	-	-	+	+	+/-	-	-
C. baratii, toxigenic	+	+	+	+/-	+/-	-	-	-	-	+
C. butyricum, toxigenic	+	+	+	+	+	-	-	-	-	-

[a] Symbols: +, positive; −, negative; +/−, weak or variable.

Table 18.4 Primary hosts and locations of toxin genes in *C. botulinum* serotypes A through G

Serotype	Susceptible species	Location of neurotoxin gene
A	Humans, horsesgene A	Chromosome, large plasmids in some strains
B	Humans, horses, swine, primates	Chromosome, large plasmids in some strains
C	Birds, horses, cattle, minks, foxes, dogs, turtles	Pseudolysogenic bacteriophage, plasmid
D	Cattle	Pseudolysogenic bacteriophage, plasmid
E	Fish, waterfowl	Chromosome, large plasmids in some strains
F	Humans (rare)	Chromosome, large plasmids in some strains
G	None known	Large plasmid

Group III includes *C. botulinum* types C and D, which are pathogenic for many wild and domestic animals but rarely cause human botulism (Table 18.4.) (20, 46). Organisms in this group generally digest milk proteins, meat particles, and gelatin. Toxin production is mediated by temperate phages in group III (143) (Table 18.4.). Glucose and mannose are fermented, whereas sucrose and trehalose are not. The optimum temperature for growth is 30 to 37°C, and the growth range is 15 to 40°C. Growth is inhibited by 3% salt. The minimum pH for growth is 5.0 to 5.2. Group III *C. botulinum* is variable in BoNT formation because the gene for BoNT is

present on an unstable temperate bacteriophage. On loss of the BoNT-containing phage, the organism is indistinguishable from *C. novyi* (143).

Group IV consists of *C. botulinum* type G, which has been taxonomically defined as *C. argentinense* for toxigenic and nontoxigenic strains (118). The gene encoding BoNT lies on a large plasmid (130). Gelatin and milk are readily digested, and meat is digested slowly. Ammonia and hydrogen sulfide are produced. Growth of *C. argentinense* relies on digestion of protein and the availability of amino acids for growth. Glucose, fructose, sucrose, maltose, mannose, and trehalose are not fermented. The optimum growth temperature is 30 to 37°C, and the minimum growth temperature is 12°C. *C. argentinense* is inhibited by 6.5% NaCl.

Sporadic strains of neurotoxigenic *C. butyricum* and *C. baratii* producing BoNT E and F, respectively, were isolated from infant botulism cases in the 1980s, and more recently, these two species have been involved in rare foodborne-botulism outbreaks (23, 123). The gene for BoNT F resides on the chromosome and/or on a plasmid. *C. butyricum* does not digest meat, but it does utilize pectin and starch. It ferments numerous sugars, including glucose, fructose, lactose, and sucrose. The optimum temperature range for growth is 30 to 37°C, and some strains will grow at 10°C. Growth is inhibited by 6.5% salt. The minimum pH for growth is 4.8. The thermal resistance of spores is low, with a *D* value of <0.1 at 100°C.

Neurotoxigenic strains of *C. baratii* have been involved in infant and foodborne-botulism cases (1, 23). *C. baratii* does not digest meat, and starch hydrolysis is variable. It ferments glucose, fructose, maltose, and cellobiose, whereas lactose fermentation is weak and trehalose is not fermented. Optimum growth occurs at 25 to 35°C, and some strains grow up to 45°C. The minimum temperature for growth is 10 to 15°C. The growth and resistance properties of neurotoxigenic *C. butyricum* and *C. baratii* (92) require further study, particularly as they are increasingly being associated with infant botulism and food botulism. The surrogate organisms are nontoxigenic strains of *C. butyricum* and *C. baratii* strains. Characteristics of the groups I to IV and neurotoxigenic *C. baratii* and *C. butyricum*, particularly pertaining to nutritional utilization, growth, and survival in foods, are summarized in Tables 18.2 and 18.3.

GENOMICS OF NEUROTOXIGENIC CLOSTRIDIA

Owing to advances in whole-genome sequencing and bioinformatics, full genomic sequences of several toxigenic *Clostridium* species, such as *C. tetani*, *C. difficile*, *C. perfringens*, *C. acetobutylicum*, and *C. botulinum*, have been determined and deposited in GenBank and other genomic databases. The genomic sequences of more than 200 strains of *C. botulinum* have been determined. These genome sequences have led to an in-depth examination of *C. botulinum* phylogenomics (122,144–146) and have also identified genes important in resistance properties in foods, spore formation, germination, and outgrowth, and BoNT expression and properties. The availability of the genomic sequences of *C. botulinum* and other botulinogenic clostridial species will enable future rational approaches for identification of genes and proteins for development of inhibitors and novel antimicrobials that target *C. botulinum* for enhancement of food safety (145).

The genes encoding BoNT and other proteins in the BoNT progenitor complexes are present on the chromosome, plasmids, and bacteriophages (Table 18.4.). It has long been realized that in *C. botulinum* type C and D, the *bont* genes can be mobilized and transferred between these serotypes and to *C. novyi* (143). More recently, plasmids containing BoNT genes that occur in *C. botulinum* groups I and II have been found, and certain of these plasmids have conjugative ability (131, 133, 147). Several of the plasmids harboring genes encoding BoNT have not been evaluated for lateral gene transfer (131), and the spread of genes encoding BoNTs may be wider

than currently realized. This hypothesis is supported by the recent finding of BoNT gene clusters in bacterial species distantly related to *C. botulinum* (98) that appear to produce BoNT-like proteins but have not been shown to have the specific toxicities (10^7 to 10^8 mouse LD_{50}s per mg) of authentic neurotoxic clostridia. The lateral transfer of genes encoding BoNTs could have a significant impact on food safety and public health.

ECOLOGY OF BOTULINOGENIC CLOSTRIDIA

BoNT-producing clostridia are widely dispersed in nature by virtue of their ability to form resistant endospores that can persist in nature for decades (see chapter 2). Unlike many other foodborne pathogenic bacteria, neurotoxigenic clostridia do not have an obligatory relationship with an animal host, and spores exist free-living in the environment and only adventitiously colonize animals as carriers.

The distribution of *C. botulinum* spores worldwide has been reviewed (20, 53, 148, 149). Although *C. botulinum* spores are distributed widely throughout the world, the different serotypes are mainly found in specific geographic regions and habitats. *C. botulinum* causing human foodborne botulism is primarily found in northern regions of the world. Proteolytic group I type A spores have been found in temperate regions, including soils of the western United States, southern and eastern European countries, and China. Group I type A *C. botulinum* spore distribution favors neutral to alkaline soils with low organic content; hence, they are mainly absent from the eastern region of the United States, as well as from western European soils. In the continental United States, proteolytic type A is most common west of the Mississippi River, whereas proteolytic type B is most common east of the Mississippi River. Twentyfour percent of the soils tested in the United States were found to harbor *C. botulinum* spores in an early survey (53). Distribution of type B spores appears to be more uniform worldwide. *C. botulinum* type B strains found in western Europe are usually nonproteolytic (group II). However, proteolytic type B spores (group I) have also been found in western European countries such as France, in Asian countries including Japan and China, and eastern European countries such as Poland and Russia. Type E spores are found mainly in aquatic sediments of colder regions of the northern hemisphere, such as Alaska, Canada, Scandinavia, Poland, Russia, and Japan. Type E levels are correlated with low sediment oxygen content, increased depth of the sediment, and absence of bioturbation activity.

C. botulinum spores have a relatively low prevalence in certain countries, including Great Britain, Sweden, The Netherlands, Switzerland, Austria, Greece, Australia, New Zealand, Mexico, South Korea, and many countries in the South American and African continents (53, 148, 150). *C. botulinum* spores have been isolated from the intestinal tracts of healthy fish, birds, mammals (but not humans), and certain invertebrates. *C. botulinum* is often associated with the decomposition of plants and animals, in which an anaerobic environment and rich source of nutrients promote the formation of spores. Spores are distributed in nature by fomites, including invertebrates and other organisms, and by natural and farming-related environmental processes, including dispersal of spores in soil by wind, rain, and runoff, bioturbation, farming practices, watershed management, and other routes (149, 151). Accordingly, the spores contaminate many foods as well as animals, soil and dust, buildings, and households. Recent investigations using metagenomics have revealed that various genera of sporeformers are more prevalent in soils and in the human microbiome than previously assumed (152). The future discovery of the presence of *C. botulinum* in previously barren regions could open new insights for their importance in disease.

The biological functions of *C. botulinum* and BoNTs in nature remain unknown, and their role remains speculative. The formation of BoNT is not necessary for growth and survival of *C. botulinum* in the laboratory, since inactivation of the toxin genes or deletion of the BoNT gene region does not alter growth, although it appears to increase sporulation (75, 153, 154). *C. botulinum* has been isolated from rotting vegetation and from animal carcasses (149, 155). Neurotoxigenic clostridia are generally considered saprophytic and do not have an obligatory relationship with an animal host (50). Due to their formation of the deadly BoNT with action on warm-blooded vertebrates, *C. botulinum* can be considered predatory in some ecological niches, and by killing the prey, it initiates the complex process of animal decomposition, important for recycling of animal biomass. The initial killing of prey is followed by a complex progression of a microbiome involved in decomposition (156–158). Considerable research has been conducted on the decomposition of plant biomass, which is wide-spread and mostly stationary, static, and abundant. However, comparatively little research has been performed on the ecology of decomposition of animal biomass in the wild, due to the mobility and spatial movement of animals prior to death, and probably the unpleasantness of studying rotting carcasses. Recently, detailed studies have been performed on the succession of the microbiome in already-dead decomposing animals and human carcasses (156–158). These studies have not defined the initial killing event that enables decomposition, and *C. botulinum* may provide this killing trigger in certain animals and humans.

PROPERTIES OF BoNTs

The exceptional feature of the neurotoxic clostridia is the production of a distinctive neurotoxin of extraordinary potency for humans and animals. BoNTs are the most potent protein toxins known for humans and many vertebrate animals (5–7, 42), having estimated human LD_{50}s of 0.5 to 3 ng per kg body weight by intravenous and intramuscular exposure, 0.10 to 1.5 μg per kg by the oral route, and 10 to 15 ng per kg by inhalation. Due to their extreme toxicity, laboratory research on *C. botulinum* and BoNTs is tightly regulated in the United States by select agent rules (159) (http://www.selectagents.gov).

BoNTs are produced as single-chain molecules of ca. 150 kDa that achieve their characteristic high toxicities of 10^7 to 10^8 mouse LD_{50}s per mg by posttranslational proteolytic cleavage and processing to form a dichain molecule composed of a light chain (~50 kDa) and a heavy chain (~100 kDa) linked by a disulfide bond (82, 160). Proteolytic strains of *C. botulinum* produce the proteolytic enzyme(s) for activation, whereas nonproteolytic strains depend on exogenous proteases (e.g., in food or in the gut) for activation. However, in certain BoNTs from proteolytic *C. botulinum* strains, such as type B strains, only partial activation (60 to 70%) occurs in culture, compared to >95% activation in type A cultures. Other factors are involved in posttranslational activation and stabilization or degradation of the BoNT component in cultures and during manufacture for pharmaceutical use (161) (Johnson lab, unpublished results).

The 150-kDa BoNTs exist naturally as components of large progenitor protein complexes ranging from 300 to 900 kDa in size and varying in complexity (42, 82, 83, 85). In these complexes, the 150-kDa neurotoxin component is associated with nontoxic proteins and RNA. The nontoxic proteins in the complexes provide protection of BoNTs during oral ingestion and passage and absorption in the gastrointestinal tract, experimental manipulations, and food processing (8, 42, 162).

Seven BoNT serotypes (A, B, C1, D, E, F, and G) are currently recognized (10, 82). Serotypes are experimentally distinguished by the neutralization in the mouse bioassay using serotype-specific antitoxins raised against purified neurotoxins in rabbits, horses, mice, and other

animals (10). The seven serotypes differ in potency in animal models and in humans, as gleaned from case studies of foodborne botulism in humans (2). Studies in animals and analyses of case studies have revealed that BoNTs from proteolytic serotype A cause the most severe and long-lasting botulism. Type B also causes severe foodborne and infant botulism but appears to be less potent than type A. Type E causes a milder botulism, but intoxication with type E can still require intensive care and be fatal. Foodborne botulism caused by type F has been extremely rare (2, 59). Infant botulism is mainly caused by BoNTs from proteolytic (group I) strains (59). Infant botulism has also been caused by rare strains of *C. butyricum* and *C. baratii* producing BoNTs E and F, respectively (59). Interestingly, several strains of *C. botulinum* isolated from infant botulism cases produce more than one serotype of BoNT, suggesting that transfer of genes encoding BoNTs may be promulgated in the infant intestinal environment. Wound botulism, which mainly occurs through injections of street drugs or by inhalation of cocaine (18, 163), is caused mainly by proteolytic strains of *C. botulinum* (164).

A landmark achievement in understanding the molecular pathology of botulism was the determination of the three-dimensional structure of BoNT A1 (89), which revealed that the 150-kDa BoNT consists of three primary domains. These three primary functional domains of approximately 50 kDa each include (i) H_C, the receptorbinding domain located in the C-terminal region of the heavy chain; (ii) H_N, the translocation domain residing in the N-terminal region of the heavy chain that enables entry of BoNT into neurons; and (iii) the light chain, which is the catalytic domain that has endopeptidase activity on neuronal substrates and cleaves substrates of the SNARE protein translocation apparatus, thus preventing release of neurotransmitter from neuronal cells (36, 38). Subsequently, the structures of BoNTs serotypes B and E were also found to have three primary domains (165). The importance of the discoveries of structures and associated functions in advancing the field of BoNT study cannot be overemphasized.

Studies during the past decade have revealed that subtypes (isoforms) of BoNTs occur within the serotypes, in which the 150-kDa proteins differ in amino acid sequence by >2.6%, as derived from amino acid sequences (121, 166). More than 40 subtypes of BoNT types A, B, and E have been detected by DNA sequencing, and sub types A1 to A6, which differ by 3 to 12% in amino acid sequence, have been purified and characterized (166, 167). Only a few subtypes of serotypes BoNT B, E, and F have been expressed, purified, and studied for toxicity, antibody neutralization by antitoxins, and other properties. Properties of BoNT subtypes related to foods, such as oral toxicity, stability in foods, production in foods, and other aspects, have not yet been studied.

In 2015, it was suggested that a new BoNT serotype, type H, was produced by an isolate of *C. botulinum* from a case of infant botulism (168). Definitive characterization of the purified BoNT FA "H" revealed that its activity was neutralized by antisera to BoNT A (169, 170). Therefore, by definition of a serotype, demonstration of its neutralization by antibodies against BoNT A precluded BoNT FA "H" as a new serotype.

CLINICAL ASPECTS AND CATEGORIES OF HUMAN BOTULISM

Botulism is a true toxemia, and BoNT is solely responsible for the illness. After being absorbed into the circulation, BoNT binds to and is internalized in the nerve terminals. The primary target is the neuromuscular junction, which has been termed "the most vulnerable synapse" (16). The symptoms of foodborne, infant, and wound botulism are clinically similar. The hallmark clinical symptoms of botulism are a bilateral and descending weakening and paralysis of skeletal muscles (2, 18). An adult patient with characteristic cranial flaccid paralysis is illustrated in Fig 18.2. The Working Group on Civilian Biodefense has described a classic triad of symptoms of botulism: (i) symmetric, descending flaccid paralysis with prominent bulbar palsies; (ii) lack of

fever; and (iii) a clear sensorium in the patient (7). The bulbar palsies can be summarized as the "four Ds": diplopia, dysphagia, dysarthria, and dysphonia. Autonomic dysfunctions, including constipation, urine retention, and mydriasis, also occur, particularly with certain serotypes of BoNTs (171). A recent in-depth review outlined the primary symptoms in adult and infant botulism (18). Intoxication by BoNTs can be exacerbated by the presence of underlying neuromuscular diseases in the patient, such as myasthenia gravis, and by neuromuscular blocking agents, such as aminoglycoside antibiotics (2).

In most cases of botulism, cranial nerves are first affected, particularly those innervating the eyes, and the first symptoms are blurred and double vision, dilated pupils, and drooping eyelids (Fig 18.2) (2). The eyes respond slowly to light in a darkened room. These abnormalities are followed by other symptoms in the cranium, including difficulty in swallowing, weakness of the neck and mouth, dysphagia (drooling and difficulty swallowing), and problems in speaking (18). As the paralysis descends, weakness of the upper limbs and the torso occurs, and in severe cases, muscles affecting respiration are weakened, and mechanical ventilation to prevent death by suffocation is required (2, 18). Generally, the patient's hearing remains normal, consciousness is not lost, and the victim is cognizant of the progression of the disease. Other symptoms may occur, including nausea or vomiting, dizziness or vertigo, diarrhea or constipation, dry mucous membranes in the mouth and throat, sore throat, and paresthesia, which may be related to botulinal neurotoxin or other toxins or pathogens in the contaminated foods (18). Infant botulism has certain distinct features, including constipation, weakness and hypotonia, poor sucking and feeding, weak cry, lack of head control, and cardiovascular abnormalities (hypotension and tachycardia) (172, 173). Certain cases of botulism have been associated with abnormalities of autonomic and sensory functions, such as constipation, dry mouth, and difficulty in urination. A patient's awareness of weakening of muscle activity and ensuing paralysis can lead to considerable emotional distress, including anxiety and depression (2). The most severe and longlasting type of botulism is foodborne botulism, which generally occurs with type A. In general, the duration of foodborne botulism illness follows the pattern A ≥ C > B > F > E in animal models and in humans (2). In infant botulism, nearly all cases are caused by group I strains of serotypes A and B, and rare cases are caused by type F. Recently, cases of type C and E infant botulism were documented (5). The fatality rate from foodborne botulism has decreased from 50 to 70% in the 1800s and 1900s to 5 to 10% in the past 20 to 30 years (5, 19, 174). Convalescence and recovery from botulism are usually prolonged, requiring weeks to months depending on the serotype of toxin and the quantity consumed. Recovery is usually complete, and patients regain full normal function (2).



Figure 18.2 Portrayal of a person with the flaccid paralysis symptoms characteristic of botulism. Drawing prepared by James K. Archer, Centers for Disease Control and Prevention, Atlanta, GA.

Several categories of botulism are recognized (2, 5). Foodborne botulism occurs following ingestion of preformed BoNT in foods. Infant botulism results from the growth and toxin production by *C. botulinum* in the infant (≤12 months of age) intestinal tract. In humans more than 12 months of age, intestinal botulism can also occur particularly after surgery, antibiotic treatment, and other predisposing conditions. *C. botulinum* can colonize skin punctures and incisions and cause wound botulism through infection of the wounds, with formation of BoNT that enters the systemic circulation. Inhalation botulism has been observed experimentally in mice, guinea pigs, and primate animal models (175, 176). Inhalation botulism has rarely occurred in humans, and one documented incident involved transfer of BoNT from the fur of laboratory animals (177). Iatrogenic botulism with systemic symptoms results from inappropriate injection of BoNT for medical use or by counterfeit BoNT preparations (17, 71, 73) and appears to be increasing in incidence (72). An "undetermined classification" of botulism is listed by the CDC and refers to cases of diagnosed botulism for which no plausible food vehicle or intestinal colonization by *C. botulinum* can be determined (2, 5, 14, 178). Intentional botulism through bioterrorist activities has been inflicted in humans, particularly during World War II (7, 179), but attempts to induce botulism in humans by transmission in water, foods, and aerosols have failed. Even so, concerns and possible scenarios of intentionally caused botulism associated with terrorist activity have periodically reemerged, including those concerning the supply of milk for school children (179, 180). Overall, foodborne and infant botulism appear to be the most common forms in humans worldwide, but the incidence of botulism is quite low.

Most botulism cases worldwide are caused by BoNT types A, B, and E, and rarely by F (2, 54, 59). The incubation time for onset of symptoms of foodborne botulism varies with BoNT serotype and with the quantity of neurotoxin that is ingested and reaches target nerves. Foodborne botulism occurs following the consumption of food contaminated with preformed BoNT, and probably with *C. botulinum* cells present in the food. It has been observed in certain foodborne-botulism cases that the food consumed has viable *C. botulinum* cells and spores, and it is possible that these contribute to the incidence and duration of the disease during ingestion and in the intestinal tract, but this has not been adequately studied (5). In foodborne-botulism cases, the onset time is usually 12 to 36 h following consumption of the toxic food (2). The incubation period can be as short as 2 h when large concentrations of toxin are ingested or as long as 2 to 14 days with serotypes B, E, or F or ingestion of small amounts of BoNT (2). Wound botulism usually has a relatively long incubation period of 4 to 14 days, reflecting the time needed for

neurotoxigenic clostridia to colonize the wound and produce neurotoxin (18). Infant botulism has an incubation time of 6 to 8 hours to several days (2), although a more rapid onset has occasionally been reported (2). It is controversial whether rapid onset of fulminant botulism is a cause of sudden infant death syndrome (181, 182). Intestinal botulism has also occurred concurrently with other clostridial intestinal infections, such as that caused by *Clostridioides* (formerly *Clostridium*) *difficile* (2). It is likely that the microbiome affects the ability of *C. botulinum* to colonize in intestinal botulism, and this field is only beginning to be explored (183).

DIAGNOSIS OF BOTULISM

Diagnosis of botulism involves a physician's assessment of clinical signs, including the initial visual disturbances and other cranial effects, skeletal muscle weakness, and detection of BoNT from appropriate specimens by the mouse bioassay (2, 18, 28, 29, 184, 185). Electrodiagnostic testing can provide presumptive diagnosis of botulism (18, 186). Electrophysiologic testing is also useful for differential diagnosis of other causes of acute flaccid paralysis syndromes, such as Guillain-Barré syndrome, myasthenia gravis, tick paralysis, and Lambert-Eaton syndrome (2, 186). Although clinical signs can provide a presumptive diagnosis of botulism, the definitive diagnosis is the detection of BoNT in the patient's serum, stool, wound, or vomitus and/or in suspect contaminated food (10, 14, 29). However, there are caveats related to the detection of BoNT in foods and clinical samples. The mouse bioassay may be negative for detection of BoNT in clinical samples, including serum and feces after 48 h of exposure, and foods may be unavailable for analysis (5, 10). False-positive results may result from lethal compounds in foods other than BoNT (5, 10). These limitations and their resolution have been carefully described (10).

The accepted procedure for BoNT detection is the mouse bioassay, which consists of two essential steps: (i) determining whether a food substrate or an extract in gel-phosphate (0.1 M sodium phosphate–0.2% gelatin, pH 6.2) is lethal on intraperitoneal or intravenous injection into mice and (ii) confirming the lethal agent as BoNT by neutralization with specific botulinum antitoxin (2, 10). When BoNT of a nonproteolytic *C. botulinum* strain is tested, it is necessary to activate the toxin to the light and heavy chains by limited proteolysis using trypsin (2, 10). A problem often encountered in testing of clinical and food samples is that these specimens may contain nonbotulinum substances that are lethal to mice and cause nonspecific deaths. These can be detected by evaluation of characteristic botulism symptoms and the onset time of symptoms in mice (generally >3 to 4 hours in mice injected intraperitoneally), by "diluting out" these substances, which generally have a lower toxicity than botulinal neurotoxin, and most definitely by use of serotype-specific antitoxins in a subset of the samples used for injection (2, 10). Another difficulty is that samples may contain more than one serotype of toxin due to a *C. botulinum* strain producing more than one serotype of BoNT or due to multiple strains forming BoNTs. These difficulties in BoNT analysis and their solutions have been described (2, 10). When delays in specimen collection and analysis occur, or if mouse-lethal substances other than BoNT are present in samples, laboratory detection of BoNT may not be conclusive. In clinical cases of botulism, a positive test may not be obtained more than 2 days after human exposure to neurotoxin. Therefore, it is important to process samples rapidly and to culture neurotoxigenic clostridia from the foods as described below.

Recently, our laboratory and others have developed neuronal cell lines sensitive to BoNTs, including primary spinal cord neurons and human stem cell pluripotent neurons, and these have outstanding potential for assay of and determination of the mechanisms of BoNTs (115, 187). Nonanimal cell assays that depend on all of the steps in the intoxication mechanism, including

receptor binding, internalization, and catalytic cleavage of neuronal substrates, such as cell-based assays (115), are excellent systems for the determination of toxin titers (115) as well as antibodies in patients' sera (188). Of particular promise is the use of human neuronal cell cultures, because toxicity to these cells depends on all domains of botulinal neurotoxin and the steps needed for intoxication (115). However, many neuronal cell lines are relatively insensitive to BoNTs, particularly cell lines derived from neuroblastomas. Recently, our laboratory and others have developed sensitive cell lines, including primary spinal cord neurons and human stem cell pluripotent neurons (91, 99–101), and these have outstanding potential for assay of and determination of the mechanisms of BoNTs. Recently, the FDA approved a cell-based assay for determination of botulinum type A toxin for use in medicine (115; www.fda.gov).

TREATMENT OF BOTULISM

Intoxication by BoNT at the neuromuscular junction of motoneurons prevents the release of acetylcholine to muscles innervating the eyes, face, and mouth, and then the toxicity descends bilaterally to motor nerves of the torso, causing a generalized paralysis. Currently, there is no antidote for botulism once it enters nerves, and the only treatment of the disease is thorough nursing care and, in severe cases, mechanical ventilation, parenteral feeding, and rehabilitation. Serum therapy by administration of human (BabyBIG) (189) in infants or heptavalent equine antibodies (currently heptavalent botulinum antitoxin) (30, 190) into the circulation of poisoned toddlers and adults (2, 191) can decrease the severity of the paralysis and duration of the illness. Antitoxin can effectively neutralize unbound toxin in the circulation, but it will not prevent the disease once receptor binding and the internalization process of BoNT are under way, while BoNT remains in the bloodstream.

CONTROL OF *C. BOTULINUM* AND FORMATION OF BoNTs IN FOODS

Botulism in the United States typically occurs in certain classes of foods, and these are subjected to certain processing and handling practices (Table 18.5.). Prevention of botulism is primarily dependent on proper food preparation, processing, formulation, and storage. The elimination of spores from raw ingredients is not practical, but spores can be eliminated by processing technologies (see chapter 2). Foodborne botulism is very rare, and the food industry and researchers in academia have extensively studied the control of *C. botulinum*, yet outbreaks continue to occur.

The primary factors that control the growth of *C. botulinum* in foods are temperature, pH and acidity, a_w, redox potential, nutrient sufficiency, the presence of antimicrobials, and competitive microbiotas (139) (Table 18.6.). In foods formulated to be botulism safe, inhibition relies on the inhibitory activities of combinations of factors (24, 25) (Table 18.6.). Among the most important inhibitory factors are an acidic pH and low a_w. For group I *C. botulinum*, the critical pH for botulism safety of foods is pH 4.6, below which *C. botulinum* spores will not germinate, outgrow, and form BoNT. Food products with a pH of <4.6 are termed high-acid foods and have an excellent record of botulism safety (24). Low-acid foods are those having an equilibrium pH of >4.6 and an a_w of >0.85. These foods require suitable processing or preservation procedures to prevent the survival and growth of *C. botulinum* (see chapter 2). For group II *C. botulinum*, the critical pH is 5.0 for prevention of spore germination and outgrowth with formation of BoNTs. Short-chain organic acids (e.g., lactic, acetate, and malic acid) with pK_a values in the range of 3.0 to 5.0 are generally used as acidulants, as these are more inhibitory than mineral

acids at equivalent pH values. When acids are used to adjust the pH of foods, adequate time and mixing are required for diffusion of the acids throughout the foods, occasionally several hours to days. If the foods will not be subsequently processed, then these acidulated foods should be refrigerated until equilibrium is reached.

Table 18.5 Typical foods associated with foodborne botulism

Fermented fish products
Home-canned vegetables (green beans, mushrooms, olives)
Foods preserved in oil (garlic, onions)
Cooked foods stored at room temperature (potatoes, onions)
Foods packaged in air-tight or modified-atmosphere containers with inappropriate refrigeration

The classification of foods into groups with high and low acidity has been a useful criterion for ensuring the botulism safety of most foods (24, 139). However, botulism outbreaks have been attributed to high-acid foods, particularly tomato products and certain homeprepared foods (24). Certain conditions can enable toxin formation in high-acid foods, including inadequate penetration of acids into larger pieces of food, thus creating microenvironments with higher pH. A second important factor has been termed metabiosis, whereby yeasts, molds, or bacteria can metabolize acids and other components such as proteins and raise the pH to a level at which *C. botulinum* can grow. For example, fungal mats can form at the surfaces of certain foods, thereby raising the pH under the mat and creating permissive conditions for *C. botulinum* growth and BoNT formation. Growth of *C. botulinum* has been reported in acid media in which precipitated protein or meat particles are present, probably forming a permissive microenvironment pocket for *C. botulinum* proliferation (24). Upper pH limits for growth of *C. botulinum* are in the range of pH 8 to 9, but inhibition by alkalinization is not practical in most foods.

Table 18.6 Primary physical treatments and intrinsic factors used in formulation of botulism-safe foods[a]

Thermal treatment
pH and acidity
a_w and solute composition
Presence of antimicrobials and competitive microbiotas
Organic acids
Nitrites, sulfites, phenolic compounds
Polyphosphates
Fatty acids and esters
Gas composition
Naturally occurring antimicrobials
Competitive microbiotas
Indirect antimicrobials

[a]Modified from reference 46.

Control of a_w, for example by addition of NaCl, is also critical for preventing growth of *C. botulinum* formation of BoNTs (24). Food preservation by brining is mainly due to the reduction of a_w, whereby adequate free water is not available to the organism for growth. Brining is the

most common practice for reducing a_w in food preservation. The limiting values for inhibition by brine alone are 10% and 5% for group I and group II *C. botulinum*, respectively (24). High concentrations of sucrose can also inhibit *C. botulinum* by reducing the a_w, but approximate concentrations of 30% and 15% sucrose are required to inhibit group I and group II strains, respectively. Similarly, other compounds, including glycerol, organic polymers, ions such as potassium, and other food components, can bind free water and reduce the a_w, but their efficacy in inhibiting *C. botulinum* growth is generally relatively poor on a weight percent basis compared to that of NaCl (24, 139). Therefore, when these compounds are used as preservatives, it is essential to measure the a_w of the food during its formulation and to conduct challenge studies of the food during development and before commercialization.

Refrigeration is commonly used to prevent *C. botulinum* growth and BoNT formation in foods. The minimum temperature for growth of group I strains is commonly accepted to be 10°C (50°F), whereas group II strains can grow at temperatures as low as 3.3°C (38°F). Growth at lower temperatures can require weeks to months due to the slowing of metabolism and lowering of the growth rate. The upper temperature limits for growth for group I and group II strains are 45 to 50°C (113 to 122°F) and 40 to 45°C (104 to 113°F), respectively. Refrigeration without processing or inclusion of preservatives is increasingly being used in the food industry for prevention of *C. botulinum* growth and BoNT formation, as many consumers desire “healthy” and “organic” foods that have minimal processing and contain low levels of salt or other preservatives. Certain minimally processed foods depend on refrigeration alone for botulism safety, yet this practice can result in *C. botulinum* growth and formation of botulinal toxin because of poor temperature control or temperature abuse in food stores, in food service establishments, or by consumers. Temperature abuse is one of the most common mishandling practices that result in BoNT production and botulism outbreaks. Therefore, it is not recommended to rely solely on low temperature for botulism safety, and adequate processing and/or formulation by inclusion of secondary barriers such as antimicrobials is recommended (Table 18.6.).

Since *C. botulinum* is a strict anaerobe, a permissive redox potential (E_h) is required for germination of spores and growth of vegetative cells (139). However, relatively high E_hs of approximately +200 mV can also allow germination and growth initiation of spores under certain conditions (192). The critical level of oxygen that prevents germination and growth by *C. botulinum* is approximately 1 to 2%. However, the level of oxygen in a food is extremely difficult to control, due to vaporization and growth of the microbiota, which can alter oxygen levels. Carbon dioxide can also be inhibitory to *C. botulinum*, partly due to lowering of the acidity in the food. Although reduced oxygen and increased carbon dioxide levels may be permissive for *C. botulinum* growth and toxin production, the safety risk depends not only on the gas environment but also on the product, storage temperature, packaging film used, and inherent competitive flora.

Modified-atmosphere packaging and high-barrier films can be used to effectively extend the shelf life of foods under refrigeration conditions (5, 192). Reduced oxygen levels and increased concentrations of carbon dioxide in refrigerated modified-atmosphere-packaged foods can decrease oxidative and chemical deterioration, as well as inhibiting common aerobic spoilage microbes, such as fungi and many bacteria (5). Concerns have been raised that these conditions may also select for anaerobic psychrotrophic microbes, such as group II *C. botulinum*, and increase the botulism risk. The combination of high-barrier films and respiring foods such as vegetables may also lead to decreased oxygen levels for packaged foods in an ambient atmosphere. The reduced oxygen content, in turn, may enhance the growth of *C. botulinum*. In packaged mushrooms, a high-barrier plastic film reduced oxygen concentration through

respiration of the mushrooms and permitted botulinum toxin production (193). Toxin was detected in prepackaged mushrooms after 3 days of storage, although mushrooms were still considered organoleptically acceptable. A simple solution to prevent botulinum toxin formation was to increase gas exchange by introducing three or four holes in the wrap covering the mushrooms. Similarly, packaging of garlic in oil and relying solely on refrigeration for botulism safety resulted in outbreaks and changes in regulatory formulation requirements. In summary, the presence of oxygen at low concentrations alone cannot be relied upon to inhibit botulinum growth and toxin production. The effect of food components, such as sulfhydryls or competitive microbiotas, may also reduce oxidative-reduction potential to a level at which *C. botulinum* can grow. Therefore, similar to relying solely on temperature to control *C. botulinum* growth, neither redox-potential nor enhanced oxygen transfer is recommended on its own to prevent the growth of *C. botulinum*; hence, suitable processing and inclusion of secondary barriers are recommended.

An important factor for prevention of *C. botulinum* growth and toxin formation in many foods is the presence of a competitive microbiota. Most foods have low levels of *C. botulinum* spores, and the pathogen is often inhibited by a competitive microbiota, such as lactic acid bacteria or yeasts, that ferments sugars and other substrates in the food with production of inhibitory levels of organic acids, alcohols, and bacteriocins. A competitive microbiota or fermentates derived from cultures have been included in food formulations to control growth of *C. botulinum* (24).

Preservatives such as nitrite have traditionally been used for control of *C. botulinum* in foods (Table 18.6.) (24). Recently, there has been a trend to use “natural” inhibitors that generally have lower efficacy than traditional preservatives. For many foods, the use of a high level of a single inhibitor at high concentrations to control *C. botulinum* growth often diminishes the organoleptic acceptability of the food. Therefore, many foods are formulated to be organoleptically desirable by using combinations of inhibitory factors at sublethal levels, and they often receive minimal thermal processing or high-pressure treatment. The individual actions of these inhibitors often have cumulative or occasionally synergistic effects for inhibiting *C. botulinum* growth. An example of a low-acid food prepared in a botulism-safe way by using a combination of treatments and antimicrobials is cured meats, in which minimal heat processing is combined with the addition of nitrite (53). Another example is pasteurized processed cheese, in which sublethal heat processing is combined with subinhibitory levels of a_w (moisture and NaCl content), acid, and phosphate salts (194). Processed cheese and related products have an excellent safety record relative to botulism. Extensive studies on processed cheese at the University of Wisconsin Food Research Institute (Madison, WI) led to one of the first empirical models for botulism-safe product formulation (194). Strategies for controlling *C. botulinum* by using combinations of physical treatments and antimicrobials in various classes of foods have been described.

The nutrient composition of a food is also important in the control of *C. botulinum*. The minimal nutrient requirements have been determined for growth of *C. botulinum* types A, B, and E in chemically defined media (100). Certain nutrients are required in high quantities for growth of *C. botulinum*, such as arginine for group I *C. botulinum* and tryptophan for type E. Increasing the arginine content promotes growth of group I *C. botulinum* (195) in otherwise inhibitory foods, probably through active metabolism with release of ammonia and an increase in pH. The presence of glutamic acid in growth medium increases the salt tolerance in group I *C. botulinum*, possibly by serving as a compatible solute or as a precursor to a protective osmolyte (196). Substrate utilization as well as protein and lipid degradation varies among the different BoNT-producing clostridial groups (Table 18.2.). *C. botulinum* groups I to III can utilize glucose as a primary carbon source. Depending on the group, certain strains can ferment fructose, maltose, sucrose, and sorbitol to varying degrees. While *C. baratii* and *C. butyricum* utilize

lactose, insignificant acid production and growth from lactose are observed with *C. botulinum* groups I to IV, which is likely important in the botulism safety of dairy products and related foods containing lactose. The limitation of substrates required for growth and BoNT formation is valuable in the formulation of botulism-safe foods. Summaries of substrate characteristics are presented in Tables 18.2 and 18.3. An overview of food-related parameters used for control of *C. botulinum* is presented in Table 18.6..

An area that is increasingly gaining attention is the food microbiome and the roles of competitor bacteria and fungi in antagonizing *C. botulinum* and other pathogens and preventing toxin formation and foodborne infections (197). In practice, it has been realized for many years that competitive microbiotas are extremely important in controlling *C. botulinum* growth and BoNT formation in foods. A precise understanding of the species of microorganisms in foods should lead to new methods and formulations to control *C. botulinum* growth and BoNT formation. In addition, whole-genome sequencing and bioinformatics will be utilized to provide an in-depth understanding of the genome composition and the presence of genes involved in growth and toxin formation. The methods for identifying the microbiome and its role in *C. botulinum* control are described in chapter 2.

New genetic tools such as CRISPR-Cas for manipulation of the genome can be utilized to identify genes essential in the growth of *C. botulinum* and BoNT formation. Identification of these essential genes could provide a focused and advanced method for discovery of inhibitors that specifically inhibit the critical enzymes and prevent growth. “-Omics” technologies, including metabolomics, are advancing rapidly, and these technologies will be valuable for understanding the physiology of *C. botulinum* and BoNT formation and providing a foundation for control of the organism in foods.

In summary, prevention of BoNT formation in foods can be achieved by (i) avoiding contamination of foods by spores; (ii) inactivating spores that are present in foods; (iii) preventing spores from germination and vegetative cell growth, resulting in botulinal neurotoxin formation; and (iv) inactivation of botulinal neurotoxins in a food. Practically, it is difficult to prevent contamination of many foods by spores due to their widespread distribution and contamination of foods during harvesting, formulation, and processing. Hence, considerable research and development have been devoted to spore inactivation and prevention of growth in foods (see chapter 2).

Thermal and other processing methods for inactivating *C. botulinum* spores are described in chapter 2. The excellent safety record of botulism prevention in commercial, low-acid canned foods attests to the efficacy of industry thermal processing practices (198). In addition to thermal processing, newer food preservation methods under development, including high pressure, pulsed electric fields, ohmic heating, and high-intensity light and sound treatments, are in preliminary stages of evaluation for inactivating spores, particularly *C. botulinum* spores (see chapter 2).

BoNT INACTIVATION

The instability of BoNTs in foods and clinical samples is an important factor contributing to the safety of foods from botulism (199). In general, BoNTs are labile to heat, alkali, chlorine, and certain other physical and chemical treatments (199). Heat has long been recognized to be highly effective for inactivation of BoNTs. BoNTs are most stable to heat under acidic conditions such as pH 3.5 to 5 and in the presence of organic acids, proteins, and certain ions, such as Ca^{2+} and Mg^{2+}. Heating at 70°C for 1 h, heating at 80°C for 30 min, or boiling for 5 minutes inactivated toxicity in buffers and foods (199). However, the rate of thermal inactivation does

not follow a linear curve, and considerable tailing has been observed during heat inactivation of BoNT in various buffers and foods. The biphasic curves observed preclude the use of *D* values to model thermal inactivation of botulinal neurotoxin, and it has been proposed that heat resistance be expressed as the time required for reduction of activity to a minimal mouse LD_{50} (*F* value) (199). The basis for heat resistance of a fraction of the total content of BoNT is unknown, and it has been suggested that it could be due to the formation of protein complexes, aggregation, changes in physical shape, or renaturation on cooling. The tailing observed could affect the degree of inactivation of high levels of BoNT during pasteurization processes used for various foods, such as milk, and the possibility for retention of toxicity is a consideration in pasteurization procedures.

The stability of BoNTs in drinking water, lake water, and other liquids depends on the presence of organic matter, higher acidity, and hypochlorite (199). The BoNT A complex is stable in distilled water for 7 days, and stability is enhanced at lower pH values such as 4 to 5 and in the presence of proteins. BoNTs are sensitive to chlorine, and the concentrations of chlorine used in drinking water are generally adequate for inactivation. Contaminated objects or surfaces can be decontaminated with 0.1 to 0.5% hypochlorite solution. For routine sanitation and cleansing of surfaces, exposure to 0.1% hypochlorite (undiluted commercial bleach is about 5.25% hypochlorite) is adequate, and for higher levels of contamination, 0.5% should be used with 20 to 30 min of exposure, followed by rinsing with distilled water. For decontamination of spills, the toxin solution on a surface should be covered with absorbent material to prevent aerosolization. The BoNT A toxin complex is inactivated by alkali at pH values of >9.5 to 10 (ca. 0.1 M NaOH) (199). Limited studies have revealed that the BoNT A complex is inactivated by 250 ppm ozone (199).

BoNTs are not generally inactivated by freezing, particularly in the presence of proteins and organic acids at pH values of 5 to 6.5. The notable exception to stabilization by organic acids during freezing is acetate buffer, in which all toxic activity is lost for BoNT A (112). BoNTs are not significantly inactivated by gamma irradiation from a ^{60}Co source (199). Drying by vacuum drying or lyophilization can stabilize BoNTs, especially in the presence of proteins such as gelatin or albumin and excipients such as trehalose, which is used in commercial preparations of BoNTs for pharmaceutical uses (42). Overall, the effects of physical and chemical treatments on BoNT and its protein complexes have not been thoroughly studied, although such information would be useful to enhance the safety of foods.

SURVEILLANCE AND REGULATORY ACTIONS

In the United States, foodborne disease surveillance and epidemiology are conducted through the Foodborne Disease Outbreak Surveillance System (FDOSS) of the Centers for Disease Control and Prevention (CDC) and the National Outbreak Reporting System (reviewed in reference 5). Investigations of the causes and factors contributing to outbreaks of foodborne disease provide important insights for their prevention. The CDC provides monthly and annual summaries of outbreaks. FDOSS also provides information on the types of foods that are most commonly associated with outbreaks and provides insights into the routes and causes of contamination, including processing and handling of foods. This information can be used to apply preventive measures and controls to prevent outbreaks. Regulatory actions in response to a commercial foodborne-botulism outbreak have recently been reviewed (29).

Beginning in 1996, the CDC developed experimental procedures to facilitate a tracking program and national database, called PulseNet, for assisting in studying the epidemiology of foodborne disease outbreaks in real time (reviewed in reference 5). PulseNet involves a network of public

health national databases with different laboratories throughout the United States. The CDC and collaborators developed a standardized PFGE program for characterization of *C. botulinum* strains involved in foodborne outbreaks. The pulsotype method has certain drawbacks and cannot distinguish between certain closely related strains, particularly dual toxin producers. Due to these limitations, PFGE is gradually being succeeded by whole-genome sequencing and bioinformatics analyses, which are able to distinguish among closely related strains (200).

USE OF PREDICTIVE MODELING AND CHALLENGE STUDIES FOR EVALUATION OF *CLOSTRIDIUM BOTULINUM* NEUROTOXIN FORMATION

Predictive modeling can be useful in developing product formulations and assessing the processing and formulation parameters for a botulism-safe food. Among the earliest applications of predictive modeling in food microbiology was the development of processing models to determine commercial sterility in canned foods (see chapter 2). During the past 2 decades, advances in computing, software development, and bioinformatics have led to significant advances in the field of predictive microbiology (201, 202). These advances have led to the development and availability of predictive modeling tools (24, 202). These modeling approaches have provided valuable insights into the combined effects of biological and environmental factors on the growth, survival, and death of *C. botulinum*.

SAFETY PRECAUTIONS FOR WORKING WITH *CLOSTRIDIUM BOTULINUM* AND BoNT

BoNTs are extremely toxic molecules; they are considered the most potent poisons known (5) and are regulated by select agent rules (11). Because the consequences of an accidental intoxication are so severe, safety must be a primary concern of scientists studying these toxins (5, 13, 14). The CDC recommends biosafety level 3 primary containment and personnel precautions for facilities producing large quantities of BoNTs and in working with high-toxin-producing *C. botulinum* strains (18, 65). The CDC has defined an “exempt” quantity of BoNT as 1.0 mg, which can be used in biosafety level 2 laboratories. All personnel who work in the laboratory should be thoroughly educated on the hazards of working with *C. botulinum* and its toxins. They must have knowledge of spill control and toxin inactivation (0.1 to 0.5% hypochlorite or 0.1 M NaOH). Operations should be performed to prevent the formation of aerosols (104, 114), e.g., use of closed containers during centrifugation, avoiding pressurized containers containing active toxin, and application of absorbent materials to spills prior to decontamination. Proper personal protective equipment must be used, including eye protection, gloves, and lab coats. Personnel who work with large concentrations of toxin (1.0 mg and/or high-toxin-producing cultures) should be immunized. Formerly, pentavalent (A through E) toxoid was available from the CDC, but it has been phased out and a heptavalent toxoid is under evaluation. A biosafety manual should be posted in the laboratory and should contain the proper emergency phone numbers and procedures for emergency response, spill control, and decontamination. When possible, culture and toxin handling and manipulation should be performed in a class II or III biological safety cabinet with appropriate respiratory protection. The use of needles and syringes for bioassays requires extreme caution. Beginning in 1997, *C. botulinum* cultures and toxins were included in a group of select agents whose transfer is controlled by the CDC and for which restricted and secure working areas are mandatory (11). To transfer select agents, both the laboratory and personnel sending and receiving cultures and

toxins must be registered with the USDA and/or CDC and exchange the appropriate approval forms through their biosafety offices. The laboratory and principal investigator should maintain excellent and frequent communication with the biosafety office and responsible official within the organization.

CONCLUDING COMMENTS

Botulism is a rare and yet potentially fatal disease and is of high importance in food safety. Botulism is a true toxemia, since BoNT is solely responsible for the disease. BoNT is the most poisonous toxin known to humankind, and prevention of its formation in foods is of paramount importance to the food industry. BoNTs are produced by *C. botulinum* and rare strains of *C. butyricum* and *C. baratii*. These clostridia produce highly resistant endospores that are distributed throughout the earth and contaminate many foods. Remarkable advances have been achieved during the past 2 decades in elucidating the biochemistry, structure, and pharmacological mechanisms of BoNTs. Structural and biochemical studies of these potent neurotoxins have provided much insight into the mechanisms of substrate catalysis, neurospecific binding, and trafficking of botulinal neurotoxin to their neuronal targets. These advances have contributed to the remarkable success in using BoNT as a pharmacological agent for the treatment of various neuronal diseases and may lead to improved vaccines and countermeasures. The availability of genomic sequences and bioinformatic analyses has revealed that the genes encoding BoNT are often present as mobile genetic elements (Table 18.4.), and their lateral gene transfer is potentially a new hazard for the food industry and medicine. The development of genetic tools such as efficient gene replacement techniques will be invaluable in elucidating pathogenic mechanisms of BoNTproducing clostridia and the development of inhibitors targeted to essential functions.

Considerable information is available regarding the microbiological features of botulinogenic clostridia and preservation and formulation strategies for their control in foods. Nonetheless, botulism continues to occur through the consumption of foods, and new technologies and research are needed to enhance control. Newer processing procedures such as pulsed electric fields, ohmic heating, high pressure, and high-intensity light and sound require careful evaluation before they are widely applied in the food industry. Predictive models combined with physiological and genomic information will be valuable resources in the control of *C. botulinum* and the formation of BoNTs. Research for control of BoNT-producing clostridia in food systems has contributed to fundamental and applied knowledge in the food industry, and *C. botulinum* and BoNT formation serve as an excellent model for enhancement of the safety of foods.

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19
Clostridium perfringens

THE ORGANISM

Clostridium perfringens (Fig 19.1) belongs to the genus *Clostridium*, which comprises a diverse group of sporeforming bacteria that are unable to grow in the presence of oxygen. *C. perfringens* was first described as *Bacillus aerogenes* in 1892 and was later named *Clostridium welchii* (1). *C. perfringens* is a Gram-positive, nonmotile rod that forms large, regular, round, slightly opaque and shiny colonies on the surface of agar plates. Although this organism is an anaerobe, it is capable of growing at E_h (redox potential) values of +350 mV and reducing its environment to less than −400 mV. Most strains grow at an optimum temperature of 37 to 45°C, but they can also grow in the range of 15 to 50°C, with growth temperatures as low as 6°C (1).

This bacterium is the most prolific toxin-producing species within the clostridial group. The pathogenic potential of *C. perfringens* is facilitated by the production of more than 20 toxins and enzymes, which can be classified into four broad categories: membrane-damaging enzymes, pore-forming toxins, intracellular toxins, and hydrolytic enzymes. Fortunately, these toxins are not produced by all strains (2). *C. perfringens* produces various toxins that are associated with diseases. Alpha toxin (CPA) has phospholipase C and sphingomyelinase activity, and it affects host signaling. Beta toxin (CPB), epsilon toxin (ETX), perfringolysin O (PFO; also known as theta toxin), necrotic enteritis toxin B-like toxin (NetB), and enterotoxin (CPE) are pore-forming toxins. Iota toxin (ITX) is

an actin-specific ADP-ribosyltransferase and toxin. *C. perfringens* large cytotoxin (TpeL) is a Ras-specific monoglucosyltransferase (3).

The toxins lecithinase, hemolysins, hyaluronidase, collagenase, DNase, and amylase allow the bacterium to scavenge nutrients from the environment. The bacterium lacks the ability to produce 13 of the 20 essential amino acids; therefore, it obtains these amino acids by breaking down host tissue using its toxins and digestive enzymes.

Generation times for CPE-positive *C. perfringens* strains grown between 41 and 46°C can be less than 8 min when the organism is cultured in autoclaved ground beef (2). Experimentally obtained values for generation times at 43°C were as low as 8.7 and 6.3 min in thioglycolate broth for CPE^+ and CPE^- strains, respectively. For autoclaved ground beef, the shortest generation times (7.1 min and 9.2 min) were obtained at 41°C and 46°C for two CPE^+ strains, respectively. For CPE^- strains cultured in autoclaved ground beef, a generation time of 6.6 min was observed at 43°C (4). CPE and heat-resistant spores were produced at similar concentrations, but they were generated more quickly at 43°C than at 37°C (5). In addition, there is a direct relationship between spore heat resistance and sporulation temperature (6).

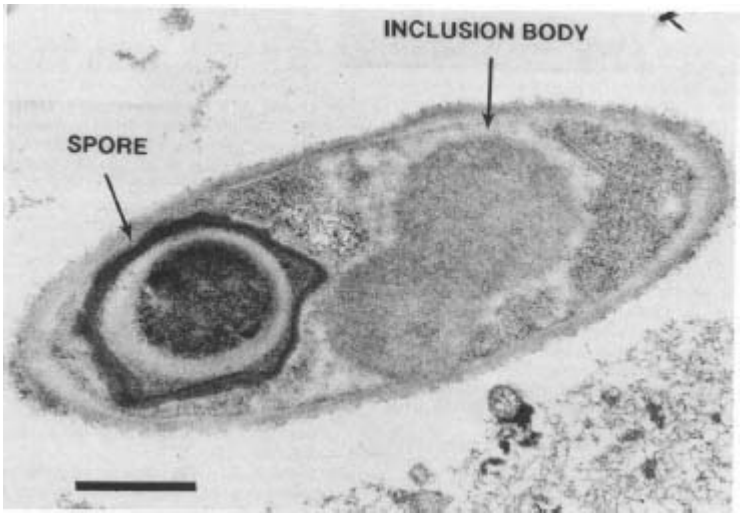


Figure 19.1 Electron micrograph of thin sections of *C. perfringens* FD-1041. Arrows indicate a spore and a CPE containing round inclusion body. Magnification, × 40,000. Bar, 0.5 μ. Reproduced with permission from reference 5.

C. perfringens causes several diseases, including gas gangrene (clostridial myonecrosis in infected wounds), food poisoning, antibiotic-associated diarrhea, enteritis necroticans (also called pigbel or Darmbrand; a serious enteric infection in humans), and other less severe diseases. Although the association of this organism with foodborne illness was first proposed approximately 100 years ago, it was not until the 1960s and 1970s that conclusive evidence accumulated showing that an enterotoxin is associated with the sporulation of *C. perfringens* in the intestines of affected individuals (2).

Various animal diseases are also caused by *C. perfringens* (type G in the new proposed toxinotyping scheme; CPA^+ $NetB^+$), including canine gastrointestinal disease and necrotic enteritis of poultry. The latter is an economically significant disease that has resulted in extremely high costs to the global poultry industry because of losses and control measures (7). *C. perfringens* type B (CPA^+ CPB^+ ETX^+) causes necrohemorrhagic enteritis in sheep (lamb dysentery), type C (CPA^+ CPB^+) causes necrotic enteritis in several neonatal animal species (horse, cattle, sheep, and pigs), type D (CPA^+ ETX^+) causes

enterotoxemia in sheep and goats, and type E (CPE^+ ITX^+) is suspected to cause gastrointestinal diseases in cattle, sheep, and rabbits (although this has not been confirmed) (7).

Classification

For many years *C. perfringens* was classified into five toxinotypes (A, B, C, D, and E) based on the production of four major toxins: CPA, CPB, ETX, and ITX (1). An updated toxinotyping system (Table 19.1.) based on the production of six major toxins (CPA, CPB, ETX, ITX, CPE, and NetB) has been proposed that incorporates two new toxinotypes (F and G). In this new proposed classification, *C. perfringens* type A strains are associated with gas gangrene but not with human food poisoning. *C. perfringens* type B, C, and D strains are mostly associated with enteric diseases in animals. *C. perfringens* type F consists of isolates that produce CPE but not CPB, ETX, or ITX and are responsible for *C. perfringens*-mediated human food poisoning and antibiotic-associated diarrhea. *C. perfringens* type G comprises isolates that produce NetB toxin and cause necrotic enteritis in chickens (8).

C. perfringens is a genetically diverse organism. The diverse nature of the *C. perfringens* pangenome likely stems from the fact that the organism prospers in multiple host species and environmental niches, interacting with many other bacterial species. Only a few bacterial studies have reported extreme pangenomic variation (9).

Ecology

C. perfringens is widely distributed in many environments, including soil, sewage, water, and the intestinal tract, and on almost every natural environmental surface examined thus far. The organism occurs in the intestine in both the vegetative and spore forms together with a complex mixture of microbes (10). Bacterial establishment in the neonatal gastrointestinal tract begins during birth when the neonate comes into contact with the maternal cervical and vaginal flora. Within 2 days after birth, *C. perfringens* is

found at higher numbers in newborns delivered by caesarean section. *C. perfringens* seems to be a precursor for the colonization of other anaerobic bacteria, such as *Bacteroides* and *Clostridium* spp. (11).

Table 19.1 *C. perfringens* toxin-based typing scheme based on the production of seven toxins[a]

			Presence of toxin[b]			
C. perfringens toxinotype	CPA	CPB	ETX	ITX	CPE	NetB
A	+++	–	–	–	–	–
B	+	++	+	–	–	–
C	+	++	–	–	±	–
D	+	–	++	–	±	–
E	+	–	–	+	±	–
F	+	–	–	–	+	–
G	+	–	–	–	–	+

[a]Modified from references 1 and 8.

[b]+, low production; ++, medium production; +++, high production; ±, some strains produce the toxin.

Heat-resistant spores of *C. perfringens* can survive in the environment for years (10). It is known that *C. perfringens* survives the longest in polluted waters and is, therefore, an excellent indicator of fecal contamination. The metabolic dormancy and resistance of bacterial spores to harsh environmental conditions are crucial to the ecological role of spores as survival structures (12).

VIRULENCE FACTORS

Signaling for Virulence through an Agr-Like QS System

C. perfringens strains encode a functional Agr-like quorumsensing (QS) system (referred to for simplicity as CpAL) that controls production of most *C. perfringens* toxins (13–15). CpAL also regulates sporulation (16) and formation of biofilm-like structures (17). As with several other QS systems, regulation of *C. perfringens* virulence factors via CpAL is mediated by population densityinduced autoinducer signaling molecules. At the structural and functional levels, CpAL is related to the Agr system from *Staphylococcus aureus* and other Grampositive organisms (13, 18). In *S. aureus*, the *agr* locus is a four-gene operon carrying in tandem the genes for a two-component system (TCS) comprising a transcriptional regulator (AgrA) and the membrane sensor (AgrC), the gene for the AgrD signaling peptide, and the gene encoding an AgrB transmembrane protein involved in AgrD processing (19) (Fig 19.2). Once secreted, extracellular AgrD binds to and activates AgrC, which then phosphorylates AgrA. In CpAL, the *agrB* and *agrD* genes are located in CPE1561 (strain 13 nomenclature) and within an intergenic region between CPE1561 and CPE1560, respectively (13, 18). Unlike the *S. aureus* Agr system, neither genes upstream nor downstream of *agrBD* in *C. perfringens* encode a TCS or are required to upregulate toxin gene transcription (13, 18).

Mechanistic Basis of CpAL Signaling

As mentioned, CpAL signals through a small secreted pheromone encoded by a short *agrD* gene, which is processed to a functional cyclic peptide by an integral transmembrane endopeptidase encoded by the *agrB* gene (Fig 19.2) (13, 18, 20). The pheromone is processed to an eight-amino-acid peptide containing a thiolactone ring, called 8-R. A linear version of this peptide, 8-L, was unable to activate production of *C. perfringens* CPB in a type C Δ*agrB* background (21). Further studies demonstrated that a pentameric peptide, containing only the thiolactone ring (5-R), was enough to activate signaling through CpAL to produce CPB in both type B and type C strains (22).



Figure 19.2 CpAL regulation of *C. perfringens* virulence factors (13, 15, 16, 18, 20–28, 36–40). CpAL is encoded by *agrD* and *agrB*, whose products are the AgrD signaling peptide (inset) and a transmembrane protein (AgrB) involved in its processing. The AgrD peptide is sensed by the membrane sensor (VirS), which in turn phosphorylates VirR, the response regulator. Upon CpAL activation, VirR directly upregulates transcription of several toxin genes or a small VR-RNA which ultimately activates expression of *plc* and *colA*. Whether VirR upregulates transcription of the CpAL operon is not clear.

In vitro studies have shown that CpAL regulates production of *C. perfringens* CPA, CPB, CPE, ETX, NetB, beta2 toxin (CPB2), and PFO in *C. perfringens* type A, B, C, and D strains. Regulation occurs at the transcription level (13, 15, 16, 18, 21). Two toxins that appear to escape from CpAL regulation, ETX and beta2 toxin, are produced by type B strains in which a mutation in genes encoding CpAL did not affect secretion of toxins into the supernatants (23).

A major role for CpAL in *C. perfringens* virulence was demonstrated using two animal models of necrotic enteritis. A rabbit ileal loop

model and a chicken necrotic enteritis model both showed that CpAL was required for full virulence and for production of CPB in the intestines of rabbits infected with a type C strain (21) or NetB in chicken infected with a type A (type G in the new toxinotype scheme) strain (15), respectively.

By the time when CpAL was discovered, it was well known that a TCS called VirSR was needed to regulate, at the transcriptional level, *in vitro* production of toxins and expression of several virulence genes, including the toxin genes *pfoA* and *cpa*, in type A strains (24–26). VirSR, where VirS is a membrane kinase and VirR is its cognate response regulator, was therefore the obvious candidate (18, 27–30). *virS* and *virR* form an operon (Fig 19.2) that is encoded by all sequenced *C. perfringens* toxigenic types (31–33). VirR can either bind to specific DNA sites via its C terminal DNA binding domain (34, 35) or regulate an RNA regulatory molecule, known as VR-RNA (VirSRregulated RNA), to regulate transcription (18, 36–38). Experiments demonstrated that the membrane sensor kinase, VirS, is activated by AgrD and this in turns activates the transcriptional regulator VirR (27, 28, 39, 40). As expected, both CpAL and VirSR are necessary for full virulence in animal models of *C. perfringens* necrotic enteritis (27, 41).

C. perfringens Toxins

C. perfringens produces several different toxins in various combinations. Besides the four well-known typing toxins, CPA, CPB, ETX, and ITX, this microorganism can produce a variety of other toxins of human and veterinary importance, such as PFO, CPE, and CPB2. The two toxins directly implicated in foodborne disease in humans are CPB and CPE, which are discussed below (42, 43).

CPB

CPB is a lethal toxin produced only by *C. perfringens* type B and type C isolates (44–46). Type B isolates cause an often fatal hemorrhagic dysentery in sheep, and possibly in other species,

while type C isolates cause enteritis necroticans in humans and enteritis and enterotoxemias in almost all livestock species. As a common trait, both type B and type C diseases are often accompanied by sudden death or acute neurological signs (46–49).

CPB mechanism of action

It was experimentally demonstrated that CPB is sufficient to cause type C pathology in a rabbit ileal loop model (44, 50). Type C disease has also been linked to endogenous trypsin deficiency or the presence, in the intestines, of trypsin inhibitors from the diet, as explained below. This results in persistency of CPB and maybe other toxins in the intestine (46, 50–52). CPB targets human endothelial cells, as evidenced by its presence in the lamina propria and submucosa in necrotic small-intestinal sections. CPB is also cytotoxic for *in vitro* human platelets, forming oligomers that result in a time-and dose-dependent death (53, 54).

Necrotizing enteritis in Papua New Guinea results from an increased consumption of a dish containing pork along with sweet potato containing trypsin inhibitors (called “kaw-kaw”) (46, 55). As CPB is highly sensitive to trypsin, the presence of trypsin inhibitors in the diet renders the innate response unable to inactivate CPB, allowing the intestinal colonization of type C bacteria, present in contaminated pork, with the subsequent intestinal damage caused by CPB (46, 50, 55). Prior to vaccination against this foodborne pathogen, which was undertaken in the 1980s, type C-induced necrotizing enteritis was the most prevalent cause of death in children over the age of 12 months in the highlands of Papua New Guinea (56, 57).

CPE

Characteristics of CPE

Nearly 5% of *C. perfringens* strains produce the toxin CPE (*C. perfringens* enterotoxin) (42). Evidence has been provided to link

CPE with *C. perfringens* type A (type F in the new proposed toxinotype scheme) foodborne illness. For example, there is a strong positive correlation between illness and the presence of CPE in feces, in contrast to the absence of CPE in feces of healthy individuals. CPE from the feces of food poisoning victims causes intestinal damage in animal experiments. Furthermore, when healthy volunteer adults were fed purified CPE, they developed the characteristic gastrointestinal symptoms, including diarrhea and cramping, of natural *C. perfringens* food poisoning (42, 58, 59).

CPE is a 319-amino-acid (~35-kDa) single polypeptide with a unique primary amino acid sequence. Although most CPE-producing stains belong to type A(F), some type C and type D strains carry the enterotoxin gene *cpe* and produce a CPE nearly identical to that of type A(F) strains (42, 45). The primary amino acid sequence of CPE is highly conserved among CPE-producing type A(F), C, and D strains, but a few type E strains carry a *cpe* gene variant that encodes a toxin with 10 amino acid differences (60, 61). Whereas the plasmid-borne *cpe* locus in type C isolates resembles that of type A(F) strains carrying *cpe* on the chromosome, in type D strains the chromosomal *cpe* locus contains two copies of an open reading frame likely encoding a transposase and thus presents substantial variation (60).

Molecular basis of CPE toxicity

As explained below, CPE is produced in sporulating cultures, and the toxin is released upon their lysis (62). Lysates from sporulating, but not vegetative, cultures of CPE-producing food poisoning strains cause gastrointestinal pathology, including unidirectional fluxes of sodium and Ca^{2+} and histopathological damage of the intestinal villi in animal models (63, 64). Sporulating lysates from a *cpe*-null mutant of a food poisoning strain failed to cause any intestinal pathology in animal models, while complementation reversed it to the wild-type phenotype (Fig 19.3) (65). In addition, physiologically relevant concentrations of highly purified CPE are sufficient to fully

recapitulate, in animal models, the gastrointestinal effects caused by sporulating culture lysates of CPE-producing type A(F) food poisoning strains (17, 18).

As briefly mentioned above, full-length CPE (~35 kDa) is cytotoxic for *in vitro* cultures of humans and animal cells (58, 66, 67). The first 44 amino acids of CPE, however, are not required for cytotoxicity (68, 69). These amino acids are not a signal peptide, as the toxin is not secreted but rather produced inside the spores and released upon lysis. Once CPE is released in the intestinal lumen, the toxin binds its receptor on enterocyte proteins that form the tight junction belt on epithelial and endothelial cells and belong to the family of claudins (58, 70). There are 27 different claudins; the topology of all claudins is similar, and they are composed of four transmembrane domains and two extracellular loops (ECL), named ECL-1 and ECL-2 (71). Whereas claudins 3 and 4 were initially identified as CPE receptors and have exhibited the highest affinity for the toxin, claudins 6, 7, 8, 9, 14 (55, 69–71), and 19 (71) also serve as receptors for CPE.

Regarding CPE, amino acids 290 to 319 are required for binding to claudins, while several residues, including Y306, Y310, Y312, and L315, are key for CPE to bind to its receptor (72, 73). On the other hand, the C-terminal half of claudins plays an essential role in CPE binding and action; this was elegantly demonstrated by using chimeric CPE-sensitive claudins expressed in fibroblasts (74). More recent studies by Robertson et al. (75) specifically mapped the CPE-binding region to the residue N149 of ECL-2 of claudin 4 (76).

After binding, CPE forms a small protein complex of ~90 kDa in plasma membranes of sensitive cells; this complex contains CPE and both receptor and nonreceptor claudins (42, 58). Formation of the small complex proceeds to oligomerization of the small complex to form a large complex of ~450 kDa that contains, besides receptor and nonreceptor claudins, CPE hexamers (76). The large complex is inserted into the membrane to form a pore, inducing cytotoxicity by permeabilizing the plasma membrane and allowing the influx of

Ca^{2+} into the cells, which triggers cell death (77–79). When low doses of toxin are incubated with sensitive host cells, CPE-induced Ca^{2+} influx can lead to apoptosis via activation of caspase-3, and at high doses of toxin, the massive Ca^{2+} influx produces a form of necrotic cell death (i.e., oncosis) (42, 80).

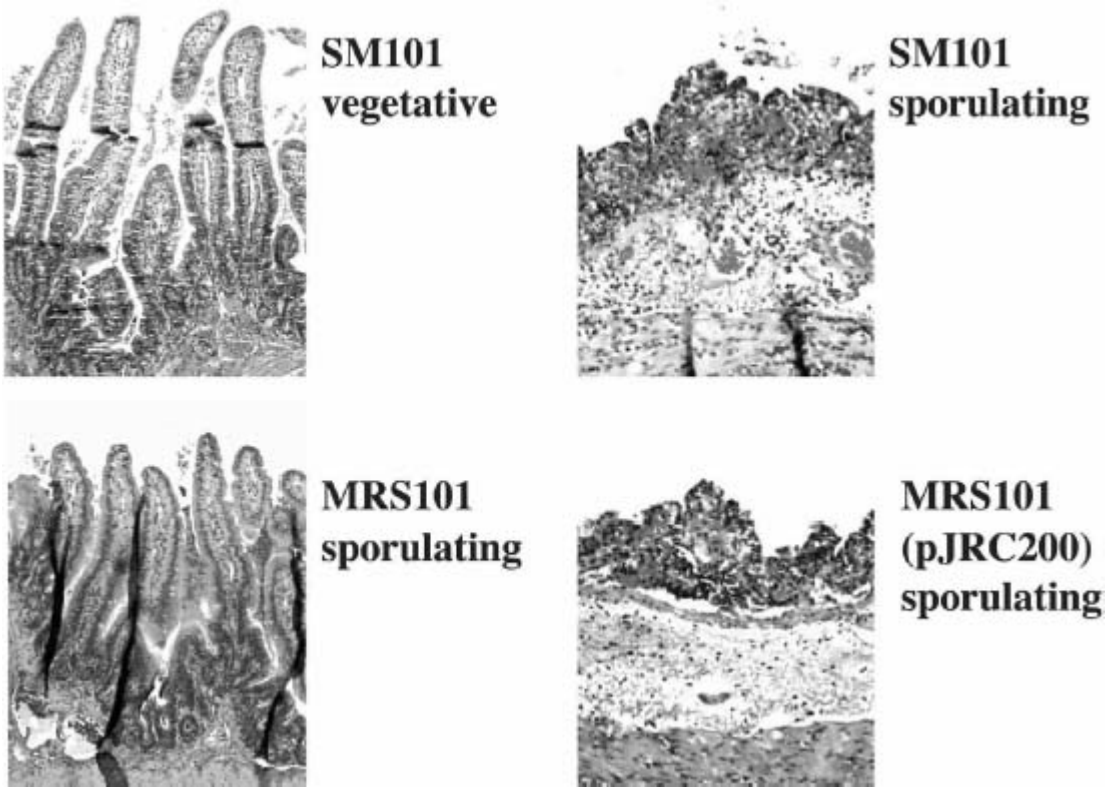


Figure 19.3 Histological damage is induced by *C. perfringens* lysates. Tissue specimens shown were collected from rabbit ileal loops treated with either concentrated vegetative (FTG) or concentrated sporulating (DS) culture lysates of *C. perfringens* wild-type, mutant, or complemented strains. Tissue specimens shown were treated with 50-fold-concentrated DS or FTG (as indicated) lysates of wild-type SM101, *cpe* knockout mutant MRS101, or complemented strain MRS101(pJRC200). Tissue specimens treated with 50-fold-concentrated FTG lysates prepared from either MRS101 or complemented strain MRS101(pJRC200) were indistinguishable from specimens treated with FTG lysates of SM101 (data not shown).

CPE causes histological damage to the human ileum epithelium which has been reproduced in different animal models (64, 81–86). A decrease in transepithelial resistance observed in cell culture models, including *ex vivo* cultures of human ileal cells, and fluid accumulation in rabbit ileal loops correlate with diarrhea observed in human volunteers fed up to 12 mg of CPE (59) and with clinical signs in patients who have suffered food poisoning where a mild and self-limiting diarrhea is observed (84).

Genetics of CPE

The CPE gene, *cpe*, is prevalent in <5% of *C. perfringens* strains, but these strains cause nearly 1 million cases of food poisoning cases every year in the United States (85). The CPE gene can be carried on the chromosome or on a large plasmid but has never been identified in both locations (i.e., in duplicate copies) (86, 87). There is an epidemiological correlation between the CPE-associated gastrointestinal disease and the genomic location of the *cpe* gene. Isolates carrying *cpe* on the chromosome have been linked to food poisoning cases, whereas isolates with plasmidborne *cpe* are strongly associated with antibiotic-associated diarrhea and sporadic diarrhea. Although *cpe*-positive type A(F) nonfoodborne human isolates and *cpe*-positive food poisoning isolates were found to produce similar amounts of spores and CPE, spores produced by food poisoning isolates are more resistant to low and high temperatures (explained in detail in "Sporulation" below) (58, 88).

Most *C. perfringens* type A(F) food poisoning isolates (~70%) that carry a copy of *cpe* on the chromosome have been associated with insertion sequences, including the IS*200*-like element IS*1469* and two copies of IS*1470* (a member of the IS*30* family) located upstream and downstream; the *cpe* gene, therefore, has been proposed to correspond to a 6.3-kb Tn*5565*-like transposon (89, 90). This putative transposon also includes open reading frame 3, located downstream of the *cpe* gene (89, 91). The remaining foodborne and nonfoodborne isolates carry the *cpe* gene on a large (70-to 75-kDa) plasmid that also includes the IS*1469* element and

IS*1470*-like elements along with a cytosine methylase gene (*dcm*) (92). The IS*1469*-like elements are present opposite to IS*1470*, which is found in foodborne type A(F) isolates carrying the *cpe* gene on the chromosome and appears to be defective (92). The prevalence of plasmid-borne *cpe*-positive isolates appears to be driven by vertical transmission rather than by transposition and recombination events which may occur in strains carrying a chromosomal *cpe* (42).

Synthesis and regulation of CPE

CPE is produced only during sporulation, resulting in a mature spore that, in food poisoning type A(F) isolates carrying *cpe* on the chromosome, is extremely resistant. Production of CPE is linked to sporulation, whose regulatory cascade is explained below (93, 94). During sporulation, chemical signs in the microenvironment, such as inorganic phosphate or bile salts, activate a master regulator of sporulation known as Spo0A. This regulator is also essential for formation of spores by several other C*lostridium* species and *Bacillus* species. Spo0A activates transcription of sigma factors, which in turn upregulate transcription of the *cpe* gene via three upstream promoters (95). The same promoters are present in the *cpe* gene whether it is plasmid borne or carried on the chromosome.

There are other regulators required for production of CPE, although the specific pathways have not been solved yet. Among them, for example, the catabolite control protein A (CcpA), which regulates utilization of carbon and nitrogen, is required for CPE production (96). CcpA regulation of sporulation does not appear to be linked to carbohydrate regulation, since the presence of glucose (5 mM) represses sporulation to the same extent in both a wild-type strain and an isogenic *ccpA* mutant (96). The CpAL QS system, found in most *C. perfringens* isolates whose genomes have been sequenced, is also involved in production of CPE and sporulation by upregulating transcription of genes encoding Spo0A and two sigma factors required to activate *cpe* transcription, SigF and SigE (16). Whether CpAL directly or indirectly upregulates *spo0A*

transcription remains to be elucidated. Another regulator, CodY, was recently linked to production of CPE and sporulation in type A(F) and type D strains (97, 98).

Sporulation

Sporulation is a long-term survival mechanism allowing the cells to survive until a more suitable environment is established (99). *C. perfringens*, like many other clostridia, initiates sporulation when unfavorable conditions (e.g., nutrient deprivation and changes in cell density) are encountered in the environment. Differentiation and spore morphogenesis are regulated by a fine mechanism involving transcriptional and posttranslational changes in several sigma factors, transcription factors, proteases, and phosphatases (100).

During sporulation, *C. perfringens* cells undergo significant morphological changes that can be divided into stages. In stage 0, bacteria experience vegetative reproduction by binary fission. Once bacteria enter sporulation in stage I, *C. perfringens* cells form an axial filament; the cells can revert to a vegetative state or continue the sporulation process. In stage II, cells initiate sporulation with asymmetric division of the cytoplasm membrane, where the asymmetric septum will become the endospore. This compartment is often termed the forespore, while the large compartment is called the mother cell. Stage III is engulfment, when the cells are fully committed to sporulation. Stage IV involves cortex formation, in stage V the deposition of spore coat proteins is observed, and in stage VI the endospore matures. Finally, the mother cell lyses in stage VII, releasing the spore into the environment (100, 101). Unlike other species, *C. perfringens* spores lack an exosporium, having instead a spore coat (101). The outer membrane is located immediately beneath the spore coat. The outer membrane is essential for spore formation.

Sporulation in *C. perfringens* requires inorganic phosphate (P_i). In the presence of P_i, the sigma factor Spo0A upregulates its

transcription, leading to spore formation. Unlike spores produced by nonpathogenic bacteria, *C. perfringens* spores not only are essential for survival in the environment but also contribute to pathogenicity, as sporulation leads to production of CPE, which is important for foodborne disease (101). During sporulation, Spo0A is phosphorylated, perhaps by orphan histidine kinases that have yet to be identified in *C. perfringens*. The phosphorylated form of Spo0A triggers transcriptional regulation of several genes involved in sporulation (101). In *Bacillus subtilis*, four sigma factors in addition to Spo0A are required for spore formation, named SigF (σ^F), SigE (σ^E), SigG (σ^G), and SigK (σ^K). *C. perfringens* carries genes encoding homologs of these four sporulation-associated sigma factors, and, as in *B. subtilis*, these sigma factors were found required for formation of mature spores in *C. perfringens*. Mutants carrying mutations in each gene were unable to sporulate, and the phenotype was rescued by genetic complementation (93, 94).

The CpAL system is required for sporulation at an early stage. An *agrB* mutant prepared in a spore-producing strain F5603 reduced sporulation by more than three orders of magnitude with concurrent reduction of Spo0A and SigF (16). The details of CpAL control of sporulation and CPE production remain to be determined.

Spore Resistance

Spores produced by *C. perfringens* are extremely resistant to conditions usually utilized in the food industry to reduce bacterial load, including exposure to low or high temperature, osmotic pressure, radiation, desiccation, chemical preservatives, and low or high pH (99, 101). For example, spores made by some strains remained viable after up to 60 min of incubation at a temperature of 100°C. Importantly, spores produced by some food poisoning isolates with chromosomally carried *cpe* survived for ~2 h in boiling temperatures (102, 103). Heat resistance appears to be an intrinsic mechanism of *C. perfringens* type A(F) strains carrying *cpe* on the

chromosome, which are associated with food poisoning, as it favors survival in incompletely cooked food.

Studies addressing the molecular basis of heat resistance of spores have concluded that it is influenced by both environmental factors and genetic factors. For example, food poisoning isolates that carry a chromosomal *cpe* gene were found to have a 60-fold-increased capacity to resist heat compared to *C. perfringens* isolates carrying a plasmid-borne *cpe* gene (103). Food poisoning isolates from the United Kingdom and Finland and strains from retail meats in Japan have shown a similar association between heat resistance and the presence of a chromosomal *cpe* gene (104–106). Therefore, rapid identification of isolates carrying the CPE gene and determination of its genomic location are important for outbreak investigation. As with the heat resistance phenotype, *C. perfringens* spores produced by isolates carrying a chromosomal *cpe* gene are considerably more cold resistant than vegetative cells or plasmid *cpe* isolates (107). The average log reduction of *cpe*-negative type A strains and isolates with plasmid-borne *cpe* was >3-fold greater than that of type A(F) strains carrying a *cpe* gene on the chromosome.

The extreme heat or cold resistance of type A(F) isolates carrying a chromosomal *cpe* gene is mediated by small acid-soluble protein 4 (SASP4). SASP4 is produced by most *C. perfringens* strains. Bioinformatics studies conducted by Li and McClane identified a SASP4 variant that contains an Asp at position 36 instead of Gly in *C. perfringens* strains producing heat-sensitive spores (108). This Asp36 variant of SASP4 binds more tightly to DNA, especially to AT-rich DNA, than Gly36 and was enough to confer heat, cold, and sodium nitrite resistance (108, 109).

Biofilm Formation

Biofilms are bacterial communities surrounded by a matrix which provides mechanical stability and mediates their adhesion to surfaces. The matrix ultimately forms a cohesive three-dimensional polymer network that protects bacteria from the host response and

antibiotics (110, 111). *C. perfringens* strains form biofilm-like structures on host tissues, epithelia, and abiotic surfaces (17, 112, 113). Biofilms are particularly important to food microbiology, as these structures allow bacteria to survive in the environment and thus to constitute a main source of food contamination (114). Recent studies demonstrated that most *C. perfringens* strains produced biofilm-like structures *in vitro* on abiotic surfaces such as glass and polystyrene (17, 112, 113). Moreover, *C. perfringens* biofilms have been harvested from different substrates, including steel, concrete, and polyvinyl chloride, thus representing a source of contamination of water for human consumption (112, 115, 116).

Evidence that *C. perfringens* Biofilms Are a Risk for Human Disease

A study in southern Africa reported that increased contamination of water was due to biofilms harboring heterotrophic bacteria, including coliforms and *C. perfringens* strains (117). In this study, authors found that mixed biofilms containing *C. perfringens* strains developed during the storage of water in containers prior to its distribution, rendering the water unsafe for human consumption (117). More evidence for the importance of *C. perfringens* biofilms was provided in a study conducted in Romania. Biofilms with a density of up to ~3.4×10^2 CFU/g were detected in their public water system. These *C. perfringens* biofilms were specifically isolated from steel and concrete walls in a clarifier (115). *C. perfringens* biofilms have been also isolated from wound tissues using a drip-flow reactor incubated for 7 days (118). The reactor was also utilized to simulate formation of multispecies biofilms, where a strain of *C. perfringens*, a strain of methicillin-resistant *S. aureus*, and a *Pseudomonas aeruginosa* isolate were inoculated and demonstrated to form mucoid biofilms with gas bubbles present throughout the biofilm structure. In this model, *C. perfringens* biofilms reached a density of ~1×10^7 CFU per membrane of drip-flow reactor (118).

Despite the interest in *C. perfringens* biofilms, we have limited information on antibiofilm approaches. A recent study by Charlebois and colleagues showed that potassium monopersulfate, quaternary ammonium chloride, hydrogen peroxide, and glutaraldehyde solutions reduced *C. perfringens* biofilms from a density of $>10^6$ CFU/ml to undetectable levels, including *C. perfringens* grown in dual-species biofilms with either *Escherichia coli* or *S. aureus* (119). These disinfectants are commonly used in farms and the food industry (120–122).

The *C. perfringens* Biofilm Matrix

As in biofilms produced by other pathogenic bacterial species, the matrix of *C. perfringens* biofilms is made of various proteins, including the hemolysins CPA and PFO, β-1,4-linked polysaccharides, and extracellular DNA (Fig 19.4) (17, 113, 123). Type IV pili are also required for biofilm formation; these pili are located on the biofilm matrix (112). More recently, Obana et al. (123) reported that biofilms formed at 37°C have an increased biomass and a phenotypically different biofilm matrix in comparison to those formed at 25°C. Furthermore, incubation at 37°C, but not at 25°C, upregulated levels of *pilA2* mRNA, which encodes the main pilin subunit of the matrix-associated *C. perfringens* type IV pilus (123). Moreover, chicken necrotic enteritis strain CP4 differentially regulated ~25% of its genome to adapt to changes at 44°C in biofilms, in contrast to planktonic bacteria (124). These data clearly indicate that *C. perfringens* biofilms are differentially regulated when they are produced at environmental temperatures versus that maintained in the human body. This was further clarified in a study conducted with QS mutants in a type A strain and a type C strain. QS CpAL mutants were not able to produce biofilms, and this was directly related to the downregulation, in the mutants compared to wild-type strains, of genes encoding the hemolysins CPA and PFO (17).

DISEASE

Food Poisoning

Symptoms associated with *C. perfringens* type A(F) food poisoning appear within 6 to 24 h and include stomach cramps and diarrhea. Although vomiting and nausea are uncommon in isolated cases, these symptoms have been reported during outbreaks of food poisoning (125–127). *C. perfringens* type A(F) *cpe*$^+$ isolates are the etiologic agents of food poisoning. As mentioned above, ~75% of type A(F) food poisoning cases are caused by isolates that carry the *cpe* gene on the chromosome, whereas the other ~25% are caused by strains carrying *cpe* on plasmids.

A typical pathogenesis cycle of *C. perfringens* type A(F) food poisoning is illustrated in Fig 19.5. Given that *C. perfringens* is ubiquitous, spores of *cpe*-positive *C. perfringens* type A(F) isolates can potentially contaminate food items at any given point of the food processing and serving chain at a variable density. Due to mishandling of food items from preparation through ingestion, along with the pathogenic properties of heat-resistant spores produced by type A(F) strains, the density of *C. perfringens* can reach ≥10^5 CFU/g of food; this infectious dose has been reported in suspected meals linked to food poisoning cases (126).

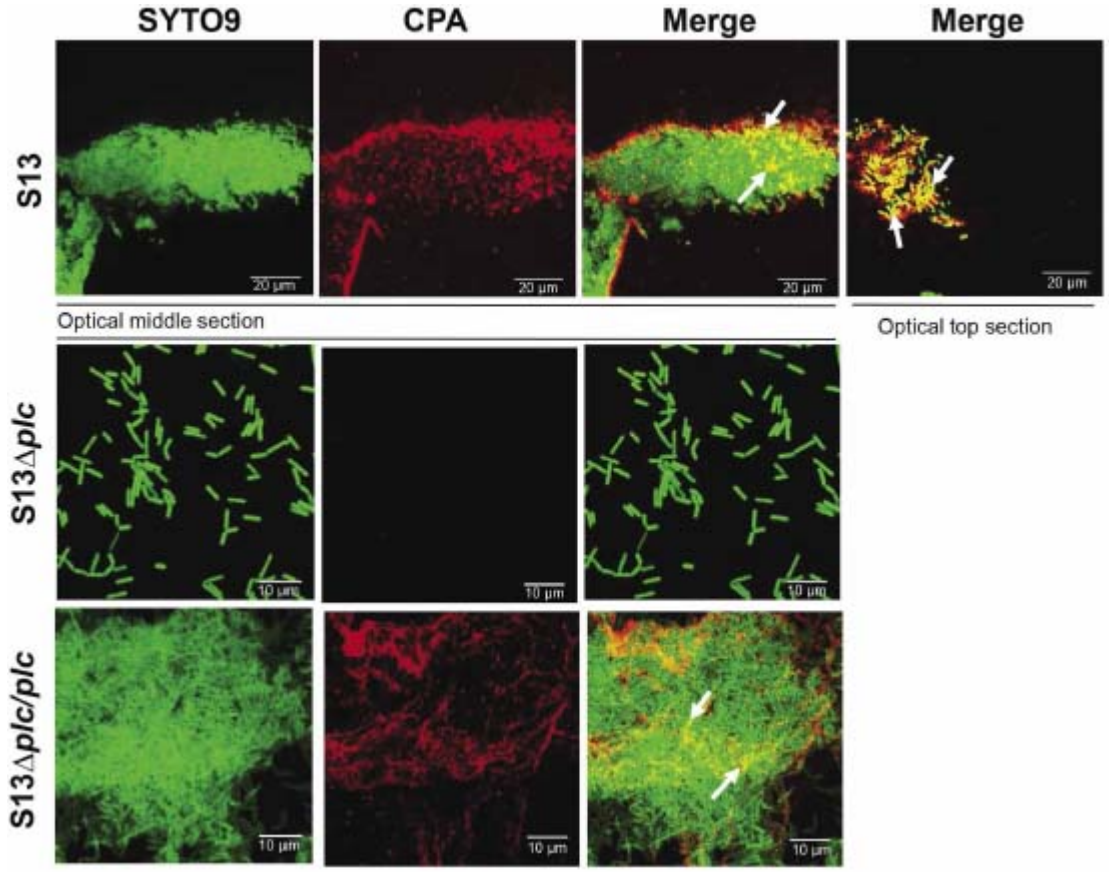


Figure 19.4 Detection of CPA on *C. perfringens* biofilms. Strain S13Δplc or S13Δplc/plc was inoculated into a four-well chamber slide containing tryptone glucose yeast extract, followed by incubation for 24 h at 37°C. Bacteria were stained with SYTO9, and CPA was detected using rabbit polyclonal anti-*C. perfringens* CPA antibodies, followed by goat anti-rabbit immunoglobulin secondary antibodies conjugated to Alexa Fluor 555. Optical middle and top sections were obtained with a confocal microscope. Arrows point to areas of colocalization.

Importantly, during outbreaks of food poisoning, the attack rate can be as high as 70% (126, 127). When food is undercooked (127), stored at abusive temperatures (126), or slowly cooled after cooking, bacteria can rapidly multiply until they are consumed with the contaminated food. Upon ingestion of *C. perfringens* vegetative cells, bacteria will probably die when exposed to stomach acidity. If the food vehicle, however, is contaminated with $>10^5$ *C. perfringens*

cells per g, some ingested bacteria survive the passage through the stomach and remain viable when entering the small intestine, where they multiply and sporulate. CPE is produced within these sporulating *C. perfringens* cells and is eventually released into the intestinal lumen, where the sporulating cells lyse to release their endospores. Once released, CPE quickly binds to intestinal epithelial cells and exerts its action, which induces intestinal tissue damage. This CPE-induced intestinal tissue damage initiates intestinal fluid loss, which clinically manifests as diarrhea.

Diarrhea, the hallmark of type A(F) food poisoning, is observed in nearly 100% of patients, whereas abdominal cramps occur in 95 to 70% of those who have eaten food contaminated with *cpe*$^+$ type A(F) strains. The onset of symptoms is generally observed within 10 h after ingestion of the contaminated food (125, 126). Because type A(F)-associated food poisoning requires bacteria to infect the intestines and then sporulate to cause the symptoms, the disease spontaneously resolves within 24 h after onset, likely because the toxin is expulsed from the intestines through diarrheic feces.

The largest outbreak of *C. perfringens* type A(F) food poisoning so far reported occurred in Washington State in 1978; in this outbreak, more than 500 people became ill after eating lobster Newburg at a restaurant (128). Fatalities due to type A(F) food poisoning are very rare, occurring in <0.03% of cases. Death usually is caused by dehydration and occurs among the very young, the elderly, and patients who have an immunodeficiency or are debilitated by illness. For example, there were reports of severe cases in patients from psychiatric hospitals in Oklahoma and Louisiana that were intoxicated with CPE and developed a severe form of food poisoning. These patients suffered secondary fecal impaction (i.e., constipation) due to drug therapy and therefore were unable to excrete CPE from the intestine, resulting in colonic perforation which in turn led to death (127, 129).

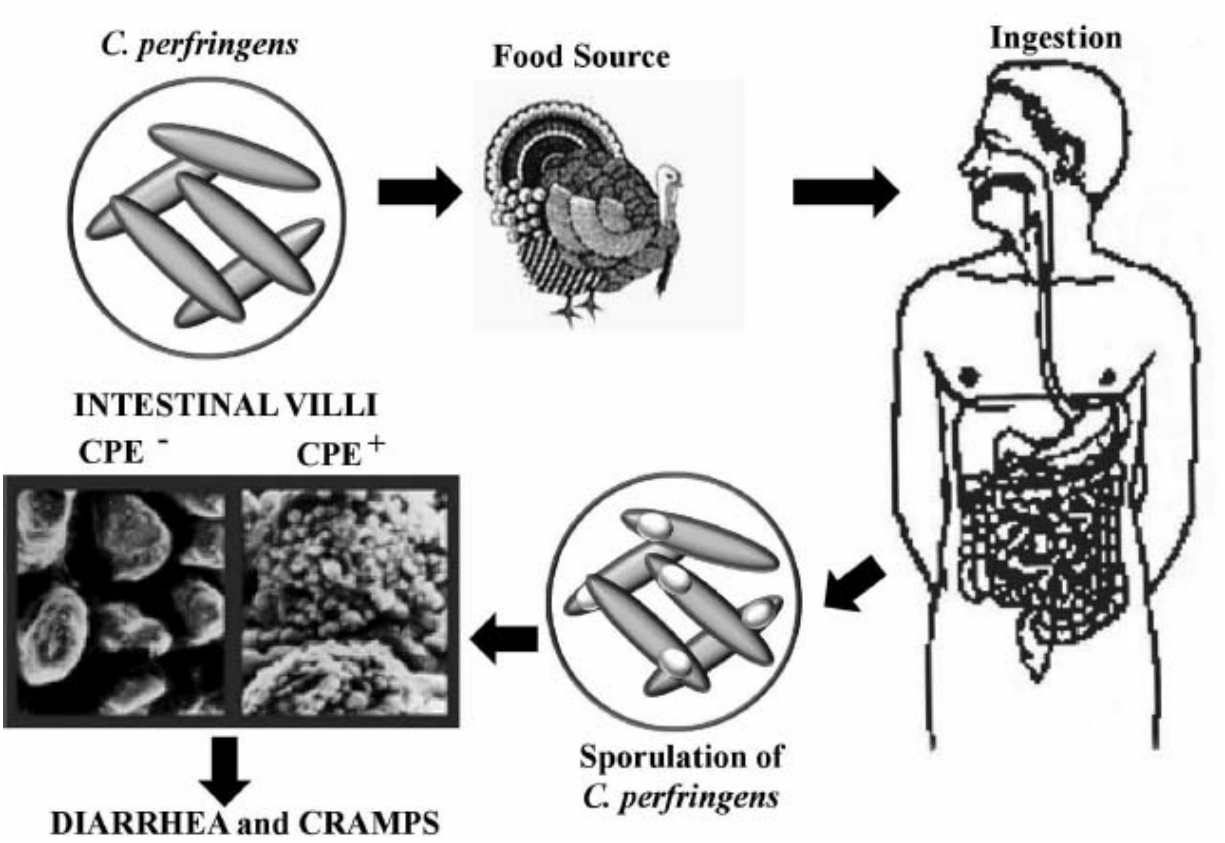


Figure 19.5 Pathogenesis of *C. perfringens* type A food poisoning. Vegetative cells of an enterotoxin (CPE)-producing *C. perfringens* strain multiply rapidly in contaminated food (usually a meat or poultry product) and, after ingestion, sporulate in the small intestine. Sporulated *C. perfringens* cells then produce CPE, which is released at the completion of sporulation, when the mother cell lyses to release its endospore. CPE then causes morphologic damage to the small intestine, resulting in diarrhea and abdominal cramps. Modified and reproduced with permission from reference 236.

The two most common symptoms of type A(F) food poisoning, diarrhea and cramps, correlate with the physiopathology induced by CPE in the intestines. Studies have clearly documented that electrolyte and fluid loss (i.e., diarrhea) is observed only with a dose of CPE high enough to cause intestinal damage. Cytotoxic damage, with fluid accumulation, in the intestinal epithelium has been reproduced in different animal models, although the most common is a rabbit intestinal model (64, 81–83). In rabbits, CPE-induced

intestinal damage includes villous shortening, along with epithelial necrosis and desquamation. CPE affects mainly the mucosa of the jejunum and ileum, with less damage of the duodenum; the colon is also sensitive to purified CPE, where it causes fluid accumulation, necrosis, and hemorrhage (43, 83). Several lines of evidence indicate that CPE-induced intestinal damage appears to be an important, if not essential, contributor to fluid and electrolyte secretion and therefore diarrhea. For example, in rabbit small-intestinal loops, a close temporal association exists between the onset of CPE-induced histologic damage and the start of fluid and electrolyte loss.

Other Human Diseases

Besides food poisoning, *C. perfringens* strains cause other diseases of biomedical importance, including human and animal myonecrosis (gas gangrene), nonfoodborne gastrointestinal disease, canine gastrointestinal disease, and necrotic enteritis in poultry. For example, type B strains can cause necrohemorrhagic enteritis in sheep (lamb dysentery), whereas type C isolates cause human necrotic enteritis (also called enteritis necroticans or pigbel) and necrotic enteritis in neonates of several animal species (horse, cattle, sheep, and pigs). Type D produces CPA and ETX, and it causes enterotoxemia in sheep and goats. Type E produces CPE and ITX. Although it has no known association with any human disease, it is suspected to be associated with gastrointestinal diseases in cattle, sheep, and rabbits. All types of *C. perfringens* may also produce several other toxins, including (but not limited to) CPB2, PFO, and TpeL (7).

Antibiotic-Associated Diarrhea

Although *C. perfringens* is not a major inhabitant of the intestinal flora, fecal carriage of enterotoxigenic strains appears to be important in the development of infectious diarrhea. The disease occurs in patients undergoing prolonged antibiotic therapy, which disrupts the stable intestinal microbiota and provokes an

overgrowth of enterotoxigenic strains. The average duration of infectious diarrhea is 11 days, and the elderly are most susceptible. Some studies report that *C. perfringens* is responsible for 10 to 15% of all cases of antibiotic-associated diarrhea (130, 131).

Gas Gangrene (Clostridial Myonecrosis)

C. perfringens cannot synthesize various amino acids because it lacks many genes related to amino acid biosynthesis. When infecting an organism, *C. perfringens* degrades host tissues to obtain nutrients for growth. During this process, *C. perfringens* produces abundant gas, which makes the conditions more suitable for further growth (20).

C. perfringens type A causes gas gangrene, which is an acute, rapidly progressive histotoxic infection in humans and animals that affects muscle tissue. It also causes infections of the fascia and skin, which are characterized by marked tissue destruction, sepsis, and massive tissue death. It develops after tissue injury or in patients suffering from various illnesses, such as diabetes or colon cancer. In these cases, vegetative cells or germinated spores of *C. perfringens* grow rapidly, invading and killing tissues, in addition to producing gas that causes an anoxic environment with reduced blood flow. This ultimately reduces the efficacy of antibiotic therapy. CPA and PFO, whose genes are carried on the chromosome, are essential for the development of gangrene. They act synergistically to provide essential nutrients to the microbe (7). The Agr-like QS system and the VirS/VirR system regulate the production of both toxins. However, PFO expression is directly modulated by the VirR response regulator, whereas CPA production is controlled by the VirR-regulated VR-RNA molecule (38).

Enteritis Necroticans

C. perfringens type C causes enteritis necroticans in humans, which is characterized by abdominal cramps, shock, bloody diarrhea, acute inflammation, and a pronounced necrosis of the intestinal mucosa

(132, 133). The disease is a life-threatening necrotizing infection of the jejunum and ileum. A high mortality rate is observed if the disease is not diagnosed early and treated with antibiotics. Infected persons can suffer from toxemia and segmental necrotizing enteritis, which is visible in the small intestines. This requires surgical excision of the necrotic small bowel and antibiotic treatment (2, 134). Necrosis of the intestinal epithelium always coincides with the presence of Gram-positive rods, characteristic of *C. perfringens*, attached to the surface of the necrotic mucosa, forming a biofilm-like structure (135, 136). CPB, the most important toxin for developing enteritis necroticans (50, 134, 137), is found underneath the attached bacteria, indicating that *in situ* production of this toxin is important for pathogenesis (136).

The disease was called Darmbrand ("fire bowels") during outbreaks in Germany following World War II. It is also called pigbel because of its association with the pig feasts of the aboriginal people in Papua New Guinea, which include huge quantities of sweet potatoes and inadequately cooked pork contaminated with pig intestines (138). Other foods associated with type C disease include poorly preserved pork sausage (139), turkey sausage (140), pork (132, 135), chicken stew, salmon, and crab (136).

Enteritis necroticans is particularly aggressive in diabetics and immunocompromised patients in developed countries (they survive less than 48 h after the onset of symptoms) (84, 132, 135, 136, 139, 140). Malnourishment, protein deprivation, poor food hygiene, uncooked food containing trypsin inhibitors, and pancreatic disorders are risk factors for developing this disease. Epidemiological studies have indicated that this disease accounted for 10% of all deaths and 50% of deaths in children between 6 and 10 years of age in Papua New Guinea. However, since 1980, immunization with a *C. perfringens* type C toxoid has reduced mortality and morbidity dramatically. Improved nutrition has provided proteolytic protection against CPB, making immunization unnecessary (138, 140).

The disease occurs sporadically in parts of Asia, Africa, and the South Pacific, where it primarily affects children with severe protein malnutrition. Although less common, this disease also occurs in developed countries, where it has been seen primarily in diabetics (138).

CONTAMINATION OF AND GROWTH ON FOODS

C. perfringens contaminates food at a variety of points in production and processing. Foods rich in protein are ideal for this organism. *C. perfringens* requires amino acids and several vitamins for its growth, which are typically present in meat. Of the 144 outbreaks caused by *C. perfringens* in the United States from 1998 to 2010, beef was the most commonly contaminated product (66 outbreaks; 46%), followed by poultry (43 outbreaks; 30%) and pork (23 outbreaks; 16%). Meat-and poultry-related outbreaks accounted for 92% of outbreaks that involved an identified single food commodity (84).

The following sections contain data regarding the detection of *C. perfringens* in a wide variety of foods all over the world. These sections also discuss the ability of this organism to thrive in a diversity of environments and nutrient conditions.

Meats

Animal carcasses and cuts of meat can become contaminated with *C. perfringens* following contact with soil or animal feces. Contamination can also occur during slaughtering and processing. Beef and beef products have been the most commonly contaminated food causing *C. perfringens* infection outbreaks in various countries, suggesting that beef is the primary source of *C. perfringens* infection in cases of foodborne poisoning in humans.

C. perfringens was found in beef from four types of markets in Seoul from June to September 2015. Eightytwo beef samples were

collected from six department stores (n = 12), 14 butcher shops (n = 28), 16 traditional markets (n = 32), and five supermarkets (n = 10). Beef samples from the department stores had the highest prevalence of *C. perfringens* (16.67%), followed by the butcher shops (3.57%) and traditional markets (3.12%); *C. perfringens* strains were not isolated from samples collected at a supermarket (0%) ($P > 0.05$). All isolates were type A, and they were negative for the CPE gene. For *C. perfringens* detection, a standard culture method and real-time PCR assay were used, and the authors concluded that the real-time PCR assay was useful for rapid detection and screening of this pathogen in beef (141).

The presence of *C. perfringens* was assayed in 300 meat samples in Turkey. *C. perfringens* strains were isolated from 95 (31.7%) samples. Of the 95 *C. perfringens* isolates, 40 (40%), 31 (31%), and 24 (24%) were isolated from beef, chicken, and turkey samples, respectively. The *cpa* gene was detected in all *C. perfringens* isolates by PCR. Furthermore, 84 of 95 *C. perfringens* isolates were type A, while two expressed both the *cpa* and *cpe* genes [type A(F)-cpe], and eight (five from beef, two from chicken meat, and one from turkey meat) expressed the *cpa* and *cpb2* genes (type A-cpb2). None of the strains expressed both the *cpe* and *cpb2* genes. *cpe*-positive *C. perfringens* type A(F) was isolated from one beef sample (1%) and one (1%) chicken sample. Also, of the 95 *C. perfringens* isolates, six, three, and two were genotyped as type C, type D, and type E, respectively. One of the six type C strains and one of the three type D strains were positive for the *cpb2* gene (142).

To evaluate the ability of *C. perfringens* to survive in Korean traditional seasoned beef rib prepared sous vide (galbi-jjim), spores of this organism were inoculated into the samples, and then germination and growth of the spores were assayed at different temperatures for several days. Processed samples were stored for 24 days at 4 and 10°C and for 4 days at 20°C. To evaluate the effect of temperature abuse, samples stored at 4°C for 6, 12, and 18 days were transferred to 10°C and stored for an additional 6 days. The samples originally stored at 4°C for 6 days were also transferred to 20°C and

stored for 1 day. The results indicated that there was no difference in the water activity or pH of the samples during storage at any temperature. However, the salt content of the samples significantly increased as storage time increased, and storage temperature affected the salt content. *C. perfringens* did not grow at 4°C or 10°C for 24 days; however, bacterial growth was detected at 20°C after 2 days of storage (143).

In the United States, a total of 445 whole-muscle and ground or emulsified raw pork, beef, and chicken product mixtures acquired from industry sources were analyzed over a 10-month period for *C. perfringens*. Samples that were positive after a 15-min heat shock at 75°C were considered presumptively positive for spores. Of the 194 cured whole-muscle samples, 1.6% were positive for the microorganism, but spores were not detected in those samples. Populations of vegetative cells did not exceed 1.70 $\log_{10}$ CFU/g, and they averaged 1.56 $\log_{10}$ CFU/g. Of the 152 cured ground or emulsified samples, 48.7% and 5.3% were positive for spores, respectively. Populations of vegetative cells did not exceed 2.72 $\log_{10}$ CFU/g, and they averaged 1.98 $\log_{10}$ CFU/g. Spores did not exceed 2.00 $\log_{10}$ CFU/g, and they averaged 1.56 $\log_{10}$ CFU/g (144).

The presence of *C. perfringens* in chicken meat parts (breast, wing, drumstick, and leg quarters) from Turkey and the expression of the *cpa, cpb, etx, iA, cpe*, and *cpb2* toxin genes in the isolates were determined. Two hundred samples of raw chicken breasts (n = 50), wings (n = 50), drumsticks (n = 50), and leg quarters (n = 50) were collected from various retail stores. Forty-seven (94%) wing samples, 40 (80%) leg quarter samples, 34 (66%) drumstick samples, and 33 (66%) breast samples were contaminated with *C. perfringens*. Furthermore, 558 isolates were identified as *C. perfringens*. Of these, 545 (97.6%) carried only the *cpa* toxin gene (type A), 12 (2.1%) carried both the *cpa* and *cpb2* toxin genes (type A-cpb2), and one (0.1%) carried both the *cpa* and *cpe* toxin genes [type A(F)-cpe] (145).

Pâté is a food prepared from a minced mixture of offal (particularly liver), muscle, fat, vegetables, herbs, and spices. Products from different animal species are used in pâté production. This mainly includes pigs or poultry (e.g., chickens and ducks), although fish and shellfish can also be used. A study investigated the microbiological quality of liver pâté in England. From 2012 to 2013, a total of 870 samples unrelated to the investigation of food poisoning outbreaks were collected either at retail (46%), during catering (53%), or at the point of manufacture (1%). Of the 870 samples collected, *C. perfringens* was present at a satisfactory level (<10 CFU/g) in 868 samples (99%) and at a borderline level (≥10 to 10^4 CFU/g) in only two samples. The bacterium was not present at unsatisfactory levels (≥10^4 CFU/g) in any samples. There was no evidence of differences in the distributions of *C. perfringens* between samples collected at retail and those from catering events (146).

The prevalence and molecular characterization of *C. perfringens* in edible offal of broiler in Ankara, Turkey, were determined. A total of 90 samples, including 45 hearts and livers and 45 gizzards, were obtained from different supermarkets. Twenty-one (46.7%) of the 45 hearts and livers and eight (17.7%) of the 45 gizzards were contaminated with *C. perfringens*. All isolates expressed the *cpa* gene, and none were positive for the *cpe* gene. Only two gizzard isolates were positive for the *cpb2* gene (147).

Fish and Seafood

Seafood-borne pathogenic bacteria can be grouped based upon their ecology and origin based upon whether they are (i) indigenous to an aquatic environment and naturally present in fish, (ii) indigenous to multiple environments and frequently found on seafood, or (iii) found on the outer and inner surfaces of diseased or asymptomatic animal or human carriers (148). The second group includes *Listeria monocytogenes*, *Clostridium botulinum* (proteolytic types A and B),

C. perfringens (type A from soil and types B, C, and D from animals), and *Bacillus* spp. (149).

C. perfringens can be isolated from seafood, but there appear to be no systematic data concerning its incidence. Although fish are less commonly involved in *C. perfringens* food poisoning outbreaks (1), the organism has been determined to be the cause of outbreaks of food poisoning after the consumption of boiled salmon and tuna salad. Seafood has also been involved in two *C. perfringens* outbreaks reported in Spain from 1993 to 1998 (150). *C. perfringens* was confirmed in 17 (5%) of 347 fresh and processed retail seafood samples in the United States. Bacteria were found at an abundance corresponding to a most probable number of 3.6 to 240 per g, and only one of the *C. perfringens* isolates possessed the CPE gene, as determined by PCR (151).

Although various authors have argued that *C. perfringens* contaminates fish and shellfish during processing, Rahmati and Labbe (151) and Oka et al. (152) detected the organism on the body surfaces and in the alimentary canals of several fish species (153). Enterotoxigenic *C. perfringens* was detected in freshwater fish, marine fish, and marine shellfish in southern Hokkaido. This organism was detected in 31.1% (19/61) of isolates from the intestinal tracts of freshwater fish and in 14.8% (8/54) of isolates from the intestinal tracts of marine fish. In addition, 6.3% (4/63) of the isolates from marine shellfish were also enterotoxigenic (152). Other studies indicated that *C. perfringens* was more frequently detected on the external surfaces of fish rather than in the intestinal contents, regardless of whether fresh fish were collected from aquaculture ponds or from markets in Egypt. No significant differences were found in the contents of the intestinal tract between fish from aquaculture ponds (9.1% of positive samples) and those from markets (6.7% of positive samples). Furthermore, a higher percentage of fish collected from markets (45.6%) had *C. perfringens* on the external surface than of those from aquaculture ponds (31.8%). When the hands of handlers, aquaculture water, and water tanks in markets were analyzed, *C. perfringens* was found in

63% of samples taken from the handlers' hands and 27.3% of samples from the water, suggesting that hands may be the primary contaminating factor. Multiplexed PCR indicated that CPE-negative *C. perfringens* type A was the predominant isolate, although types C and E were also detected (154).

When *C. perfringens* was inoculated into fish fillets of jack mackerel and subsequently packaged under an air or a controlled CO_2 atmosphere (40% CO_2–60% N_2) followed by incubation at 15°C and 30°C, the bacteria were unable to grow at the lower temperature during the 3 days of storage. However, all samples were spoiled. Interestingly, *C. perfringens* rapidly grew after a 2-to 4-h lag phase at 30°C (abuse temperature) regardless of the package type, but growth was significantly faster under the controlled atmosphere within 6 h of storage (155). No growth of *C. perfringens* was detected in these foods when they were incubated at 5°C in air or under a controlled atmosphere for 6 days (156).

Although *C. perfringens* has been reported to be a potentially important histamine producer in suboptimal temperatures under anaerobic conditions, a study indicated that histamine production by *C. perfringens* in scombrotoxin-forming fish was minimal compared to that of Gram-negative histamine-producing bacteria. Thus, *C. perfringens* may not be significantly associated with scombrotoxin fish poisoning (157).

Spices

Culinary herbs and spices are minor food components that have widespread use. Their low water activity is not a barrier to the survival of some spore-forming microorganisms, including *C. perfringens* (158). Studies in different countries of the microbiology of herbs and spices have demonstrated the presence of important foodborne bacteria and toxigenic molds, including *Salmonella, E. coli*, *C. perfringens*, *Bacillus cereus*, and aflatoxigenic *Aspergillus* (159). The addition of microbially contaminated herbs and spices to

ready-to-eat foods in combination with improper food storage can pose a serious health risk to the consumer.

Several foodborne disease outbreaks have been associated with microbially contaminated spices. These foods are thought to have been contaminated with spores of CPE$^+$ *C. perfringens* as a result of the addition of spices. Because of its spore-forming ability and rapid generation time, improper heating, cooling, and holding conditions can lead to elevated levels of *C. perfringens* in foods, which can subsequently cause a foodborne disease (158).

Various studies have been conducted in many parts of the world to determine the presence of *C. perfringens* in spices. Three hundred eighty samples of spices and herbs (cumin seeds, black pepper, oregano, garlic powder, and bay leaves) widely used in Mexico were analyzed for the presence of *C. perfringens*, while the potential enterotoxigenicity of the isolates was determined by detecting *cpe* using a dot blot technique. The spices were stored in three different commercial presentations (polyethylene bag, glass container, and nonpackaged) and were purchased from retail markets in Monterrey, Mexico. *C. perfringens* counts varied from <100 to 500 CFU/g in garlic powder, from <100 to 200 CFU/g in black pepper, from <100 to 433 CFU/g in cumin seed, from <100 to 340 CFU/g in oregano, and from <100 to 450 CFU/g in bay leaves. Significant differences in the extent of contamination were observed among the three different commercial presentations. Nonpackaged samples of garlic powder, cumin seed, and oregano were more extensively contaminated than those packaged in polyethylene bags, perhaps because of sanitary deficiencies during collection, storage, transportation, packaging, and sale. No detectable contamination by *C. perfringens* was found in the 30 samples stored in glass containers, probably because of a pretreatment of the spices. The dot blot technique detected the *cpe* gene in eight (4.25%) of 188 confirmed isolates of *C. perfringens* (160).

The abundance and characteristics of the spores of *C. perfringens* from 247 samples of spices in U.S. retail were determined. Forty-

three confirmed isolates from 17% of the samples were observed. The microorganism was present at levels ranging from 3.6 to 2,400 CFU/g. Twenty-seven (63%) of the *C. perfringens* isolates were positive for the *cpe* gene. Seven random spice isolates produced CPE at levels between 4 and 16 ng/ml, which were lower than the levels produced by each of three outbreak (control) strains, which were >1,024 ng/ml. Five of the 43 isolates expressed *etx* as well as *cpe* (158).

Other Foods

C. perfringens has been isolated from milk and milk products in India. A study revealed that of the 55 milk (cow) and milk products sampled, isolates were recovered from 11 (20%). Of these, 10 were isolated from raw milk, and the remaining one was obtained from paneer. All of the isolates were found to be type A based on the expression of the *cpa* gene. One of the isolates was positive for the *cpe* gene (161). Other surveys indicated an incidence (percent positive samples) of *C. perfringens* in raw milk of 1.4% (Normandy, France) and 9.3% (various raw-milk surveys, 1990 to 1995) (162).

The contamination of cereals, vegetables, and fruits by spores of pathogenic bacteria present in the soil (such as *C. perfringens*) is not uncommon. Bacterial pathogens that cause disease by contaminating cereal grains and cereal products include *C. perfringens* (163). However, this microorganism is usually of concern only when produce is handled under conditions that enable germination of the spores and growth of the vegetative cells.

In a study conducted in Mexico, the microbial contamination of 300 fresh produce samples from six different products (green onions, parsley, tomatoes, serrano peppers, jalapeño peppers, and cantaloupe) in retail stores was assessed. Fifty samples of each item were obtained, including 25 from popular markets and 25 from supermarkets. *C. perfringens* was detected in only six samples of parsley (2% of the total and 20% of the parsley samples). However, PCR did not detect any CPE genes in the isolates (164). *C.*

perfringens in contaminated refried beans in Mexican food has been associated with foodborne outbreaks in the United States (165).

Factors Contributing to Growth and Survival

Various physical, chemical, and biological factors contribute to the growth of *C. perfringens* in food. The optimal growth temperature of this organism is 43 to 46°C, resulting in generation times as short as 7.1 min. The ability to grow quickly in foods allows the bacteria to reach an abundance necessary to cause food poisoning. The microorganism can grow at temperatures ranging from 12 to 50°C, and spores can survive at even higher temperatures. However, *C. perfringens* is unusually sensitive to refrigeration and freezing. At temperatures below 10°C, no growth is detected, although some strains grow in food maintained at 12°C. During cooking or processing of foods, many organisms that compete with *C. perfringens* are killed. However, the spores of this microorganism can be difficult to eliminate. Instead, they can be heat activated, facilitating germination when temperatures become favorable for growth. Furthermore, during cooking, E_h values drop, favoring the growth of *C. perfringens* (1).

It has been shown that the thermotolerance of vegetative cells and spores increases as the incubation temperature of the culture is raised. Heat-shocked cells of *C. perfringens* are more heat tolerant than controls. It has also been demonstrated that vegetative cells and spores can survive various cooking procedures (166). At 55°C, the vegetative cells of chromosomal-*cpe* isolates are twice as resistant as isolates expressing the *cpe* gene from a plasmid. Furthermore, cells expressing the chromosomal *cpe* gene produce spores that are 60-fold more heat resistant than those of cells expressing the *cpe* gene from a plasmid (42). $D_{95°C}$ values (i.e., the time needed to reduce the population by 1 log at 95°C) of 1.0 to 3.3 min for spores from four randomly selected spice isolates suggest the presence of a plasmid-expressed *cpe* gene, while one isolate had a $D_{95°C}$ (>45 min) consistent with a chromosomal *cpe* gene (158).

Heat-resistant chromosomal-*cpe* strains showed less growth potential in vacuum-packed ground beef stored at 12°C than the plasmidic-*cpe* and *cpe*-negative strains. Meanwhile, no significant differences were observed at 25°C. These results suggest that the latter strains may have a competitive advantage over chromosomal-*cpe* strains at reduced temperatures during food storage that supports the growth of *C. perfringens* (167). Isolates of *C. perfringens* that grow, sporulate, and produce more heat-resistant vegetative cells and spores that are capable of growth over a wide range of temperatures would be of major concern. This could constitute a major source of food poisoning and other diseases (166).

The use of water-depressing solutes other than salt affects minimal a_w (water activity) levels during the growth of *C. perfringens*. For example, this bacterium grows at an a_w of 0.97 to 0.95 in laboratory media in which a_w is adjusted using sucrose or sodium chloride and at 0.93 in media adjusted with glycerol. Growth at low temperatures allows *C. perfringens* to respond by reducing fatty acid chain lengths rather than increasing unsaturated fatty acids (168).

The ability of *C. perfringens* to produce a biofilm protects the bacterium from exposure to atmospheric oxygen and high concentrations of antibiotics or other agents (124). It has been suggested that an extracellular polysaccharide matrix of biofilm could play a major role in the initial attachment of *C. perfringens* to chicken intestinal cells, and this could be linked to the persistence of the bacterium in chickens' intestines despite antibiotic treatment of the environment (124).

Predictive Modeling of Growth in Foods

Microbial pathogen computer modeling programs can be valuable analytical tools, helping develop critical limits and evaluate the relative severity of problems caused by process deviations (169). Accordingly, predictive microbial models for *C. perfringens* growth from spores have been developed to estimate growth during the

cooling of cooked or heat-treated meat and poultry products (i.e., under nonisothermal conditions or in continuously varying temperature). These cooling models are the tools most commonly used by regulatory agencies and the meat and poultry industry when evaluating the risk of *C. perfringens* growth during cooling (stabilization) deviations, which can occur because of malfunctions in refrigeration equipment or electrical outages. The models are also used by meat processors to develop customized or alternative cooling methods when they cannot follow the stabilization compliance guidelines for the cooling of cooked or heat-treated meat and poultry products issued by the Food Safety and Inspection Service of the U.S. Department of Agriculture (USDA FSIS).

Juneja et al. (170) provided the first critical assessment of the effect of cooling rates on the outgrowth of *C. perfringens*, which demonstrated the effectiveness and validity of the square root model under nonisothermal conditions. This work also established the safe cooling rate for cooked beef. A number of cooling models have been developed that take into account the effect of temperature in order to predict the relative growth of *C. perfringens* from spores at temperatures relevant to the cooling of cooked meat products (171–189). Amézquita et al. (171) developed an integrated model for the heat transfer phenomenon and dynamics of *C. perfringens* growth during the cooling of cooked boneless cured ham. To take into account other environmental factors, Le Marc et al. (190) developed a dynamic model for the effects of temperature, pH, and NaCl concentration on the growth of *C. perfringens* during the cooling of bulk meat.

Another dynamic model for the growth of *C. perfringens* spores during the cooling of cooked beef in the presence of NaCl (0 to 3% [wt/wt]), sodium pyrophosphate (0 to 0.3% [wt/wt]), and sodium nitrite (0 or 200 ppm [wt/wt]) as controlling factors was developed (191). Predictive models for *C. perfringens* growth from spores related to the cooling of cooked pea soup (192), refried beans (165), and rice (193) have also been published. Fazil et al. (194) conducted a probabilistic analysis of the growth of *C. perfringens* during food

service operation and reported that hot holding is the most critical step for the growth of this pathogen. However, growth during inadequate chilling occurs more frequently. In a study by Juneja et al. (195), the effects and interaction of NaCl, pH, sodium pyrophosphate, and temperature were assessed in order to develop quadratic and cubic models that could be used to predict the behavior of *C. perfringens* vegetative cells. This latter study suggested that sodium pyrophosphate exhibits bacteriostatic activity against *C. perfringens*, particularly when used in combination with low pH, high NaCl concentrations, and adequate refrigeration.

The published models have been incorporated into user-friendly computer programs (e.g., the USDA Pathogen Modeling Program [PMP]). Mohr et al. (196) evaluated the relative performance of six cooling models (the PMP 7.0 broth model; Predictive Microbiology Information Portal [PMIP] uncured beef, chicken, and pork models; Smith-Schaffner [version 3]; and the United Kingdom Institute of Food Research's ComBase Perfringens Predictor) for predicting *C. perfringens* growth using 188 datasets that represented various single-rate and dual-rate exponential cooling conditions, in addition to the extended chilling of cooked meat, uncured meat, and poultry products. The performance of the models was evaluated using three sets of criteria based on different definitions for accurate, fail-safe, and fail-dangerous predictions. The first criterion used to evaluate model performance (i.e., an accurate prediction is when the residual, observed minus predicted value is −1 log to +0.5 log; a fail-safe prediction is when the residual is <−1.0 log; and a fail-dangerous prediction is when the residual is >+0.5 log) was based upon the acceptable prediction zone method (197). The boundaries for the second criterion (i.e., an accurate prediction is when the residual is −1 log to +1.0 log; a fail-safe prediction is when the residual is <−1.0 log; and a fail-dangerous prediction is when the residual is >+1.0 log) were based on the extent of microbial growth that an expert food microbiologist would not consider significant. The boundaries for the third criterion (i.e., an accurate prediction is when the residual is −0.5 log to +0.5 log; a fail-safe prediction is

when the residual is <−0.5 log; and a faildangerous prediction is when the residual is >+0.5 log) were based on a half-log resolution limit generally accepted for microbial testing. The combined percentages of accurate and fail-safe predictions based on the three performance criteria during single-rate exponential cooling were 34.66 to 42.61% for the PMP 7.0 beef broth model, 100% for the PMIP cooling models for uncured beef, uncured pork, and uncured chicken, 80.11 to 93.18% for the Smith-Schaffner cooling model, and 74.43 to 85.23% for the ComBase Perfringens Predictor. Mohr et al. (196) concluded that, with the exception of the PMP 7.0 broth model, the cooling models were reliable tools for estimating *C. perfringens* growth in cooked meat, uncured meat, and poultry products in the event of cooling deviations. These models were also useful for developing customized cooling schedules.

METHODS FOR DETECTING *C. PERFRINGENS*

To implicate *C. perfringens* as a cause of food poisoning, at least one of the following must be found: (i) CPE-producing *C. perfringens* present in epidemiologically implicated food at a significant abundance (usually more than 10^5/g, provided that the specimen is properly handled); (ii) more than 10^6 organisms/g of stool from two or more sick persons; or (iii) CPE in the stools of two or more sick individuals (1, 198, 199). One problem associated with implicating this bacterium as a cause of disease is that most laboratories do not routinely test for *C. perfringens* because of the labor requirements and high cost. Although various less expensive methods are available (such as PCR), detection often requires anaerobic culturing, which requires more labor and resources (200). In general, the available methods for detecting CPE-producing *C. perfringens* are classified as metabolite-based biochemical assays, antigen-based immunological methods (mainly for CPE), or nucleic acid-based molecular techniques to detect *cpe* (3).

To identify and enumerate *C. perfringens* in food or feces, the recommended commercially available selectiveplating medium is tryptose sulfite cycloserine (TSC) agar, and plating is followed by incubation in an oxygen-free environment (1). Confirmation using biochemical tests (lactose fermentation, nitrate reduction, and motility assay) from 10 presumptive black colonies via plating is recommended (1). When egg yolk emulsion is added to TSC agar, *C. perfringens* produces an opaque white zone surrounding the colonies as a result of lecithinase activity (3). TSC agar has various advantages over other selective media developed for the isolation and quantification of *C. perfringens*, including iron sulfite agar, Shahidi-Ferguson perfringens agar, sulfite cycloserine azide, differential clostridial agar, and oleandomycin polymyxin sulfadiazine perfringens agar (3).

For the detection of *C. perfringens* in water samples, the membrane filtration technique using a modified form of TSC agar (which lacks sodium metabisulfite but is supplemented with sodium pyruvate) is recommended in order to improve recovery. This procedure allows the isolation and confirmation of *C. perfringens* within 18 to 24 h (201). Furthermore, the m-CP agar base with a selective supplement allows the differentiation of *C. perfringens* strains based upon the microorganism's ability to ferment sucrose and produce yellow colonies (3).

Recently, a colorimetric paper-based one-step assay for *C. perfringens* was reported. It detects nitrophenyl liberated by the hydrolysis of paranitrophenyl phosphatidyl choline by phospholipase C (202). PCR is also a well-accepted, rapid, and sensitive technique for detecting *C. perfringens*. Moreover, it is efficient under circumstances in which bacteria are present in low numbers. Multiplex PCR techniques have been developed that permit the simultaneous detection of *cpe* and other extracellular toxin genes. This assay is commonly used for toxigenic typing of the organism, and it has been useful for epidemiological purposes (1, 142). Other available methods for subtyping the bacterium include

pulsedfield gel electrophoresis, randomly amplified polymorphic DNA analysis, and ribotyping (1).

To determine whether a *C. perfringens* isolate is the etiological agent of an outbreak, it is useful to serotype or molecular genotype samples using pulsed-field gel electrophoresis for microbial source tracking (203). However, this technique does not reflect genetic sequence diversity, although it does pinpoint the clonal lineage of outbreak strains (204). More recently, multilocus sequence typing has emerged as a more precise technique to investigate relationships among isolates, diseases, and/or host preferences (204).

Since only a small subpopulation of *C. perfringens* (usually less than 5%) harbors the *cpe* gene, the presence of this bacterium in food does not always indicate that it will cause disease. Unfortunately, an assay for CPE production, which could help in identifying dangerous isolates, is not included in standard diagnostic procedures. Furthermore, an assay for CPE can be inaccurate, because this toxin is not always present at high enough concentrations for detection (205). Since CPE-positive and CPE-negative *C. perfringens* strains are usually present in low numbers in food ingredients or contaminated environments, an enrichment culture step can increase the number of *C. perfringens* cells. In the case of molecular methods, diluting out interfering substances present in the food matrix can also help with the assay (206).

Although not common, bioassays can be used to determine the action of CPE. The intestinal loops of rabbits and mice are most commonly used, where severe necrosis of the small intestinal epithelium is observed along with fluid accumulation. Other animal models used to study the effects of this toxin include rats, nonhuman primates, sheep, and cattle (43).

Since bacterial sporulation is required for CPE production (5), the detection of CPE in isolates of *C. perfringens* can prove difficult, because not all strains sporulate well in certain media and not all occur in large enough amounts to be detected. To overcome this

problem, various media have been developed to increase bacterial sporulation, and several enzyme-linked immunosorbent assay procedures for CPE detection are commercially available that have limits of detection in the range of several nanograms per gram of sample (1).

Data provided by whole-genome sequencing of a chromosomal-*cpe* strain and several strains harboring large *cpe*-expressing plasmids have allowed the development of molecular approaches to more precisely study the circulating strains (204). Several molecular methods have been developed to detect CPE and CPE-producing *C. perfringens* in various matrices if they are present at levels of 10^3 cells/g or greater. This assay distinguishes between the *cpe* locus located on a chromosome versus that carried on a plasmid. These techniques include PCR, nested PCR, real-time PCR, and loop-mediated isothermal amplification (206). The sensitivity of these methods can be improved if they are combined with enrichment culture, allowing the detection of fewer than three viable CPEpositive *C. perfringens* cells in the original sample (204).

EPIDEMIOLOGY OF *C. PERFRINGENS*

The high prevalence of CPE-positive *C. perfringens* in the intestines of animals used for food (10 to 40%) and in foods of animal origin (up to 12%) has been reported (207). It is well known that *C. perfringens* is highly prevalent in the intestinal tract of poultry, and it is detected in the feces of 80% of the animals (208). The bacteria are associated with different phases of poultry growth and production.

Additionally, the intestinal tracts of others animals are considered a reservoir for CPE-positive *C. perfringens*, and they constitute potential contamination sources. The presence of this bacterium has been demonstrated in domestic pets, including dogs (31%) and cats (20%), and 17% and 3% of all isolates, respectively, express the *cpe* gene (209). Additionally, the organism has been isolated from

24% of feces samples from horses (14% *cpe* positive), 36% of feces samples from cattle (10% *cpe* positive), and 2% of feces samples from pigs (208). It has also been detected in the feces of captive wild animals from India, such as ruminants (34.1%), nonruminants (36%), and birds (22.5%), and in the feces of caretakers (6.7%). In these cases, the bacterium has been detected only in feces samples, and no *cpe*-positive isolates have been detected (210).

Various studies have identified healthy humans as reservoirs for CPE-positive *C. perfringens* (211). These studies suggest that CPE-producing *C. perfringens* strains are frequently present in the intestines of healthy humans (207), and this is one source of contamination for foods.

C. perfringens can be dispersed in the environment as spores, allowing it to survive under extreme conditions and germinate in raw or cooked foods (1, 84). *C. perfringens* can be isolated from a range of environments, including soil and marine and freshwater sediments (212). One report even showed that 10% of all strains isolated from water samples near shores or mouths of rivers were *cpe* positive (213).

In general, foodborne diseases are globally important because they result in considerable morbidity, mortality, and economic costs, accounting for 3.6% of the total disease burden. This results in a substantially higher impact in low-income countries and regions with poor water quality, sanitation, and food safety (214). The case fatality ratio associated with *C. perfringens* in low-mortalityrate countries is estimated to be 0.003%, and this is higher than ratios associated with the enterotoxigenic bacteria *S. aureus* (0.0025%) and *B. cereus* (0%) (9). Data available from 2010 indicate that 5,409,083 cases of bacterial intoxication (uncertainty interval, 2,187,762 to 12,929,293) occurred worldwide, and 3,998,164 cases (uncertainty interval, 837,262 to 11,529,642) were attributable to C. *perfringens* (215).

Foodborne illnesses caused by *C. perfringens* are among the most common illnesses of this type worldwide. These are the second

most common foodborne illnesses in the United States (84) and the third most common in England and Wales (216). Although many sporadic illnesses caused by *C. perfringens* are usually not reportable, it is estimated that this pathogen causes 1 million episodes of foodborne illness (or 10% of all cases) in the United States annually (200). In particular, 16 to 31 outbreaks are typically reported each year (84), resulting in an estimated medical cost of $539 per case and an annual economic loss of $382 million (84, 85).

C. perfringens outbreaks occur throughout the year. However, outbreaks are most common during November and December, when many holiday gatherings and social events occur. During these celebrations, people tend to meet in groups to eat seasonal foods, such as roasts, gravies, and poultry. These dishes are often cooked in large batches or prepared long before serving (84). The roasting or baking of meat and poultry is the most common method of preparation (48%) associated with outbreaks, followed by preparation as part of a large, solid mass of food (29%) (199). Food service establishments, such as restaurants (43%), are the most likely sites for contracting food poisoning caused by *C. perfringens*. Other settings include catering facilities (19%), private homes (16%), prisons (11%), and miscellaneous locations (10%) (1, 84).

Problems in food processing and preparation account for 93% of all causes of *C. perfringens* outbreaks. The most frequent mistakes include (i) maintaining foods at room temperature for several hours (58%), (ii) insufficient cooking times or temperatures during reheating (57%), (iii) slow cooling of prepared foods (44%), (iv) insufficient cooking times or temperatures during the initial cooking process (40%), (v) preparing foods in advance by more than half a day (33%), and (vi) insufficient times or temperatures used during hot (33%) or cold (22%) holding (199). According to USDA FSIS compliance guidelines (Appendix B of the FSIS Performance Standards for the Production of Certain Meat and Poultry Products [217]), to avoid *C. perfringens* growth, it is necessary to chill uncured meat and poultry products for no longer than 1.5 h in order

to shift from 54.4°C to 26.7°C (130°F to 80°F). Furthermore, the chilling from 26.7°C to 4.4°C (40°F) should take no more than 5 h (1). The contamination of raw products or ingredients has been a principal factor (21%) contributing to *C. perfringens* outbreaks, followed by cross-contamination in the food processing and preparation environment (13%) and contamination by a food worker (6%) (199).

METHODS OF CONTROL

Historic information gathered from reports of outbreaks provided data regarding the causes of *C. perfringens*-associated illnesses, helping to establish adequate control measures in order to avoid outbreaks. Furthermore, knowledge about environmental reservoirs for CPEproducing *C. perfringens* and factors associated with contamination of food vehicles might lead to control measures that could decrease food contamination (84). In general, *C. perfringens* outbreaks are preventable with proper food handling and preparation protocols, including cooling and hot holding, particularly in restaurants and catering facilities (1, 84).

C. perfringens is commonly found in retail meat and poultry at levels of 10 to 100 CFU/g (1). Thus, efforts must be made to avoid the growth of the bacteria that increase the risk of a foodborne out break. To ensure that vegetative cells are inactivated, they must reach temperatures above 60°C. For this, foods should be cooked or reheated to a temperature of ≥75°C (1). The refrigeration of foods at temperatures below 10°C also inhibits bacterial growth (84). Sodium nitrite (0.2%), sorbate (0.3%), and benzoate (0.1%) inhibit the vegetative growth of *C. perfringens* isolates (218). Ascorbate enhances the antimicrobial effects of nitrite on *C. perfringens* at concentrations commonly used in alternatively cured meats (219). Buffered sodium citrate alone and in combination with sodium diacetate reduces the population of *C. perfringens* vegetative cells during the abusive chilling of roast beef and pork (220).

If the spores of the bacterium are present, it is important to consider that the organism can survive cooking and multiply rapidly at 37 to 45°C (1). The heat resistance of *C. perfringens* spores varies depending on the strain and temperature at which they are produced. There is a direct relationship between spore heat resistance and sporulation temperatures (5, 6). The germination and outgrowth of *C. perfringens* spores during the improper cooling of thermally processed meat products have been reported (221). In these cases, it is not always possible to apply enough heat during food processing to kill the spores. However, other interventions can be useful, such as a combination of temperature and pressure (84), proper cleaning and disinfection with hypochlorite (pH < 8), the avoidance of bacterial overgrowth by cooking and rapid cooling, and proper reheating above 60°C throughout all parts of the food product (222).

Some chemicals widely used as flavor enhancers and/or antimicrobial agents are alternatives for the biological control of *C. perfringens*. These products inhibit *C. perfringens* spore germination and outgrowth during extended cooling periods in a variety of meat systems. These chemicals include 2% NaCl (221), sodium nitrite (223), organic acid salts (such as sodium, potassium or calcium salts of acetic, citric, or lactic acids) (224), sorbate, and benzoate (225). The organic acids added to foods exist in a pH-dependent equilibrium between the undissociated state and the dissociated state. The antimicrobial activity of organic acids primarily results from the uncharged and undissociated state of the molecule, which crosses the bacterium's plasma membrane into the cytoplasm and releases charged anions and protons. This inhibits the microbe's metabolism (224). However, although sorbate (0.3%) and benzoate (0.1%) have been reported to effectively arrest the growth of germinated *C. perfringens* spores in rich medium, these compounds could not control the growth of spores in chicken meat stored under extreme conditions (218).

The bacteriocin nisin (which is produced by *Lactococcus lactis* subsp. *lactis*) has been used as a food preservative due to its lethal

action and wide spectrum of activity (226). Other bacteriocins, such as those produced by *Pediococcus acidilactici* (227) and *Lactococcus lactis* strain WNC 20 (226), among others, also display activity against *C. perfringens*.

Many studies have explored ways to reduce, inhibit, and kill *C. perfringens* in foods, and other strategies for controlling this pathogen include reducing or eliminating *C. perfringens* colonization of the gut in reservoir animals. *Lactobacillus* has been widely used as a probiotic in feeds to improve poultry growth performance and overall health. *L. acidophilus* and *L. fermentum* inhibit the pathological effects of *C. perfringens* in intestinal epithelial cells of chicken embryos (228). The ingestion of *L. acidophilus* (229) and *B. licheniformis* (230) modulates the relative abundance of some bacteria taxa, increasing the concentrations of lactate and butyrate while decreasing the relative abundance of undesirable bacteria (such as *Escherichia*), reducing disturbances in the cecal microbiome, promoting intestinal health, and contributing to the recovery of the microbial community disrupted by a *C. perfringens* infection (229).

The inhibitory effects of plant extracts and essential oils on the growth of *C. perfringens* have also been studied. Lemon juice concentrate and vinegar, either alone or as a mixture, are effective in controlling the germination and outgrowth of *C. perfringens* spores in ground roast beef (221) and ground turkey roast (224). When used in combination with small amounts of sodium nitrite (100 ppm), the winter savory (*Satureja montana* L.) essential oil (in which the major compounds are thymol [28.99%], *p*-cymene [12.00%], linalool [11.00%], and carvacrol [10.71%]) effectively controls the growth of *C. perfringens* in mortadella (231). The essential oil and ethanoic extract of *Eugenia caryophyllata* (232), in addition to the ethanoic extracts of *Agave lechuguilla* Torr. (Agavaceae), *Baccharis glutinosa* Pers. (Compositae), and *Larrea tridentata* (DC.) Cov. (Zygophyllaceae) (233), shows growth-inhibitory activity against *C. perfringens*.

Chitosan, a natural carbohydrate polymer derived from the deacetylation of chitin, is the primary component of crustacean and insect exoskeletons and the cell walls of fungi. This carbohydrate displays strong growthinhibitory activity against *C. perfringens* in laboratory medium at 1.0 mg/g. However, to achieve a similar inhibition of spores inoculated into chicken meat, higher levels (2.0 mg/g) are required (225). Other antimicrobial replacements for antibiotics include fatty acids, such as caprylic, capric, lauric, myristic, and oleic acids (234).

To treat or prevent disease, it is important to kill the pathogen that causes the disease, thereby stopping the production of that pathogen's virulence factors. Aqueous extracts of *Psidium guajava* L., *Haematoxylon brasiletto*, and *Euphorbia prostata* are effective not only as inhibitors of *C. perfringens* growth but also against spore formation and CPE production (235).

CONCLUDING REMARKS

Significant progress has been made in the last few years towards our understanding of the epidemiology, pathogenicity, and control measures of foodborne disease, including food poisoning, caused by *C. perfringens* strains. Despite this, a significant burden of food poisoning illnesses still occurs in the United States every year, with nearly a million cases reported. The challenge for us in the field of food microbiology is to identify and design strategies to help reduce the burden of food poisoning. We have learned that food poisoning cases commonly occur as outbreaks in institutions where food is prepared in large quantities. These prepared meals become contaminated with CPE-producing *C. perfringens* strains, commonly those carrying the enterotoxin gene *cpe* on the chromosome, and contaminated food is not thoroughly cooked and also improperly stored. As a result, *C. perfringens* vegetative cells multiply and sporulate, producing enough enterotoxin to cause diarrhea once consumed. Therefore, prophylactic measures to prevent food poisoning should focus on restricting multiplication of

vegetative cells in cooked foods. Cooking at proper temperatures and for the right time, along with rapid cooling after cooking with subsequent refrigeration, is the most effective action to control the multiplication of *C. perfringens* and thus avoid food poisoning outbreaks.

The ultimate responsibility to guard against the pathogens lies with the food industry and retail food service establishments. Processors can take advantage of multiple food formulation factors or hurdles in foods, e.g., water activity, pH, and added preservatives, to restrict growth from spores in cooked foods. Predictive models have been developed to estimate growth under conditions that are relevant to food processing operations. Recent advances in molecular techniques have enabled researchers to characterize *C. perfringens* virulence factors, toxins, sporulation, and spore heat resistance, to carry out epidemiologic trace-back of foodborne illness and toxigenic typing methods, etc. Future research efforts should be directed towards efficient tracing of *C. perfringens* strains of public health significance, multiple hurdles in formulated foods, proper processing of ready-to-eat foods, and consumer awareness of handling such foods.

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20
Bacillus cereus

The *Bacillus cereus* group currently consists of nine *Bacillus* species, i.e., *B. anthracis*, *B. cereus*, *B. mycoides*, *B. pseudomycoides*, *B. thuringiensis*, *B. weihenstephanensis*, *B. cytotoxicus*, *B. bombysepticus*, and the most recently classified member of the group, *B. toyonensis*. The *B. cereus* group is often referred to as *B. cereus sensu lato*, and the species *B. cereus* is referred to as *B. cereus sensu stricto*. The species within the *B. cereus* group are so closely related that they could be one species; however, *B. anthracis* (not treated here) can be distinguished from other members of the *B. cereus* group since it is nonmotile and nonhemolytic and carries two large plasmids containing toxin and capsule genes. The broad spectrum of *B. cereus sensu lato* toxicity ranges from avirulent strains used as probiotics for humans to highly toxic strains responsible for foodrelated fatalities (1).

B. cereus sensu stricto (referred to herein as *B. cereus*) can cause two different types of foodborne illness: the diarrheal type, first recognized after a hospital outbreak associated with vanilla sauce in Norway in 1948 (2), and the emetic type, which was described about 20 years later after several outbreaks associated with fried rice in England (3). The diarrheal type is caused by an enterotoxin(s) produced during vegetative growth of *B. cereus* in the small intestine (4), whereas the emetic illness is caused by a small heat-stable emetic toxin that is preformed by *B. cereus* in the food (5). For both these types of foodborne illness, the food involved has usually been heat treated, and surviving spores are the source of the food poisoning. *B. cereus* is not a very competitive microorganism but grows well in food after cooking and cooling (<48°C; 55°C for *B. cytotoxicus*). Heat treatment induces germination of spores, and in the absence of competing flora, *B. cereus* grows well, with a generation time as short as 12 minutes for some strains under optimal conditions (6). The members of the *B. cereus* group are common soil saprophytes and are easily spread to many types of foods, especially those of plant origin (rice and pasta), but are also frequently isolated from meat, eggs, and dairy products (5). Detection of an increasing number of psychrotolerant strains, mainly *B. weihenstephanensis* strains associated with the dairy industry, has led to increased surveillance for *B. cereus* in recent years (7–11).

B. cereus foodborne illness is probably highly underreported, as both types of illness are usually relatively mild and typically last for less than 24 hours (5). However, more severe forms of *B. cereus* foodborne illness are reported occasionally, including three deaths caused by the necrotic enterotoxin CytK (12) and also fatalities due to ingestion of large amounts of emetic toxin (13–15).

CHARACTERISTICS OF *B. CEREUS SENSU LATO*

Aerobic endospore-forming bacteria have traditionally been placed in the genus *Bacillus*, belonging to the family *Bacillaceae*. Phylogenetic analyses based on 16S rRNA have revealed that the family *Bacillaceae* is paraphyletic; some genera are misassigned to the family, and some species are misassigned to the genus *Bacillus*. However, a reclassification of this often-abused taxon has so far not been done. The genus *Bacillus* has expanded over the decades and today accommodates more than 100 species, mainly divided into 11 phylogenetic subclusters, named subclusters a through d (16, 17). Subcluster d comprises the members of the *B. cereus* group: *B. anthracis*, *B. cereus*, *B. mycoides*, *B. thuringiensis*, and more recently, *B. pseudomycoides* (18), *B. weihenstephanensis* (10), *B. cytotoxicus* (19), *B. bombysepticus* (20), and *B. toyonensis* (21). These species have highly similar 16S and 23S rRNA sequences, indicating that they diverged from a common evolutionary line relatively recently (22–24).

The *B. cereus* group of bacteria can be divided into three distinct clades, where pathogenic *B. cereus* strains are evenly distributed among clades 1 and 2 (25). *B. thuringiensis* is primarily found in clade 2, while clade 3 contains *B. cereus*, *B. mycoides*, and *B. weihenstephanensis*. *B. anthracis* is a monophyletic clone derived from *B. cereus* and *B. thuringiensis* and is restricted to clade 1. Although *B. anthracis* is genetically closely related to the other species within the *B. cereus* group (24), it is the most distinctive member of this group, due to its highly virulent pathogenicity, introduced by two large plasmids (pOX1 and -2). However, strains of *B. cereus* may cause anthrax-like symptoms (26–29); hence, it is difficult to identify definite criteria to differentiate the two species. In addition, extensive genomic studies including full-genome sequencing of strains of *B. cereus* and *B. thuringiensis* have revealed that there is no taxonomic basis for separate status for these two species (24). Nevertheless, the name *B. thuringiensis* is retained for strains that synthesize a crystalline inclusion (Cry protein) or δ-endotoxin that is highly toxic to specific insects. The *cry* genes are usually located on plasmids, and without the relevant plasmid(s), the bacterium is indistinguishable from *B. cereus*.

The cells of the species in the *B. cereus* group are large (cell width, >0.9 μm) and produce central to terminal ellipsoid or cylindrical spores that do not distend the sporangia (10, 16, 18). Bacteria of these *Bacillus* species sporulate easily in most media after 1 to 3 days. *B. cereus*, *B. anthracis, B. thuringiensis*, and *B. mycoides* can be differentiated by the phenotypic traits listed in Table 20.1.. *B. weihenstephanensis* can be differentiated from *B. cereus* based on growth below 7°C and not at 43°C, by 16S RNA sequences, or by PCR targeting *cspA*, the cold shock protein A-encoding gene (10). *B. pseudomycoides* is not distinguishable from *B. mycoides* by physiological and morphological characteristics but can be clearly differentiated by fatty acid composition and 16S RNA sequences (18). *B. cytotoxicus* can be differentiated from other species of the *B. cereus* group by maximum growth at 50°C and minimum growth at 20°C, the absence of starch hydrolysis, and the absence of growth on synthetic media without tryptophan (19). *B. toyonensis* is distinguished from other *B. cereus* group members by pairwise calculation of the average nucleotide identity (21). *B. bombysepticus* produces parasporal crystal and infects the silkworm *Bombyx mori*; however, it is very closely related to *B. thuringiensis*, with a genome sequence identity of more than 96% (20, 30).

Table 20.1 Criteria to differentiate members of the *B. cereus* group[a]

Species	Colony morphology	Hemolysis	Motility	Susceptibility to penicillin	Parasporal crystal inclusion
B. cereus	White	+	+	-	-
B. anthracis	White	-	-	+	-
B. thuringiensis	White/gray	+	+	-	+
B. mycoides	Rhizoid	(+)	-	-	-
B. pseudomycoides	As for *B. mycoides* above. Differentiated from *B. mycoides* based on fatty acid composition and 16S RNA sequences (18).				
B. weihenstephanensis	As for *B. cereus* above. Differentiated from *B. cereus* based on growth at <7°C and not at 43°C; it can be identified rapidly using rRNA gene-or *cspA* (cold shock protein A gene)-targeted PCR (10).				
B. cytotoxicus	As for *B. cereus* above. Differentiated from *B. cereus* by maximum growth at 50°C and minimum growth at 20°C, by absence of starch hydrolysis, and by absence of growth on synthetic media without tryptophan (19).				
B. toyonensis	*B. toyonensis* is distinguished from other *B. cereus* group members by pairwise calculation of the average nucleotide identity (21).				

[a]Symbols: +, positive; –, negative; (+), weakly positive.

RESERVOIRS

B. cereus is widespread in nature and frequently isolated from soil and growing plants (5). From this natural environment, it is easily spread to foods, especially those of plant origin. It is frequently present in raw materials and ingredients used in the food industry, such as vegetables, starch, and spices (30% of samples contain 10^2 to 10^5 CFU/g). In addition to rice, pasta, and spices, dairy products are among the most common food vehicles for *B. cereus*. *B. cereus* is spread to milk and dairy products largely through soil and grass that come into contact with the udder of the cow and then are passed into raw milk. *B. cereus* spores survive milk pasteurization, and after germination, the cells are free from competition from other vegetative cells (31). According to the classical literature, *B. cereus* is unable to grow at temperatures below 10°C

and cannot grow in milk and milk products stored at temperatures between 4 and 8°C. However, psychrotolerant species such as *B. weihenstephanensis* can grow well at temperatures as low as 4 to 6°C (7, 8, 32). The majority (if not all) of *B. weihenstephanensis* isolates are unable to cause diarrheal food poisoning (33) and will outcompete mesophilic *B. cereus* in foods stored at temperatures below 8°C. *B. weihenstephanensis* is, however, able to produce emetic toxins at low temperature (8 to 10°C) (34, 35).

The closely related *B. thuringiensis* can produce enterotoxins (36–38) and can cause foodborne illness when administered to human volunteers (37). This may pose a problem, as spraying of this organism to protect crops against insect infestations has become a common practice in several countries. In 2005, it was shown that 31 of the 40 randomly selected *B. cereus*-like strains isolated from foods were classified as *B. thuringiensis* due to crystal production and/or carriage of *cry* genes (39). However, because the procedures normally used for identification of *B. cereus* would not differentiate between the two species of *Bacillus* (Table 20.1.), outbreaks caused by *B. thuringiensis* may be unrecognized. To ensure safe spraying of *B. thuringiensis*, the organism used should not produce enterotoxins. Although all members of the *B. cereus* group harbor genes for at least one of the enterotoxins (Nhe), some strains do not produce detectable amounts of toxin(s) (38), included the newly described species *B. toyonensis* (21).

FOODBORNE OUTBREAKS

The number of outbreaks of *B. cereus* foodborne illness is greatly underestimated. This is mainly due to the relatively short duration of both types of illness (usually <24 hours) and to the fact that complete recovery is, with a few exceptions, rapid after the symptoms subside. The dominating type of disease caused by *B. cereus* differs among countries. In Japan, the emetic type is reported about 10 times more frequently than the diarrheal type (40), whereas in Europe and North America, the diarrheal type is most frequently reported (5, 41). This is likely attributed to eating habits, although contaminated milk was reported to cause at least one large outbreak of the emetic type in Japan (42). Some patients experience both types of *B. cereus* foodborne illness concurrently (43), with about 5% of *B. cereus* strains producing both types of toxins (5, 44).

Surveillance of foodborne illnesses differs greatly among countries. Hence, it is not possible to directly compare the incidence of outbreaks reported by different countries. The percentage of outbreaks and cases attributed to *B. cereus* in Japan, North America, and Europe varies from approximately 1 to

47% for outbreaks and from approximately 0.5 to 33% for individual cases (reports are from different periods between 1960 and 2005) (5, 41). The highest numbers of reported *B. cereus* outbreaks and cases are from Iceland, The Netherlands, and Norway. However, in the past few years (2010 to 2014), *B. cereus* outbreaks have more than doubled in France, and this organism is now the second cause of foodborne outbreaks following *Staphylococcus aureus* (45). There are relatively few outbreaks of salmonellosis and *Campylobacter* enteritis reported in Norway and Iceland; these are the two most frequently reported causes of foodborne illness in most of Europe and the United States, and the increase in France is also in part due to a strong reduction in salmonellosis (45). In The Netherlands, between 1993 and 1998, *B. cereus* was responsible for 12% of outbreaks of foodborne illness for which the causative agent was identified. However, the actual incidence of *B. cereus* illnesses was only 2.0% of the total number of reported cases, because most cases of foodborne illness were of unknown etiology (41). According to the European Food Safety Authority, *B. cereus* food poisoning was caused mainly by mixed buffet meals and food of plant origin (46) (Fig 20.1). Examples of foods involved in foodborne outbreaks are shown in Table 20.2..

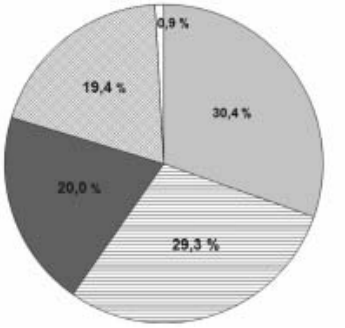


Figure 20.1 Types of foods involved in *B. cereus* food poisoning in Europe (European Food Safety Authority) (46).

CHARACTERISTICS OF DISEASE

There are two types of *B. cereus* foodborne illness. The first type, which is caused by an emetic toxin, results in vomiting, whereas the second type, which is caused by enterotoxin(s), results in diarrhea (5). In a small number of cases, both types of symptoms occur (5), likely due to production of both types of toxin. There has been some debate about whether enterotoxin(s) can be preformed in foods. In reviewing the literature, it appears that the incubation time (>6 hours; average, 12 hours) is too long for the diarrheal illness to be caused by preformed enterotoxin (5), and experiments in animal models revealed that the enterotoxin(s) is degraded as it proceeds to the ileum (47).

However, there is no doubt that *B. cereus* enterotoxins can be preformed in food, but the number of *B. cereus* cells necessary to produce enterotoxin in such food is at least 2 orders of magnitude higher than the number of cells needed to cause emetic toxin food poisoning (7, 47). Usually, foods with such large populations of *B. cereus* will no longer be acceptable to the consumer, although food containing $>10^7$ *B. cereus* cells/ml may not always appear spoiled. The characteristics of the two types of *B. cereus* foodborne illness are described in Table 20.3..

Severe illness associated with *B. cereus* food poisoning is of great concern, and deaths caused by both enterotoxin and emetic toxin have been reported. *B. cereus* cytotoxin K (CytK), similar to the β-toxin of *Clostridium perfringens*, was the causative agent of a severe outbreak of *B. cereus* foodborne illness in France in 1998 (12). Several people in this outbreak developed bloody diarrhea, and three died. This was the first recorded outbreak of *B. cereus* necrotic enteritis, although the symptoms were not nearly as severe as symptoms seen after *C. perfringens* type C foodborne illness (48). The organism isolated from this outbreak, *B. cytotoxicus*, differed from other members of the *B. cereus* group and grew at higher temperatures (thermotolerant). *B. cytotoxicus* is now recognized as a species (19).

Table 20.2 Examples of foods involved in *B. cereus* food poisoning events

Type of food	Country	No. of people involved	Type(s) of syndrome[a]
Barbecued chicken	Many countries	—[b]	E, D
Cooked noodles	Spain	13	D
Cream cake	Norway	5	D
Eclair (pastry)	Thailand	>400	E(D)
Fish soup	Norway	20	D
Hibachi steak	United States	11	E, D
Lobster pâté	United Kingdom	—	D
Meat loaf	United States	—	D
Meat with rice	Denmark	>200	D
Milk	Many countries	—	E, D
Milkshake	United States	36	?
Pea soup	The Netherlands	—	D
Sausages	Ireland, China	—	D
School lunch	Japan	1,877	E
Scrambled egg	Norway	12	D
Several rice dishes	Many countries	—	E, D
Stew	Norway	152	D
Turkey	United Kingdom, United States	—	D
Vanilla sauce	Norway (many countries)	>200	D E, D
Vegetable sprouts	United States	3	E, D
Wheat flour dessert	Bulgaria	—	D

[a] E, emetic syndrome; D, diarrheal syndrome.

[b] —, data not available.

Table 20.3 Characteristics of the two types of illness caused by *B. cereus*[a]

Characteristic	Diarrheal syndrome	Emetic syndrome
Dose causing illness	10^5–10^7 cells (total)	10^5–10^8 cells (per g in foods)
Toxin production	In the small intestine of the host	Preformed in foods
Type of toxin	Protein; enterotoxin(s)	Cyclic peptide; emetic toxin
Incubation period	8–16 h (occasionally >24 h)	0–5 h
Duration of illness	12–24 h (occasionally several days)	6–24 h
Symptoms	Abdominal pain, watery diarrhea occasionally with nausea	Nausea, vomiting, and malaise (sometimes followed by diarrhea, due to production of enterotoxin)
Foods most frequently implicated	Meat products, soups, vegetables, puddings/sauces, and milk/milk products	Fried and cooked rice, pasta, pastry, and noodles

[a]Based on references 5, 42, and 47.

In 1997, the emetic toxin cereulide was responsible for the death of a 17-year-old Swiss boy, due to fulminant liver failure (14). A large amount of *B. cereus* emetic toxin was detected in residue in the pan used to reheat the implicated food (pasta) and in the boy's liver and bile. In 2005, a 7-year-old girl died in Belgium only 13 hours after ingesting a pasta salad that had been stored for several days in a refrigerator where the temperature was 14°C. Her 2-year-old brother, who had eaten only a small amount of the salad, was hospitalized for 8 days (13). Yet another lethal case was described in Belgium in 2008 (49). The latest report of a serious outbreak of *B. cereus* emetic type is from Japan (15), where three family members began vomiting 30 minutes after consuming reheated fried rice. After 6 hours, a 1-year-old boy died of acute encephalopathy. A 2-year-old girl recovered rapidly only after plasma exchange and subsequent hemodialysis. Their mother recovered after fluid therapy.

An outbreak in Norway in 1991 associated with eating stew containing approximately 10^4 to 10^5 *B. cereus* cells per serving affected 17 people, of whom 3 were hospitalized, one for 3 weeks. The onset for these three patients was late, more than 24 h, and the illness was probably due to ingestion of spores.

DOSE AND SUSCEPTIBLE POPULATIONS

After the first recognized diarrheal outbreak of *B. cereus* foodborne illness in Oslo (vanilla sauce), S. Hauge isolated the causative agent, grew it to 4×10^6 CFU/ml, and drank 200 ml of the culture (2). Approximately 13 hours later, he developed abdominal pain and watery diarrhea that lasted for approximately 8 hours. The number of *B. cereus* cells he ingested was approximately 8×10^8. Counts of *B. cereus* ranging from 200 to 10^9 CFU/g (or ml) have been reported in foods incriminated in outbreaks (2, 5, 47, 50), indicating ingestion of a total number of 5×10^4 to 10^{11} cells. The total number of *B. cereus* cells required to cause illness is likely in the range of 10^5 to 10^8 viable cells or spores. In the lower part of this range, it is likely that only the spores, which survive the stomach acid barrier, can cause illness. Still, the wide range of infective doses is also due to differences in the amount of enterotoxin produced by different strains (47). Hence, food containing more than 10^3 *B. cereus* spores/g cannot be considered completely safe for consumption. Little is known about susceptible populations, but the more severe types of illness have occasionally involved young athletes (<19 years) or the elderly (>70 years) (12, 50, 51). More studies are needed to address this important question.

VIRULENCE FACTORS AND MECHANISMS OF PATHOGENICITY

The two types of *B. cereus* foodborne illness are caused by very different types of toxins. The emetic toxin, cereulide, is a 1.2-kDa dodecadepsipeptide synthesized by a nonribosomal peptide synthetase (44, 52, 53), whereas the diarrheal disease is caused by enterotoxins of protein nature (12, 54, 55).

Emetic Toxin

The emetic toxin (Table 20.4.) causes emesis and vomiting only, and its structure was for many years a mystery because the only detection system involved living primates (5, 56, 57). However, the discovery that the toxin could be detected by HEp-2 cells (vacuolation activity) has led to its isolation and structure determination (52, 53). The emetic toxin has been named cereulide and consists of a ring structure of three repeats of four amino acids and/or oxyacids: (D-*O*-Leu-D-Ala-l-*O*-Val-L-Val)^3. The toxin acts as a K^+ ionophore (like valinomycin), is resistant to heat, pH, and proteolysis, and is not antigenic (52, 53) (Table 20.4.).

Table 20.4 Properties of the *B. cereus* emetic toxin cereulide[a]

Trait	Property or activity
Molecular mass	1.2 kDa (many isoforms)
Structure	Ring-shaped peptide
Isoelectric point	Uncharged
Antigenic	No
Biological activity in living primates and Asian house shrew	Vomiting
Receptor	5-HT3 (stimulation of the vagus afferent)
Ileal loop tests (rabbit, mouse)	None
Cytotoxic	No
HEp-2 cells	Vacuolation activity
Stability to heat	90 min at 121°C
Stability to pH	Stable at pH 2–11
Effect of proteolysis (trypsin, pepsin)	None
Conditions under which toxin is produced	In food (rice and milk at 12–32°C)
Mechanisms of production	Produced by a nonribosomal peptide synthetase

[a]Based on references 5, 34, 42, 52, 53, 57, and 58.

Cereulide causes inhibition of mitochondrial activity (inhibition of fatty acid oxidation) and stimulates the afferent vagus nerve by binding to the 5-HT3 receptor (52). Mice injected intraperitoneally with synthetic cereulide developed histopathological changes in the liver, and massive degeneration of hepatocytes was observed (32). The levels of hepatic enzymes in mouse serum were highest on days 2 and 3 after the inoculation of cereulide and rapidly decreased thereafter. General recovery from pathological changes and regeneration of hepatocytes were observed after 4 weeks.

Cereulide is nonribosomally synthesized by the multienzyme complex Ces-NRPS, which is encoded on a megaplasmid with homology to the *B. anthracis* pXO1 plasmid (58, 59). However, the transcriptional regulators involved in *ces* synthetase regulation that have been identified so far are encoded chromosomally. The 24-kb cereulide synthetase gene cluster is transcribed as an operon, and transcription seems to be highly temporal and tightly regulated; *ces* mRNA is detectable only during mid-exponential-phase to late-exponential-

phase growth (60). Several promoters are identified within the *ces* gene cluster, although *ces*P1 has been shown to be the main promoter (61). This promoter is not subjected to regulation by the PlcR-PapR quorum-sensing system (see “Regulation of Enterotoxin Production” below) but is directly repressed by the chromosomally encoded transition state regulator AbrB (62).

The majority of the characterized emetic strains do not grow below 10°C, nor do they produce the Hbl enterotoxin (44). However, a few strains of *B. weihenstephanensis* have been reported to produce cereulide (63). Although these strains grow at lower temperatures than other members of the *B. cereus* group, the emetic toxin is not produced below 8°C (34). Recently, it was shown that Ces-NRPS synthesizes at least 17 different isoforms of cereulide which differ significantly in their cytotoxic properties (64). All emetic *B. cereus* strains produce the same set of isocereulides, although at different ratios (64, 65). The formation of the cereulide isoforms is highly temperature dependent and is regulated at posttranscriptional levels, and surprisingly, production of a particularly cytotoxic cereulide isoform was elevated at low temperatures (12 to 15°C) (66). Cereulide synthetase gene expression and cereulide toxin production are influenced by complex interactions of various intrinsic as well as extrinsic factors, and this may be of great importance in terms of food safety and predictive microbiology (67).

Enterotoxins

B. cereus produces different proteins (or protein complexes), referred to as enterotoxins, that can cause foodborne illness (Table 20.5.). Two three-component enterotoxin complexes, hemolysin BL (Hbl) and Nhe (54, 55), and a single 34-kDa protein, CytK (12), have been thoroughly described. Hbl was the first to be characterized; it causes vascular permeability, is dermonecrotic, and leads to fluid accumulation in ligated ileal loops in rabbits (68, 69). Hbl consists of the components B, L_1, and L_2, which are all necessary for maximal enterotoxin activity, and a 1:1:1 ratio of the three components gives maximum biological activity (68). It was recently shown by priming the Hbl components on Chinese hamster ovary (CHO) cells that the order of assembly on the cell surface is B, L_1, and finally L_2 (70). About 60% of *B. cereus* strains carry *hbl* genes (71, 72), while the number is slightly higher for *B. thuringiensis* strains (71, 73). Some *B. cereus* strains carry a second *hbl* operon (71, 74), and a phylogenetic study indicated that the duplication of the *hbl* operon was an ancient event which occurred early in the evolution of *B. cereus sensu lato* (71).

Table 20.5 Toxins produced by *B. cereus*

Toxin	Type/size	Food poisoning	Reference(s)
Hemolysin BL (Hbl)	Protein, 3 components	Probably	68, 69, 91, 119
Nonhemolytic enterotoxin (Nhe)	Protein, 3 components	Yes	55, 75, 78, 110
Cytotoxin K (CytK)	Protein, 1 component, 34 kDa	Yes; 3 deaths	12
Emetic toxin (cereulide)	Cyclic peptide, 1.2 kDa	Yes; several deaths	13–15, 52, 53

Hbl was previously suggested to be a primary virulence factor in *B. cereus* diarrhea; however, strains without *hbl* genes have caused food poisoning (55). The second three-component enterotoxin, Nhe, was first described by Lund and Granum in 1996 after a large foodassociated outbreak caused by a *B. cereus* strain lacking Hbl-encoding genes (55, 75). Initially, the hemolytic activity of Nhe was not detected, but more recently, Nhe was found to possess hemolytic activity, although it is not as high as that of Hbl (76). The Nhe toxin components, NheA, NheB, and NheC, are secreted as soluble proteins (77), and the Nhe pore-forming complex is most active when the ratio between NheA, NheB, and NheC is 10:10:1 (76, 78). However, NheC is expressed in lower ratios *in vivo* than the optimal ratio, and this is likely why NheC is not detected on two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (79, 80). If higher-than-optimal ratios for NheC activity are used in experiments, the biological activity is rapidly reduced, and at a 1:1:1 ratio, very little biological activity is left (76, 78). Like Hbl, Nhe requires a specific binding order of the three components for pore formation (70, 81, 82). The first step is a complex formation between NheB and NheC in solution, followed by binding of the complex to the cell surface, probably to a specific surface structure recognizing the NheB-NheC complex (82). This step is accompanied by conformational changes in NheB (83), leading to oligomerization and attachment of NheB proteins, forming a ring-shaped structure on the cell surface. NheA then binds to cellbound NheB, and a conformational rearrangement of NheA finalizes the pore formation (84).

The X-ray crystal structures of NheA and HblB have recently been solved, and the two toxin components are structurally related to each other, as well as to the hemolytic enterotoxins HlyE, ClyA, and SheA of *Escherichia coli*, *Salmonella enterica*, and *Shigella flexneri*, respectively (85, 86). They all

belong to the ClyA superfamily of α-helical pore-forming toxins, possessing an α -helix bundle and a subdomain containing a β-hairpin structure. However, in contrast to the characteristic hydrophobic β-hairpin of HblB and ClyA, NheA has an amphipathic β-hairpin structure resembling a structure of *Staphylococcus aureus* α-hemolysin, which is a β-barrel pore-forming toxin (85). Due to amino acid sequence similarities, the other components of Nhe and Hbl are predicted to share the overall three-dimensional structure with NheA and HblB, and the β-hairpin of NheC has been shown to be essential for binding of NheC to the cell membrane (81). More than 99% of all *B. cereus* strains carry genes encoding Nhe (44, 71).

The last-characterized *B. cereus* toxin associated with food poisoning is CytK. This toxin belongs to a family of β-barrel toxins and is similar to the β-toxin of *C. perfringens*; it was the cause of symptoms in a severe outbreak of *B. cereus* foodborne illness in a nursing home in France in 1998 (12). In this outbreak (*B. cereus* necrotic enteritis), several people developed bloody diarrhea and three patients died. Similar to the other members of this β-barrel toxin family, CytK is inserted into membranes as a heptamer and makes pores 7 Å in diameter in planar lipid bilayers (87).

The *Bacillus* strain that caused the fatal outbreak in France (12) is highly cytotoxic and is now the type strain for *B. cytotoxicus* (19). The CytK produced by this particular strain, CytK-1, differs from CytK produced by most other *B. cereus* strains (12, 88). *cytK* is present in about 40% of *B. cereus* strains (71), and CytK-1 is 89% identical in amino acid sequence to the more commonly occurring CytK (89). The high virulence of the *B. cytotoxicus* type strain is thought to be due to the greater cytotoxic activity of CytK-1 than CytK, and also to a high level of *cytK* expression (88). CytK-1 is about five times more toxic on epithelial cells than the other CytK, although the activities of these two toxins on erythrocytes are very similar (88). CytK-1 is produced by a few strains that are genetically remote from other strains of the *B. cereus* group (89). These strains harbor a novel gene variant encoding Nhe, which is not detected using ordinary *nhe* PCR primers or monoclonal antibodies against the Nhe proteins (89). Although studies have revealed that the total toxicity of *B. cereus* supernatant fluids for most strains is largely due to Nhe (90), Hbl and CytK cannot be excluded as contributing to diarrhea, and CytK can be fatal when produced in large amounts by some strains (12).

Regulation of Enterotoxin Production

The genomes of more than 400 *B. cereus* strains have been sequenced, and database searches reveal that the organization of both the *hbl* and *nhe* operons is very well conserved between strains. All three proteins of the Hbl complex

are transcribed from one operon (*hbl*) in a 5.5-kb polycistronic mRNA, transcribing *hblC* (component L_2), *hblD* (component L_1), and *hblA* (component B) (91). The three genes encoding the Nhe components, *nheA, nheB*, and *nheC*, constitute the *nhe* operon (Fig 20.2) and are transcribed in a 3.7-kb polycistronic mRNA (78). The properties of the three Nhe components are described in Table 20.6..

Regulation of transcription of the enterotoxin encoding genes is very complex, and the 5′ intergenic regions upstream of both the *nhe* and *hbl* operons harbor recognition sites for transcriptional regulators such as PlcR, CcpA, CodY, and SinR (Fig 20.2). The transcriptional activator PlcR was first identified as an activator required for transcription of *plcA* at the onset of stationary phase in *B. thuringiensis* (92) and was later recognized as a major regulator of *B. cereus* virulence (93). The PlcR quorumsensing system regulates transcription of 45 genes, where 22 are secreted proteins (mainly toxins, phospholipases, and proteases), 18 are cell wall proteins, and 5 are cytoplasmic proteins (93). The activity of PlcR depends on activation by PapR, a 48-amino-acid polypeptide with an N-terminal signal peptide sequence. PapR is secreted via the SecA pathway, processed, and then reimported into the bacterial cell via the oligopeptide permease (Opp) system (94). Crystal structure studies have revealed that PlcR is composed of an N-terminal helix-turn-helix DNA-binding domain and a C-terminal regulatory domain composed of five degenerated tetratricopeptide repeats (TPRs) (95). TPRs are structurally conserved helical domains involved in protein-protein or protein-peptide interactions (96), and when PapR binds to the TPR domain, the helix-turn-helix domain is rearranged, allowing binding to DNA (95). PapR-PlcR then binds to a specific DNA sequence called the PlcR box ($TATGNAN_4TNCATA$), located upstream from the controlled genes, resulting in upregulation of expression of *plcR* and *papR* as well as the other genes in the PlcR regulon (92, 93). The PlcR activation is strain specific, and four groups of PlcR-PapR pairs defining four different pherotypes have been identified: PlcRI, PlcRII, PlcRIII, and PlcRIV (97, 98). The strain specificity depends on PapR originating from the same strain or from a member of the same pherogroup. In *B. anthracis*, PlcR is inactivated by a nonsense mutation in the *plcR* gene (99), and consequently, genes regulated by PlcR are expressed at very low levels compared to those of *B. cereus*. Interestingly, most *B. weihenstephanensis* strains also produce enterotoxins, but usually at temperatures below 30°C (100). This temperature regulation is not under the control of the above-described (PlcR-PapR) system and has yet to be elucidated.

Table 20.6 Properties of the Nhe proteins[a]

Protein	Signal peptide size (aa)	Active protein size (aa)	Mol wt (active protein)	pI
NheA	26	360	41,019	5.13
NheB	30	372	39,820	5.61
NheC	30	329	36,481	5.28

[a]Based on reference 110.

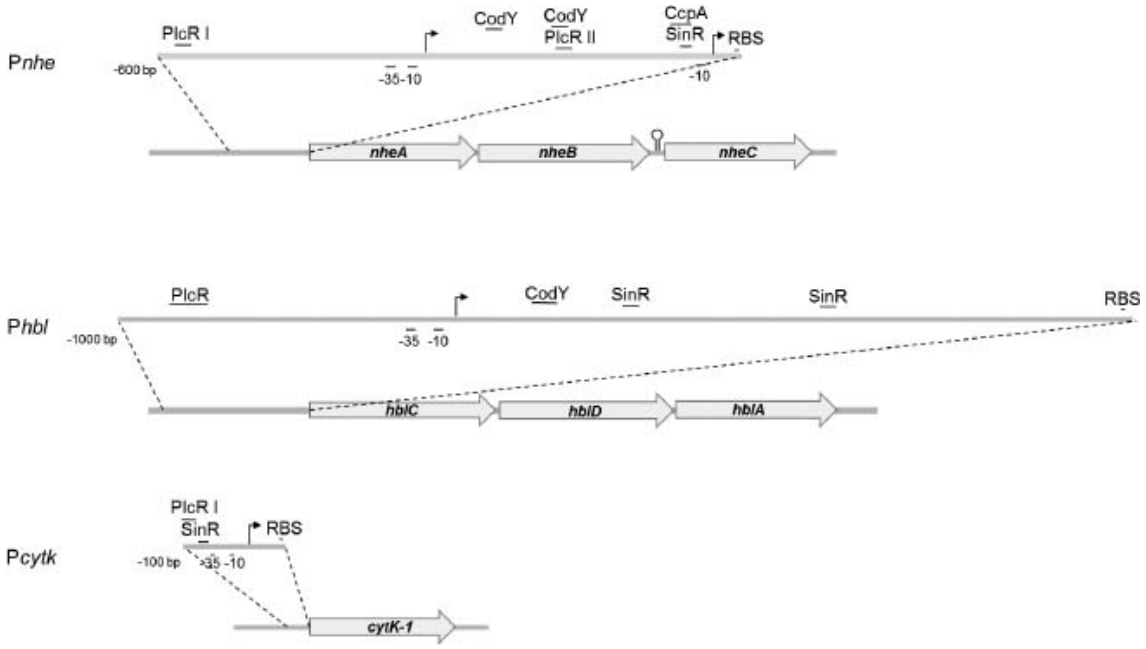


Figure 20.2 Schematic overview of the promoter regions of the three major enterotoxin operons in *B. cereus sensu lato* (109). The −35 and −10 regions and transcriptional start sites (bent arrows) (78, 92, 117, 118), CodY binding sites (109), catabolite-responsive element (Cre) (103), PlcR binding sites (78, 92, 93, 117), SinR binding sites (105, 106), and ribosomal binding sites (RBS) are indicated. The inverted repeat possibly involved in regulation of translation of *nheC* is indicated between *nheB* and *nheC* (78).

Hbl and Nhe have been suggested to be subject to catabolite repression, at least during anaerobiosis, because transcription of *hbl* is repressed by increasing concentrations of glucose (101), and growth on sucrose produces higher levels of Hbl and Nhe than growth on glucose (102). CcpA is a catabolite control protein *B. cereus* described by van der Voort et al., and they suggest that CcpA-mediated glucose sensing is involved in transcription of *nhe* and *hbl* operons (103). The position of the binding site of CcpA in the *nhe* promoter region is indicated in Fig 20.2. CcpA is also possibly involved in expression of Hbl;

however, this binding site is located in the intergenic region between *hblC* and *hblD* (103).

SinR is a master regulator of biofilm formation, and a microarray study on *B. licheniformis* biofilm revealed that SinR also controlled expression of Hbl (104). Putative SinR binding sites in the regulatory regions of the *nhe* and *hbl* operons are indicated in Fig 20.2 (105, 106).

CodY is a nutrient-sensitive regulator of transcription important for efficient production of enterotoxins in *B. cereus* (107). It has been shown that CodY activates production of the proteins of the oligopeptide permease OppABCDF in *B. thuringiensis* (108). Involvement of CodY in the control of the import of PapR allows the PlcR-PapR quorum-sensing system to integrate two types of external signals: the composition of the medium and the accumulation of the peptide. This mechanism adds another layer of complexity to the regulatory circuits which influence the expression of *B. cereus* enterotoxins. In addition, it was recently suggested that CodY may be used for strain-specific fine-tuning of enterotoxin transcription via repression in response to specific environmental conditions (Fig 20.2) (109).

Since a low concentration of NheC is crucial for full activity of the Nhe complex, regulation of *nheC* mRNA translation is of importance. The 109-bp region between *nheB* and *nheC* contains an inverted repeat of 13 bp, which most likely is important for repression of translation of *nheC* mRNA (Fig 20.2) (78, 110).

The *B. cereus* Spore

The *B. cereus* spore is an important factor in foodborne illness. First, *B. cereus* spores are more hydrophobic than spores from any other *Bacillus* sp., which enables them to adhere to several types of surfaces (111). Hence, they are difficult to remove during cleaning and a challenging target for disinfectants. *B. cereus* spores also contain appendages and/or pili (111, 112) that are, at least partly, involved in adhesion (111), although these structures also contribute to spores' ability to stick together in large aggregates. Not only can these properties of *B. cereus* spores enable them to withstand cleaning and disinfection and hence remain present on equipment surfaces to contaminate different foods, but they also contribute to adherence to epithelial cells. Studies have revealed that spores of many strains associated with foodborne outbreaks (112) can adhere to Caco-2 cells in culture and that these properties are associated with hydrophobicity and possibly with the spore's appendages (31).

Food Spoilage

As described above, *B. cereus* contamination is very difficult to avoid in many food products. Spices and other dried components (starch) typically contain from a few to more than 10^3 spores per gram; hence, many food products will be contaminated. If the final product is not sufficiently heat treated (>100°C), the spores will survive, and outgrowth will occur if the food is held at >10°C over time (over 4°C for *B. weihenstephanensis*). This is the reason for outbreaks (mainly emetic) involving heat-treated rice and pasta and even pizza stored at ambient temperatures overnight. Other lightly heattreated products may also be at risk, but most such products (e.g., those packaged sous vide) are usually stored cold and heat treated a second time at home before consumption. A special problem is pasteurized milk, in which the mesophilic strains do not grow if it is stored properly; however, *B. weihenstephanensis* grows in milk at approximately 4°C and reaches high enough cell numbers to cause what is referred to as "sweet curdling" and "bitty cream." Long before reaching this point, the number of *B. cereus* cells is high enough to cause food poisoning. However, milk is rarely associated with outbreaks of *B. cereus* food poisoning, because *B. weihenstephanensis* dominates in milk, and most of these strains (if not all) are unable to produce enterotoxin in the human gut, although they can produce the toxins at lower temperatures (100).

Commercial Methods for Detection of the *B. cereus* Toxins

No commercial kit for detection of the emetic toxin (cereulide) is yet available. However, PCR can be used for detection of the genes encoding the NRPS responsible for cereulide production (113). Screening for cereulide production by *B. cereus* strains can also easily be done by using a boar sperm motility assay (114), and a fully validated liquid chromatography-mass spectrometry detection method was recently published (115).

The Thermo Scientific Oxoid BCET-RPLA toxin detection kit is semiquantitative and measures the presence of the HblC (L_2) component in culture supernatants (116). If the kit reacts positively with the supernatant, it is likely that the strain is enterotoxin positive. If culture supernatants are also toxic for Vero cells in culture, the strains can be regarded as enterotoxin positive. At present, there is no commercial method available for detecting CytK.

CONCLUDING REMARKS

B. cereus is a normal inhabitant of soil but is also well adapted to grow in the gut of warm-blooded animals. *B. cereus* is frequently isolated from a variety of foods, including vegetables, dairy products, and meat. This bacterium causes both emetic and diarrheal food-associated illnesses that occur largely in developed countries. The diarrheal type of illness is most prevalent in the Western Hemisphere, whereas the emetic type is most prevalent in Japan and other Asian countries. Desserts, meat dishes, and dairy products are most frequently associated with diarrheal illness, whereas rice and pasta are the most common vehicles of emetic illness.

Three types of *B. cereus* enterotoxins involved in outbreaks of foodborne illness have been identified. Two of these enterotoxins, Nhe and Hbl, are structurally related three-component toxin complexes, whereas the third is a one-component protein (CytK). Some *B. cereus* strains also produce an emetic toxin, a cyclic dodecadepsipeptide, which is synthesized by a nonribosomal peptide synthetase complex. Both the emetic toxin and CytK have been involved in foodborne outbreaks with fatal outcomes.

Some strains of the *B. cereus* group are able to grow at refrigeration temperature. These variants raise concerns regarding the safety of cooked, refrigerated foods with extended shelf lives. *B. cereus* foodborne illness is likely to be highly underreported because of its relatively mild symptoms and short duration. However, precooked, chilled food products with extended shelf lives can be well suited to *B. cereus* survival and growth, and increased consumer interest in these products may increase the prominence of *B. cereus* as a foodborne pathogen.

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Staphylococcus aureus

Staphylococcal food poisoning (SFP) is among the most prevalent causes of gastroenteritis worldwide. It results from ingestion of one or more preformed staphylococcal enterotoxins (SEs) in staphylococcus-contaminated food. The etiologic agents of SFP are members of the genus *Staphylococcus*, predominantly *Staphylococcus aureus*. This form of food poisoning is considered an intoxication and does not involve infection by and growth of the bacteria in the host.

The association of staphylococci with foodborne illness was made more than a century ago. Barber, in 1914, was the first to implicate a toxin in SFP (1). He reported that repeated ingestion of contaminated milk produced symptoms typical of the illness. Barber cultured the milk, demonstrated the presence of a putative causative staphylococcal agent, and provided the first evidence that a soluble toxin was responsible for the disease. The next major advance in understanding SFP etiology was reported in 1930 by Dack et al. (2); Dack voluntarily consumed supernatant fluids from cultures of "a yellow hemolytic staphylococcus" grown from contaminated sponge cake. Upon ingestion of the filtrates, he became ill with vomiting, abdominal cramps, and diarrhea. At that time, the only other foodborne toxin that had been recognized was botulinum toxin. However, staphylococcal toxin, which exerted an effect on the gastrointestinal tract, was the first true enterotoxin described. It was particularly different from botulinum toxin because its activity was "not entirely destroyed by heating even for 30 minutes at 100°C."

S. aureus has been extensively characterized. This bacterium produces a variety of extracellular products. Many of these, including the SEs, are virulence factors which have been implicated in diseases of humans and animals. As a group, the SEs elaborate a set of biological properties that enable staphylococci to cause at least two common human diseases, toxic shock syndrome (TSS) and SFP. This chapter primarily addresses SFP; however, there is significant overlap in the histories of both diseases. Hence, TSS is also discussed in sections in which this overlap is most relevant.

CHARACTERISICS OF THE ORGANISM

Taxonomy

The term "staphylococci" describes a group of small spherical, Gram-positive bacteria. Depending on the species and culture conditions, their cells have diameters ranging from approximately 0.5 to 1.5 μm. They are catalasepositive chemoorganotrophs with a DNA guanine-plus-cytosine content of 30 to 40 mol%. Staphylococci have a typical Gram-positive cell wall containing peptidoglycan and teichoic acids. Except for clinical isolates, such as some community-acquired methicillin-resistant *S. aureus* strains (3) and strains exposed to antimicrobial therapy, most staphylococci are sensitive to β-lactams, tetracyclines, macrolides, lincosamides, novobiocin, and chloramphenicol but are resistant to polymyxin and polyene. Some differential characteristics of *S. aureus* and several other selected species of staphylococci are summarized in Table 21.1.

There have been many useful schemes for classification of the staphylococci. According to *Bergey's Manual of Systematic Bacteriology* (4), staphylococci are classified in the family *Micrococcaceae*. This family includes the genera *Micrococcus*, *Staphylococcus*, *Stomatococcus*, and *Planococcus*. The genus *Staphylococcus* is further subdivided into 53 species. Many of these are present in food as a result of human, animal, or environmental contamination. Several species of *Staphylococcus*, including both coagulase-negative and coagulase-positive isolates, can produce SEs. Although several species, including some coagulase-negative staphylococci, have the potential to cause gastroenteritis (5), nearly

all cases of SFP are attributed to *S. aureus*. This is because of the relatively high prevalence of SE production by *S. aureus* in comparison to other staphylococcal species. Although the reason for this is unknown, the SEs are superantigens (SAgs) and therefore are potential immunomodulating agents (see below). Hence, SE production is proposed to provide a selective advantage to *S. aureus*, a species that is common to both humans and animals, the two most common sources of food contamination.

Enterotoxigenic strains of staphylococci have been well characterized based on a number of genotypic and phenotypic characteristics. An extensive phage-typing system is available for *S. aureus*. Most SE-producing isolates belong to phage group I or III or are nontypeable. Although SE production by other phage groups is less common, it has been documented. Hájek and Marsálek (6) developed a classification scheme based largely on the animal host of origin. They were able to differentiate *S. aureus* into at least six biotypes. By far, SE production was most prevalent among human isolates within biotype A. SE production by other biotypes is rare, except for biotype C bovine and ovine mastitis isolates. SEs may also be produced by *Staphylococcus intermedius* and *S. hyicus* (formerly *S. aureus* biotypes E and F, respectively) and coagulase-negative staphylococci, albeit considerably less frequently than *S. aureus*.

Physiology

Some unique resistance properties of *S. aureus* facilitate its contamination and growth in food. Of the non-sporeforming human pathogens, *S. aureus* is one of the most resistant to environmental stress outside the body and can survive for extended periods in a dry state. Its survival is facilitated by organic material, which is likely to be associated with the staphylococci from an inflammatory lesion. Isolation of staphylococci from air, dust, sewage, and water is relatively easy, and environmental sources of food contamination have been documented in several outbreaks of SFP. The physiological characteristics of *S. aureus* are summarized in Table 21.2.

Table 21.1 General characteristics of selected *Staphylococcus* species[a]

Characteristic	*S. aureus*	*S. chromogenes*	*S. hyicus*	*S. intermedius*	*S. epidermidis*	*S. saprophyticus*
Coagulase	+	–	+	+	–	–
Thermostable nuclease	+	–	+	+	±	–
Clumping factor	+	–	–	+	–	–
Yellow pigment	+	+	–	–	–	±
Hemolytic activity	+	–	–	+	±	–
Phosphatase	+	+	+	+	±	–
Lysostaphin	Sensitive	Sensitive	Sensitive	Sensitive	Slightly sensitive	ND
Hyaluronidase	+	–	+	–	±	ND
Mannitol	+	±	–	±	–	±
fermentation Novobiocin resistance	–	–	–	–	–	+

[a] +, positive; –, negative; ±, variable; ND, not determined.

Table 21.2 Physiological limits for the growth of *S. aureus* and SE production (179–183)

Parameter[a]	Growth		SE production		SEs affected	Food products
	Optimal	Limit	Optimal	Limit		
Temp (°C)	35–41	6–48	34–40	10–46	SEA, SEB, SEC, SED	Milk, ham, egg products
pH	6–7	4–10	7–8	5–9.6	SEA, SEB, SEC, SED, SEE	Ham, sausage
a_w	0.99	0.83–0.99	0.99	0.86–0.99	SEA, SEB, SEC, SEH	Bacon, sausage
NaCl (%)	0	0–20	0	<12	SEA, SEB, SEC	Ham, sausage
Oxygen	Aerobic	Facultatively anaerobic	Aerobic	Facultatively anaerobic	SEA, SEB, SEC, SEH	Ham, sausage

[a] a_W, water activity.

The growth temperature for *S. aureus* ranges from 6 to 48°C, with optimum growth at 35 to 41°C. The upper limit of the growth temperature can be extended in the presence of NaCl and monosodium glutamate (7). *S. aureus* is facultatively aerobic and still can grow in the presence of 80% CO_2 (8). The pH tolerance ranges from 4 to 10, with optimal growth at pH 6 to 7. The acid tolerance of *S. aureus* is significantly decreased in the presence of glycerol, sucrose, potassium sorbate, and NaCl (9). *S. aureus* is known for acquiring genetic resistance to heavy metals and antimicrobial agents used in clinical medicine. However, the resistance of this bacterium to common food preservation methods is generally unremarkable. One noteworthy exception is its osmotolerance, which permits growth in media containing the equivalent of 3.5 M NaCl and survival at water activities less than 0.86. This is especially problematic, because other bacteria with which *S. aureus* does not compete effectively are likely to be inhibited under these conditions.

The molecular basis for staphylococcal osmotolerance has been investigated in recent years, although systems for responding to osmotic stress have been more intensively studied in less tolerant bacteria. Considering the unique resistance of staphylococci, it would not be surprising to find that they have developed a highly efficient osmoprotectant system. As in other bacteria, several compounds accumulate in the cell or enhance staphylococcal growth under osmotic stress. Glycine betaine appears to be the most important osmoprotectant for *S. aureus*. To varying degrees, other compounds, including l-proline, proline betaine, choline, and taurine, can also act as compatible solutes for this bacterium. In *S. aureus*, proline and glycine betaine accumulate to very high intracellular levels in response to increased concentrations of NaCl in the environment. Although the signal transduction pathway in staphylococci is not known, in other bacteria it involves a loss in turgor pressure in the cell and activation of required transport systems. High-affinity and low-affinity transport systems operate in *S. aureus* for both proline and glycine betaine (10, 11). The low-affinity systems are primarily stimulated by osmotic stress, have broad substrate specificity, and may be the same transporter shared by both osmoprotectants. By itself, the demonstration of a stress response system in *S. aureus* does not explain the unusual staphylococcal osmotolerance. Other, less tolerant microorganisms possess mechanisms for counteracting osmotic stress. The efficiency of the staphylococcal system may reflect an unusually high endogenous level of intracellular K^+ and lack of need for *de novo* transporter synthesis. For example, in other well-studied systems, such as that of *Escherichia coli*, changes in osmotic stress activate K^+ transport systems. The elevated intracellular K^+ levels that result are required for induction of *proU* and eventually lead to synthesis of transporters for glycine betaine and other osmoprotectants. In *S. aureus*, K^+ levels are high in unstressed cells. Therefore, the transport system is constitutively present in this bacterium and is preformed when high-salt conditions are encountered. The net result is a very rapid and efficient response. *S. aureus* cells accumulate a 21-fold increase in proline after less than 3 minutes of exposure to high salt concentrations (10).

Reservoirs

Humans are the main reservoir for staphylococci involved in human disease, including *S. aureus*. Although most species are normal inhabitants of the external regions of the body, *S. aureus* is also a leading human pathogen. Colonized individuals may be carriers and are an important source for disseminating staphylococci to others and to food. In humans, the anterior nares are the predominant site of colonization, although *S. aureus* can be present on other sites, such as the skin or perineum. Dissemination of *S. aureus* among humans and from humans to food can occur by direct contact, indirectly by skin fragments, or through respiratory tract droplet nuclei.

Today, most sources of SFP are found to be humans who contaminated food during preparation. In addition to contamination by food preparers who are carriers, *S. aureus* may also be introduced into food by contaminated equipment used in food processing, such as meat grinders, knives, storage utensils, cutting blocks, and saw blades. An analysis of more than 700 foodborne disease outbreaks revealed that the following conditions were most often associated with food poisoning: (i) inadequate refrigeration; (ii) preparation of foods far in advance; (iii) poor personal hygiene, e.g., not washing either hands or instruments properly; (iv) inadequate cooking or heating of food; and (v) prolonged use of warming plates when serving foods, a practice that promotes staphylococcal growth and SE production (12).

Animals, also an important source of *S. aureus*, are often heavily colonized with staphylococci. Predisposing factors which facilitate the survival of the bacterium are major concerns in the maintenance and processing of food animals and their products. For example, one very serious problem for the dairy industry is mastitis, an infectious disease of mammary tissue often caused by *S. aureus*. The combined losses and expenses associated with bovine mastitis make it the single most costly disease of animal agriculture in the United States. Colonization of animals by *S. aureus* is also a public health concern because it may result in contamination of milk and dairy products with *S. aureus* prior to or during processing.

It is not always possible to trace the source of staphylococcal food contamination to a human or animal origin. Regardless of its source, many studies have revealed the common presence of *S. aureus* in many different types of food products. In Canada, *S. aureus* was obtained from 10.5% (73/693) of food samples, including 29 meats, 20 prepared foods containing meat, 11 prepared foods not containing meat, 10 dairy products, and three produce samples, between 2007 and 2010 (13). In China, a study of 27,000 retail food items from 203 cities of 24 provinces revealed that 4.3% of the samples (1, 150) were positive for *S. aureus*. From this study, 9.4% of the positive samples were from poultry meats, 7.2% were from red meats, and 3% were from rice and flour products (14). In the European Union, 15.7% (136/868) of food products confiscated from travelers, including antelope, duck, guinea pig, pork, turkey, dairy, and eggs, were positive for *S. aureus* (15). In the United Kingdom, 53% of SFP incidents reported between 1969 and 1990 were associated with meat products, meat-based dishes, and especially ham; 22% of the cases were from poultry and poultry-based meals; 8% were from milk products; 7% were from fish and shellfish; and 3.5% were from eggs (16). In France, 32% of instances of SFP reported in 1999 to 2000 were associated with milk products and especially cheeses, 22% were from meats, 15% were from sausages and pies, 11% were from fish and seafood, 11% were from eggs and egg products, and 9.5% were from poultry (17). In the United States, 36% of SFP cases reported between 1975 and 1982 were associated with red meat, 12.3% with salads, 11.3% with poultry, 5.1% with pastries, and only 1.4% with milk products and seafood (18). The cell numbers of staphylococci in these sources are often low initially. However, their widespread presence provides a potential source of bacteria capable of causing SFP if conditions appropriate for SE production are provided.

CHARACTERISTICS OF SEs

Classification of SEs

Major advances in the characterization of SEs were made approximately 2 decades after Dack and colleagues (2) associated SFP with an exotoxin. Bergdoll and coworkers were the first investigators to

produce purified SE preparations and develop specific antisera. They and others, using purified or partially purified toxins, induced protective antibodies in several species of animals. Immunity was strain specific and did not provide protection against strains other than the one that was used to induce the initial immune response (19). It soon became apparent that *S. aureus* could produce multiple toxins with similar molecular weights as well as biological and physicochemical properties.

Initially, differentiation between antigenic forms of SE was based on the observation that many food isolates produce one common antigenic type of toxin, tentatively designated the F-type toxin. Most other enterotoxigenic strains, such as those from enteritis patients, also produced a second antigenic form that was classified as E toxin. The discovery of additional isolates that did not conform to this pattern prompted adoption of an improved nomenclature system. A committee assembled in 1962 established an alphabetical nomenclature (20) which, with some modification (21), is still recommended. Accordingly, SEs are sequentially assigned a letter of the alphabet in the order of their discovery. The F and E type toxins were designated SEA and SEB, respectively. Between 1962 and 1972, three additional classical SE serotypes (SEC, SED, and SEE) were reported (22). Initially, the term "SEF" was used for an exotoxin commonly produced by isolates of *S. aureus* associated with TSS. This designation was changed when the toxin was confirmed to lack emetic activity. "SEF" was retired from use in the SE nomenclature system, and this toxin is now referred to as toxic shock syndrome toxin 1 (TSST-1) (23).

Designation of SEs based on serological typing has been useful. However, sequence analysis and detailed immunological studies have produced some examples in which the antigenic characteristics of the proteins do not reflect their molecular or biological uniqueness. The best-documented examples are with SEC. It had been observed for some time that the SEC serological variant can be further divided into at least three subtypes (SEC1, SEC2, and SEC3) based on minor differences in immunological reactivity. However, within each subtype, significant sequence variability may occur. For example, the SECs produced by strains FRI-909 and FRI-913 were both designated SEC3 according to their immunological reactivity. However, it was later determined that the sequences of these toxins differ by nine residues (24). The SEC variants produced by bovine and ovine isolates of *S. aureus* have very similar sequences and are apparently indistinguishable from SEC1 in immunological assays. In contrast, they behave differently from SEC1 in biological assays. For example, although SEC-bovine differs from SEC1 by only three residues, the potencies of the two toxins differ by several orders of magnitude in lymphocyte proliferation assays (24). Examples of heterogeneity among both the classical SEs and newly described toxins are becoming more common with the rapid rate of sequence determinations.

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Compared to the rate of identification of the classical SEs (SEA through SEE), modern genomic analysis has greatly increased the rate at which previously unrecognized SEs and related proteins are discovered. As a result, classification based solely on antigenic properties is not practical. Instead, nomenclature is now based predominantly on molecular relatedness of the primary sequences.

Furthermore, it is not common to confirm the enterotoxigenic activities of putative SEs prior to publication. As a result, some toxins originally reported to be SEs were later determined to lack emetic activity. To ensure an orderly assignment for newly identified confirmed or putative toxins, investigators should contact the International Nomenclature Committee for Staphylococcal SAgs (INCSS) for guidance prior to publication (21). The INCSS also recommends that proteins related to the SEs but not confirmed to exhibit emetic activity in the monkey feeding assay (see below) be designated SE-like (SEl) until their enterotoxic activity can be confirmed. Thus far, 25 SEs and SEl proteins have been reported and designated SEA through SEZ except SEF. The primary sequences and other properties of currently reported SEs and SEl proteins are summarized in Fig. 21.1 and Table 21.3 (24–34).

Based on amino acid sequence alignment, currently known members of the SE family are divided into four groups (Fig. 21.2). Group 1 comprises the closely related toxins SEA, SEP, and SEE, as well as SEIJ, SED, SElW, SEH, SES, SEN, and SEO, which are more distantly related. Group 2 contains SEB, the SEC subtypes, and the molecular variants SEG, SER, SElU, and SElZ. Group 3 contains SEI, SEL, SEK, SEM, SElV, and SEQ. Group 4 contains SET, SElY, SElX, and TSST-1, which are smaller than the other SEs.

Most SEs, SEl proteins, and TSST-1 contain four highly conserved stretches of primary sequence (35). This suggests that there is a selective advantage in host-pathogen interactions for these bacteria to maintain certain toxin characteristics. At the same time, modification of selected regions of the proteins could allow the bacteria to broaden their host range. This molecular diversity may explain how a group of toxins with the same function but different host specificities could have arisen for the purpose of exploiting a broader repertoire of receptors.

Sequence comparisons of members within the SAg family have provided several examples where diversity among the toxins appears to have arisen through gene duplication and/or homologous recombination. For example, SEC1 is most related to SEC2 and SEC3. However, residues 14 through 26 of SEC1 (Fig. 21.1) are identical to the analogous region of SEB but substantially different from the same region in the other SEC subtypes (30). Genetic recombination between *seb* and *sec* in a strain producing both SEB and SEC2 (or SEC3) could explain the generation of SEC1.

Additional minor variabilities have resulted from point mutations; even closely related SEs display some sequence differences. This may reflect fine-tuning of the toxin sequences for interacting with cells from a variety of hosts, as observed for the SEC subtype variants. The sequences of SEC toxins produced by strains of *S. aureus* isolated from humans differ slightly (>95% identity) from sequences of SEC variants produced by bovine and ovine isolates (24).

Locations of SE Genes

Most SE and SEl-protein genes are located on accessory genetic elements, such as prophages, plasmids, staphylococcal pathogenicity islands (SaPIs), genomic islands, or staphylococcal chromosomal cassettes (Table 21.3). Most of these are mobile genetic elements which can be horizontally transferred among *S. aureus* isolates, thereby modifying their virulence and contributing to pathogenic evolution (36, 37).

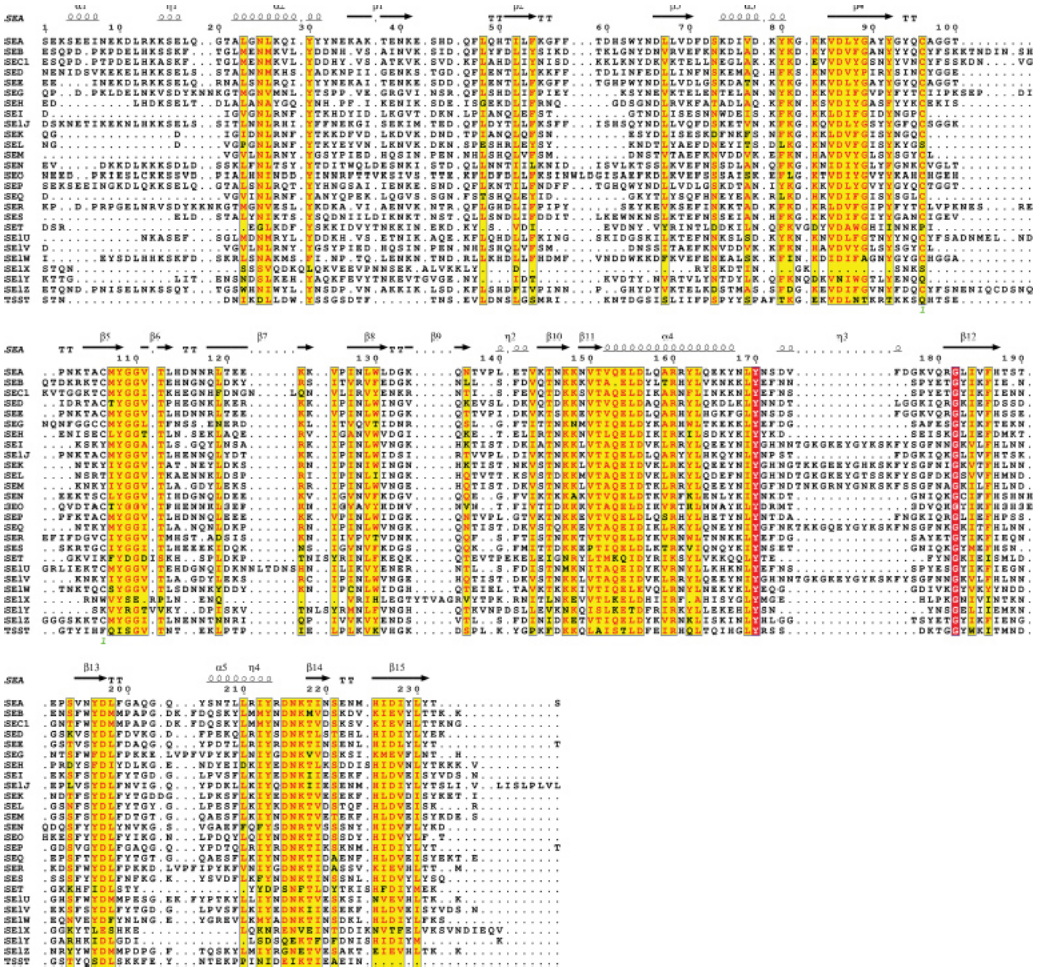


Figure 21.1 Alignment of primary sequences of mature SEs and SEl proteins in the current literature. Sequence alignment and output were conducted using the ClustalW program (177). The secondary structure was aligned to SEA (66).

Most *S. aureus* prophages carrying SEs belong to the family *Siphoviridae*, whose members have an integrase that inserts the phage genome at a specific site (the *att* site) in the bacterial chromosome. To date, at least six fully sequenced *S. aureus* strains carrying different temperate phages harboring the SE genes have been identified (38). The genes *sea*, *sek*, and *sek* are carried in ϕSa3ms and ϕSa3mw, whereas other phages carry the *sea* (ϕMu50a) or *sep* (ϕSa3n) gene alone. Plasmids are one of the main mechanisms for spreading virulence genes by bacteria. The *sed* gene was the first SE gene to be identified as plasmid encoded. Bayles and Iandolo reported that in more than 20 characterized *sed*$^+$ isolates, the *sed* structural gene is localized to a stable 27.6-kb plasmid (pIB485), which also encodes penicillin and cadmium resistance (25). Subsequent studies also revealed that pIB485-related plasmids harbor the genes for SElJ, SER, SES, and SET (39, 40).

Table 21.3 Biochemical and functional properties of SEs, SEl proteins, and TSST-1[a]

SE or SEl Protein[b]	Mol mass (kDa)	Genetic locations	Zinc site(s)	Binding to MHC α/β chain	Human TCR interactions[c]	Emesis tested animal	Emetic dose (μg/kg)	No. positive/no. tested	Referenc
SEA	27.1	Prophage (ϕSa3mu)	Cleft, domain 2	α/β	1.1, 5.3, 6.3, 6.4, 6.9, 7.3, 7.4, 9, 16, 18, 21.3, 22.1, 23	*Macaca mulatta*	5	4/16	87, 184
SED	26.9	Plasmid (pIB485)	Cleft, domain 2	α/β	1, 5.3, 6.9, 7.4, 8, 12	*Macaca mulatta*	10–20	NA	25, 87, 18
SEE	26.8	Prophage (ϕSa)	Domain 2	α/β	5.1, 6.3, 6.4, 8.1, 8.2, 13.1, 18, 21.6	NA			184
SElJ	31.2	Plasmid (pIB485, pF5)	ND	?/β	8, 21.3	ND			39, 48, 18
SEP	27.1	Prophage (ϕSa3n)	ND	ND	5.1, 8, 16, 18, 21.3	*Macaca fascicularis*	100[d]	3/6	95, 187
SEN	26.1	*egc*, chromosome	ND	ND	7, 8, 9, 17	*Macaca fascicularis*	100[d]	2/6	48, 95
SEO	26.8	*egc*, chromosome	ND	ND	5, 7, 22	*Macaca fascicularis*	100[d]	1/8	48, 95
SHE	25.1	Transposon	Domain 2	ND/β	6.7, 8, Vα10	*Macaca mulatta*	30	1/1	32, 85, 18
ESES	26.3	Plasmid (pIB485, pF5)	ND	ND	9, 16	*Macaca fascicularis*	100[d]	2/4	34
SElW	23.2	*egc*, chromosome	ND	ND	13.2, 14	ND			33
SEB	28.4	SaPI3, chromosome, plasmid	–	α/–	1, 3.2, 6.4, 12, 14, 15, 17, 20	*Macaca mulatta*	12	9/16	82, 87
SEC1	27.5	SaPI, plasmid	Cleft	α/–	3.2, 5, 6.4, 6.9, 12, 13.2,15, 17, 20	*Macaca mulatta*	3	5/6	82, 87
SEC2	26.6	SaPI, plasmid	Cleft	α/–	5, 12, 13.1, 13.2, 14, 15, 17, 20	*Macaca mulatta*	5	5/6	82
SEC3	26.6	SaPI, plasmid	Cleft	α/–	2, 3, 5, 12, 13.1, 13.2, 14, 17, 20	*Macaca mulatta*	5	5/6	82
SElU	27.2	*egc*, chromosome	ND	ND	13.2, 14	ND			186, 188
SEG	27.0	*egc*,	–	α/–	3, 12, 13.6,	*Macaca*		4/6	48, 88

		chromosome			14	*mulatta*	80[d]		
SER	27.0	Plasmid (pIB485, pF5)	ND	ND	3, 11, 12, 13.2, 14	*Macaca fascicularis*	100[d]	2/4	34, 189
SElZ	27.2	chromosome	ND	ND	ND				190
SEI	24.9	*egc*, chromosome	ND	ND/β	1, 5, 5.3, 6, 23	*Macaca mulatta*	150	1/4	48, 88
SEK	30.0	SaPI1, SaPI3	ND	ND/β	5.1, 5.2, 6.7	*Macaca fascicularis*	100[d]	2/6	89, 95, 1
SEL	26.8	SaPIbov	ND	ND/β	5, 7, 22	*Macaca fascicularis*	100[d]	1/6	48, 95
SEM	24.8	*egc*, chromosome	ND	ND/β	6, 8, 9, 18, 21.3	*Macaca fascicularis*	100[d]	1/7	48, 95
SEQ	25.0	SaPI3	ND	ND/β	2, 5.1, 21.3	*Macaca fascicularis*	100[d]	2/6	48, 95
SElV	25.0	*egc*, chromosome	ND	ND	6.7, 18, 21.3	ND			186, 191
SET	22.7	Plasmid (pIB485, pF5)	ND	ND	ND	*Macaca fascicularis*	100[d]	3/4	34
SElX	19.3	Chromosome	ND	ND/β	1, 6, 18, 21	ND			192
SElY	22.5	Chromosome	ND	ND	ND	ND			193
TSST-1	21.9	Chromosome	–	α/–	2, 8	*Macaca mulatta*	10	Not emetic	194

[a] ND, not determined; NA, not available.

[b] Nomenclature used in this table follows the new nomenclature rule of the INCSS (75).

[c] Unless otherwise indicated, numbers indicate TCR Vβ elements.

[d] Toxins were prepared from *E. coli*.

The SEC and SEB genes are also associated with transmissible penicillin resistance plasmids (41). However, it is now generally agreed that both of these toxins are chromosomally encoded and harbored on SaPIs in most strains. The SaPIs are widely distributed in *S. aureus* and have a highly conserved genetic structure. Each SaPI has a single copy of the integrase gene and a module for excision and replication in the presence of helper phages which induce excision and packaging of SaPIs into transducing phage particles, resulting in a high frequency of horizontal transfer (36, 37). Genome sequencing revealed more than 20 SaPIs, with many of them carrying SE genes. The *seb* gene is carried on SaPI3 (42), whereas the *sec*, *sel*, and *tst* genes are carried on SaPI4 and SaPIbov (43). SaPI1 carries the *sek*, *sel*, and *tst1* genes (41).

Uniquely present in the *S. aureus* chromosome are genomic islands that occupy specific loci and carry various combinations of virulence factors with either intact or remnant DNA recombinases (44, 45). Two genomic islands, vSaα and vSaβ, are present in all *S. aureus* genomes sequenced to date. vSaα carries a cluster of lipoprotein genes (*lpl* cluster) and the staphylococcal SAg-like cluster (*ssl* cluster) (46). The *ssl* cluster comprises a series of related genes (7 to 11) encoding SAglike proteins which are not superantigenic but are capable of modulating the host immune system by interaction with IgA, C5, or neutrophils (47). vSaβ carries a serine protease gene (*spl*) cluster, leucocidin D and E genes, lantibiotic biosynthesis genes (*bsa*), and/or an enterotoxin gene cluster (*egc*), which includes various

combinations of five or six SE genes (*seg*, *sei*, *sem*, *sen*, *seo*, and *selu*) (43, 48). The *egc* cluster has been proposed to be a nursery of new toxin genes that has created variant SEs, such as those encoded by *selv* and *selw*, by gene recombination (33, 49). It has long been postulated that genomic islands are not mobile; however, recent studies revealed that a temperate phage, ϕSaBov, in *S. aureus* strain RF122 induces specific excision of vSaα, vSaβ, and vSaγ genomic islands, packages them to transducing phage particles, and horizontally transfers them to other *S. aureus* strains at high frequency (50, 51). Importantly, the *egc* cluster proteins have become the most prevalent SEs in *S. aureus* isolates, suggesting an important role for phage-mediated transfer of *egc* in the pathogenic evolution of *S. aureus*.

Basic Biophysical and Structural Features

SEs and SEl proteins are globular proteins of approximately 25 kDa. SE sequence analysis, relatedness, and diversity are discussed above (Fig. 21.1 and 21.2 and Table 21,3). Although detailed studies have not been performed with every toxin, as a group they are stable molecules in many respects. Their recognition as heatstable toxins arose from early studies in which enterotoxicity and antigenicity were not completely destroyed upon boiling of crude preparations. Furthermore, less extreme temperature treatments, such as those used for the pasteurization of milk, had little or no effect on SE toxicity. The heat resistance of the SEs has been extensively studied. The general conclusion from this combined work is that SEs are difficult to inactivate by heating and have increased stability when present in high concentrations or in crude states, such as in the environment of food. Since temperatures required to inactivate SEs are much higher than those needed to kill *S. aureus* under the same environmental conditions, toxic food involved in many cases of SFP is devoid of viable staphylococci at the time of serving. One additional property of SEs that has potential significance with regard to development of SFP is their resistance to inactivation by proteases present in the gastrointestinal tract. Resistance to pepsin, especially in a relatively low-pH environment, is a key requirement for SE activity *in vivo*. Each of the SEs tested has some resistance to pepsin, a property not shared by at least one nonemetic staphylococcal pyrogenic toxin (PT), TSST-1. SEB is susceptible to degradation by pepsin at very low pH, but partial neutralization of gastric acidity by ingested food is presumed to temporarily provide a protective environment for the toxin (1).

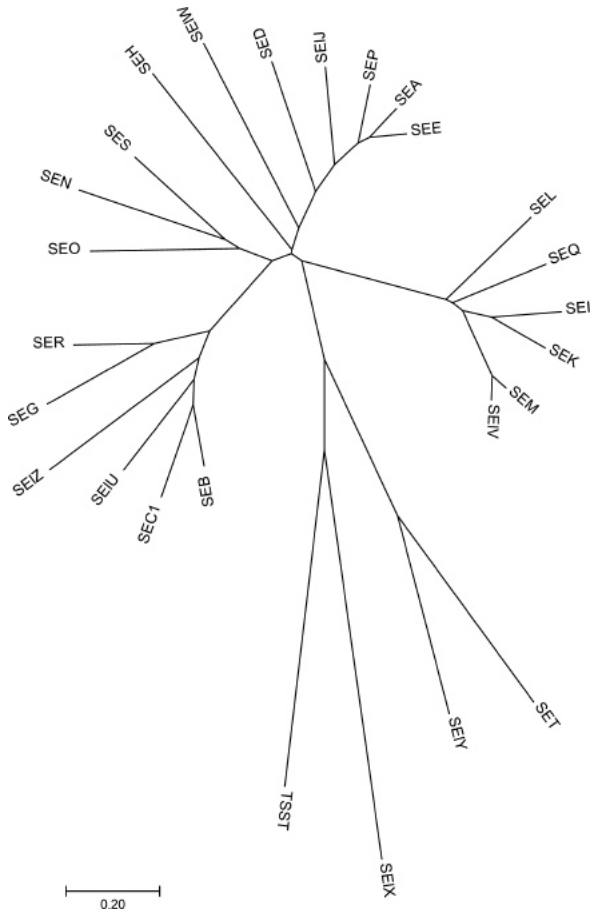


Figure 21.2 Neighbor-joining tree based on the sequences of currently known SEs, SEl proteins, and TSST-1. This tree was created with the clustering feature of Clustal W using MEGA 4.0.1 software (178).

SEs may be cleaved by other common proteases; however, unless the fragments generated are separated in the presence of denaturing agents, proteolysis alone may not be sufficient to cause a loss of biological activity. This is apparently representative of inherent SE molecular stability, which can be demonstrated by renaturing studies. For example, denaturation occurs only under strong denaturing conditions employing high concentrations of urea or guanidine hydrochloride. If the denaturing conditions are removed, SEs may spontaneously renature and regain biological activity (52). Differences in stability exist among the toxins. For example, SEB and SEC1 are approximately 50-fold more stable under denaturing conditions than SEA (53). It has been suggested that the SE disulfide bond is responsible for its inherent molecular stability. Several investigators have determined that the closed disulfide bond does contribute at least some degree of conformational stabilization but that its disruption has only minimal effects on the overall stability and activity of the molecule (54) (see below).

SEs Are SAgs and Belong to a Large PT Family

In discussing the genetics and evolution of the SEs, one must also consider other staphylococcal toxins, plus some toxins produced by other bacteria, especially group A streptococci. SEs are part of a large family of related PTs produced by *S. aureus* and *Streptococcus pyogenes* (55). Members of this family are grouped together based on shared biological and biochemical properties. The one feature common to all PTs, including SEs, is their unique ability to act as SAgs (56). SAgs are molecules that stimulate an exceptionally high percentage of T cells. The mechanism by which this occurs distinguishes them from mitogens and conventional antigens (Ags). Regarding T-cell stimulation, SAgs are bifunctional

molecules that interact with major histocompatibility complex class II (MHCII) molecules on Ag-presenting cells (APCs). Unlike with conventional Ags, this interaction does not require processing and occurs outside of the MHCII peptide-binding groove (Fig. 21.3). The MHCIISAg complex interacts with the T-cell receptor (TCR). The interaction with the TCR is also nonconventional and relatively nonspecific; for most SAgs, binding occurs at a variable location on the TCR β chain (the Vβ region). Since SAgs bind outside the area on the TCR used for Ag recognition, they activate a much higher percentage of T cells than can be activated by conventional Ags. However, compared to mitogens, which stimulate T cells in an indiscriminate manner, there is some degree of specificity in SAg action, because only certain Vβ (or rarely Vα) sequences are recognized. Hence, not all T cells are stimulated. The binding of SAgs with the TCRCD3 complex activates the Src family of protein kinases, including Lck and ZAP-70, which subsequently activates phospholipase C gamma, resulting in the activation of diacylglycerol and protein kinase C (57, 58). Protein kinase C activates downstream signaling pathways, including the mitogen-activated protein kinase, NF-κB, p38, extracellular signal-regulated kinase, and Jun N-terminal protein kinase, which induce expression of many proinflammatory cytokines and chemokines, such as interleukin 1 (IL-1), tumor necrosis factor alpha, gamma interferon, IL-2, IL-6, and monocyte chemoattractant protein 1 (59). Recent studies revealed that SAgs also directly bind to CD28, thus activating phosphatidylinositol 3-kinase–Akt–mTOR pathways, which modulate many biological processes, including cell growth, differentiation, proliferation, survival, migration, and metabolism (60–62).

The staphylococcal and streptococcal PTs are prototype microbial SAgs that exert a variety of immunomodulatory effects leading to shock, immunosuppression, and other systemic abnormalities associated with TSS. While SEs are included with the PTs, they have the unique distinction of possessing an additional ability to induce an emetic response upon oral ingestion and are thus solely responsible for SFP. It is generally agreed that many of the toxins in this family, including members of the SE family, arose from a common ancestral gene which crossed the genus barrier and became stably introduced in both the genera *Staphylococcus* and *Streptococcus*. This idea is strongly supported by the observation that the structural genes for some SEs and related streptococcal PTs are carried on discrete genetic elements (see below).

Three-Dimensional Structure

Despite their sequence diversity with only partial sequence conservation, structural studies revealed that threedimensional topology of the SEs is remarkably similar (63). The molecule has an overall ellipsoidal shape and is folded into two domains of amino- and carboxy-terminal domains which are connected by α-helices in the center (Fig. 21.4). The amino-terminal domain, domain 1, contains residues near the N terminus but not the N-terminal residues themselves. The folding conformation of this domain may have potential significance for the function of the toxin. Its topology, in which a β-barrel structure is capped at one end by an α-helix, is known as the oligonucleotide/oligosaccharide-binding (OB) fold. The internal portion of its β-barrel is rich in hydrophobic residues, and the potential oligomer binding surface is covered with mainly hydrophilic residues. This OB folding pattern is found in staphylococcal nuclease and the B subunit of AB_5 heat-labile enterotoxins, such as cholera toxin, pertussis toxin, and Shiga toxin (64), which share the feature of exerting their activity by interacting with either oligosaccharides or oligonucleotides. The other prominent feature of domain 1 is that in most toxins of this family it contains two cysteine residues responsible for forming the disulfide linkage characteristic of most SEs. This bond and the cysteine loop are located at the end of the domain opposite its α-helix cap. Crystallographic data for SEA, SEB, and SEC3 indicate that the loop regions of all three toxins are quite flexible (63, 65, 66).

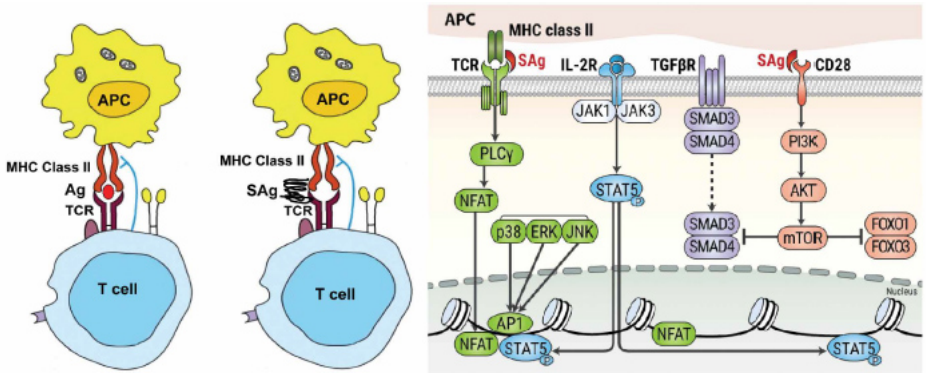


Figure 21.3 Interactions between APCs and T cells facilitated by conventional Ags and SAgs. The binding of SAg with TCR-MHCII triggers activation of signaling cascades through Lck-dependent and Lck-independent pathways, leading to the activation of transcriptional factors that induce T-cell proliferation, cytokine production, and proinflammation. TGFβR, transforming growth factor beta receptor; PLCγ, phospholipase Cγ; ERK, extracellular signal-regulated kinase; JNK, Jun N-terminal protein kinase; NFAT, nuclear factor of activated T cells; PI3K, phosphatidylinositol 3-kinase; mTOR, the mechanistic target of rapamycin; AKT, protein kinase B.

The larger carboxy-terminal domain (domain 2) can be described as a five-strand antiparallel β-sheet wall overlaid with a group of α-helices forming a β-grasp motif. It has structural similarity to the immunoglobulin-binding motifs of streptococcal proteins G and L, ubiquitin, $2F_2$-2S ferredoxin, and the Ras-binding domains of the Ser/Thr-specific protein kinase Raf-1 (67, 68). The structural similarities among SEs and other bacterial proteins suggest that SEs evolved through the recombination of these proteins (69).

Zinc Binding

Biochemical and structural studies on SEs have revealed that some SEs are dependent on zinc ions to be functional and to be able to properly bind MHCII. Fraser et al. (70) determined that SEA and SEE bind zinc via a single site, with a dissociation constant of 1 to 2 μM. The binding site was subsequently predicted by mutagenesis of SEA to be composed of a nonlinear stretch of residues (H187, H225, and D227) in the concave β-sheet within the C-terminal domain and an N-terminal serine in the metal coordination (Fig. 21.4) (66). Crystallographic analysis of SEA crystals soaked in 10 mM $ZnCl_2$ revealed a second zinc-binding site, which is similar to the SEC2 site (see below). It is coordinated by D86 and H114 (corresponding to D83 and H118 in SEC2), a water molecule replaces the second histidine residue in SEC2 (H122), and E39 is analogous to D9 in SEC2 (Fig. 21.4) (71).

SEC2 and SEC3 bind zinc through a low-affinity mechanism (65, 72). The zinc ion is tetrahedrally coordinated by D83, H118, and H122 from one molecule and D9 from a neighboring molecule. The zinc-binding site is located in a classical motif (H-E-X-X-H) at the cleft in the α5 groove of SEC3 between the two domains. This zincbinding motif is typically in the catalytic site of metalloenzymes such as thermolysin (73) and in certain other bacterial protease-dependent toxins, such as botulinum and tetanus neurotoxins (74). However, none of the SEs is known to possess protease activity. Although the function of zinc in SEC is not certain, it has been suggested that it plays a minor role in stabilizing the toxin structure.

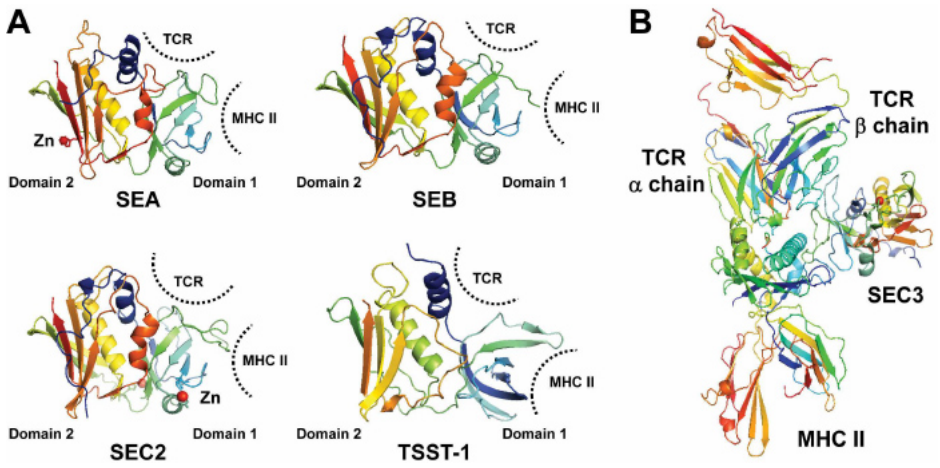


Figure 21.4 (A) Schematic diagrams of SEA, SEB, SEC2, and TSST-1 crystal structures. Domain 1 contains highly conserved, low-affinity binding sites for TCR Vβ and MHCII at generic sites. (B) Hypothetical model of the MHCII-SEC3-TCR complex based on modeling predicted from the crystal structures of the SEC3-HLA-DR1 and SEC3-Vβ complexes.

Crystallographic studies revealed that SED binds two zinc ions and forms a homodimer in a zinc-dependent manner (75). One zinc ion is tetrahedrally coordinated by D182, H220, and D222 from one molecule and H218 from the other molecule, which allows it to bind both MHCII α and β chains (see below). The second zincbinding site is similar to that in SEC2.

Binding to MHCII Molecules

Despite their structural similarities, the mechanisms by which various SEs bind MHCII are diverse. SEs interact with MHCII in three ways: (i) binding a single α chain of MHCII, (ii) binding a single β chain of MHCII, or (iii) cross-linking two MHCII molecules.

The first type of SE binding is to the α chain of MHCII. SEB, SEC1, SEC2, SEC3, and TSST-1 bind in this manner and share a common MHCII-binding site at domain 1 (referred to as the generic site), with a comparatively low affinity (0.4 to 0.7 μM) (Fig. 21.4). The crystal structure of the SEB-HLA-DR1 complex revealed that one major interaction is mediated by a salt bridge provided by Glu67 of SEB and Lys39 on HLA-DR1 (76). Although SEC2 and SEC3 are able to bind zinc, crystal structure data and site-directed mutagenesis studies revealed that the zinc ion is not involved in the MHCII interaction (77).

The second type of interaction is with the β chain of MHCII. A crystal structure of SEH complexed with HLA-DR1 revealed that toxin interacts with the β chain of the receptor, and binding requires its bound zinc ion (78). The cross-linking of the zinc ion plays an important role in the interaction of SEH and HLA-DR1 by providing a cross-link through His81 on HLA-DR1 and through His206 and Asp208 on SEH.

The third type of interaction involves SE cross-linking of two MHCII molecules. SEA has a low-affinity MHCII α-chain-binding site which overlaps that of SEB and TSST-1, plus a high-affinity site on the outside of domain 2 near the N terminus (79). The SEA highaffinity site involves coordination of zinc through three toxin residues (His187, His225, and Asp227) and His81 of the MHCII β chain. Two MHCII-binding sites per toxin allow two MHCII molecules to be cross-linked (80) (Fig. 21.4). Similar modes of action, including formation of homodimers by some toxins, have been proposed for other SEs with high-affinity zinc-binding sites on the outer face of domain 2 (SED and SEE) (75, 81).

Binding to TCRs

SEs and other SAgs interact with a characteristic repertoire of TCR sequences (Table 21.3). The TCR specificity of each SE is determined by toxin residues in the shallow cavity at the top of the molecule (82). Crystallographic analysis of a molecular complex between SEC3 and a portion of the murine TCR

β chain (83) revealed that the toxin binds to the CDR1, CDR2, and HV4 loops of Vβ. Modeling permitted the deduction of a trimolecular complex model containing the SAg bridging the T cell and APC. Using SEC3 and SEB, this model shows that these toxins form a "wedge" between the TCR and the MHCII, orienting the peptide-binding cleft away from the TCR (Fig. 21.4B). Although different SAgs are predicted to bind with variations of this theme, in general, SAg-binding results in receptor interactions significantly different from that in typical Ag presentation. The overall affinity of the entire complex determines the effectiveness of the stimulation (84), and binding by toxins with low affinity for the TCR can be compensated for by stronger binding to MHCII, and vice versa. SEH appears to have the most divergent mechanism of binding and T-cell stimulation. This toxin binds to MHCII with a mechanism that does not allow efficient interactions between SEH and the TCR Vβ, or between MHCII and Vβ. In one study (85), SEH stimulated T cells by interacting with TCR Vα (Vα10), and Vβ-specific expansion was not observed.

SE Mode of Action in Induction of Emesis and Related Symptoms in SFP

Molecular Regions of SEs Responsible for Enterotoxicity

The structural aspects of SEs that enable them to survive degradation by pepsin and other enzymes in the gastrointestinal tract are required for the toxins to induce SFP. However, stability alone is not sufficient. SEs must also be able to interact with the appropriate target, leading to emesis, diarrhea, and other gastrointestinal tract symptoms. Initial attempts to define molecular regions responsible for enterotoxicity involved testing biological activity of protease-generated fragments derived from SEA, SEB, or SEC1 (52). Three main conclusions were drawn from this work. First, only large toxin fragments containing central and C-terminal portions of the SEs retained enough of the native structure to cause emesis. Second, N-terminal residues of the SEs were not required for emesis. A form of SEC1 with the 59 N-terminal residues removed retains the ability to cause emesis in monkeys. Smaller toxin fragments from SEC1 and other SEs were inactive. The third conclusion was that emesis seemed to require preservation of structure in the conserved SE disulfide loop.

The disulfide bond is a structural feature that is characteristic of most, but not all, SEs. The disulfide bond has long been associated with emesis and is not present uniformly in nonemetic staphylococcal and streptococcal exotoxins. With a few known exceptions, SEs contain exactly two cysteine residues which could potentially form an intramolecular linkage and a spacer disulfide loop. SEG and the SEC molecular variant produced by isolates from sheep (SEC-ovine) deviate from this pattern and possess an additional third cysteine (Fig. 21.5). There is speculation regarding the importance in emesis of the disulfide bond, which is located near the center of every classical SE and most other SEs. Data from analyses of recently identified SEs and SEl proteins in the monkey feeding assay suggest that a stable disulfide bond facilitates an emetic response *in vivo*. Specifically, toxins lacking one or both cysteine residues are generally nonemetic or weakly emetic (86–91). Potential structural contributions from the Cys-Cys bond that could contribute to the SE conformation necessary for emetic activity include one or more of the following features: (i) proper positioning of cysteine residues upon formation of the disulfide bond, (ii) exposure and/orientation of crucial residues in the loop formed between the two linked cysteines, (iii) exposure and/or orientation of residues immediately adjacent to the disulfide linkage but not contained within the cysteine loop, and (iv) contributions to the overall SE conformation by the linkage of the two cysteine residues.

Each of these possibilities has been considered. The cysteine residues and the loop probably do not play a direct role in the emetic response. It has been found to be possible to replace the cysteine residues in several SEs by site-directed mutagenesis and show that neither of these two residues is required. Although most of the SEs have a cysteine loop or analogous feature, the lengths and composition of residues within the loops of different SE types vary greatly. This lack of consistency among SE loop properties suggests that they are unlikely to have a shared enterotoxigenic function (Fig. 21.5). Furthermore, proteolytic nicking of toxins in their loops has no effect on their ability to cause emesis (52). Warren et al. (92) determined that the disulfide bond contributes only minimally to overall protein conformation. Presumably, then, if the disulfide linkage is important in emesis, the

effect is likely to provide a specific orientation of residues near the disulfide linkage, but not in the loop. The most convincing evidence in support of this possibility has been provided by mutagenesis of SEC1 in which its cysteine residues were replaced by either serine or alanine (54). It was determined that mutants with serine substitutions were emetic, whereas the analogous mutants with alanine substitutions were nonemetic. Although serine and alanine are both considered conservative substitutions for cysteine, one difference between them is that serine can form hydrogen bonds. Thus, hydrogen bonding by serine may be able to replace the disulfide linkage stabilization of local structure.

	Conserved Cysteine loop	Downstream
Highly emetic		
SEA	CAGGTPNK----------TAC	MYGGVTLH
SEB	CYFSKKTNDINSHQTDKRKTC	MYGGVTEH
SEC1	CYFSSKD---NVGKVTGGKTC	MYGGITKH
SEG	CIIPKSE---PDINQNFGGCC	MYGGLTFN
SER	CLVPKNE---SREEFIFDGVC	IYGGVTMH
SET	PIG----------FVIKF	YDGDISKH
Weakly emetic		
SEI	CKS----------KY	MYGGATLS
SEK	CNT----------KY	IUGGVTAT
SEM	CLKN----------KY	IYGGVTLA
SEN	CVGLTEEK----------TSC	LYGGVTIH
SEO	CHGEHQVD----------TAC	TYGGVTPH
SEQ	CNT----------KY	MYGGITLA

Figure 21.5 Comparison of the cysteine loop and adjacent sequences between highly emetic and weakly emetic SEs in the monkey feeding assay.

Which critical local residues require proper orientation by the disulfide bond (or hydrogen bonding at the same positions) for the SEs to induce emesis is unknown. Possible candidates are those within a highly conserved stretch of residues directly adjacent to, and downstream from, the disulfide loop (Fig. 21.5). In SEC3, these residues are located on the β5 strand. An attractive hypothesis is that in addition to stability in the gut, two other structural requirements need to be met for enterotoxicity. First, the appropriate conserved residues must be present in the toxin. Many PTs, emetic and nonemetic, have a similar set of highly conserved residues in a location analogous to the β5 strand of SEC3. Second, they must be positioned properly for interacting with their target in the gut. The unique SE disulfide bond may serve this function. Of the entire PT family, two non-SE toxins produced by *Streptococcus pyogenes* (streptococcal pyrogenic exotoxin and streptococcal SAg) could potentially form a disulfide linkage (93). However, the presence of more than two cysteines in these toxins suggests that the structure in this area and degree of local stabilization by their putative disulfide linkage may not be identical to those of the SEs.

Additional studies on SEA revealed that single-site substitutions of residues close to the N terminus also influence emesis (94). This was especially the case for mutants constructed by substitution with glycine. For example, mutagenesis of residues 25, 47, and 48 cause significant reduction in the emetic potency of SEA. Although these residues are far from the disulfide bond in the SEA primary sequence, they are located near or within domain 1 of SEA and could potentially influence the area near the disulfide bond.

Recently, the emetic activity of newly discovered SEs has been determined in monkey feeding assays using the cynomolgus monkey (*Macaca fascicularis*) (95). All of the SEs tested were emetic but required a very high dose (100 μg/kg of body weight). Some SEs induced emesis in one of six animals tested. It is noteworthy that these SEs were prepared from *E. coli* protein expression systems. One of the prominent features of SEs is the enhancement of the toxic effect of lipopolysaccharide (96), which is very difficult to remove from proteins expressed by *E. coli*. Additionally, for the safety of animal handlers, animals may be sedated with ketamine-HCl. Previous studies revealed that 10 of 41 cynomolgus monkeys had an emetic response to an intramuscular injection of 2.5 to 20 mg/kg of ketamine-HCl (97), whereas the rhesus monkey (*Macaca mulatta*) did not have this response even at doses of 7.5 to 15 mg/kg, and sedation did not inhibit the emetic activity of subsequent oral administration of SEB (98). The SEs have the potential to be used as biological weapons; hence, the

SEs with confirmed emesis are classified as select agents (99). Thus, the results from recent cynomolgus monkey feeding assays need to be carefully interpreted.

SE-Induced Emesis Requires Nerve Stimulation

Except for rare SFP cases, in which massive doses of SEs are consumed, systemic dissemination of the toxins does not contribute significantly to illness. When fed to rodents, SEs do enter the circulatory system but are rapidly removed by the kidneys (100). Most studies using the simian model indicate that the SE site of action following ingestion is the abdominal viscera. Early studies into the mechanism of action of SEs tested the emetic responsiveness of animals to the toxins after disruption of well-defined neural systems or after visceral deafferentation. The characteristic emetic response resulted from stimulation of local neural receptors in the abdomen (101), which transmit impulses through the vagus and sympathetic nerves, ultimately stimulating the medullary emetic center.

Cellular Histopathology in the Gastrointestinal Tract

Information on the histological effects of oral doses of SEs in humans is extremely limited. Most of it was obtained from experiments in rhesus monkeys. Upon ingestion of the toxin, pathological changes compatible with a definition of gastroenteritis were observed in several parts of the gastrointestinal tract (102).

The primate stomach becomes hyperemic and is marked by lesions which begin with the influx of neutrophils into the lamina propria and epithelium. A mucopurulent exudate in the gastric lumen is typically observed. Also characteristic are mucus-filled surface cells, which eventually release their contents. Later in the illness, neutrophils are replaced by macrophages. Eventually, upon resolution of the symptoms, the cellular infiltrate clears.

A similar cellular infiltrate and lumen exudate occur in the small intestine, although they are less severe in sections from lower portions of the intestine than in those from upper portions. Clearly evident in the jejunum are an extension of crypts, disruption or loss of the brush border, and an extensive infiltration of neutrophils and macrophages into the lamina propria. Changes in the colon are minimal in the monkey model; only a mild cellular exudate and mucus depletion are evident. The only other significant effect in monkeys is acute lymphadenitis in the mesenteric lymph nodes.

Search for the Gastrointestinal Tract Target

The specific cells with which SEs interact in the abdomen have not been clearly identified, nor has their receptor. Evidence suggests that interaction of SEs with their target directly or indirectly causes production of inflammatory mediators that induce SFP symptoms. Jett et al. (103) determined that oral administration of SEB produced elevated levels of arachidonic acid cascade products. Specifically, they observed significant increases in prostaglandin E_2, leukotriene B4, and 5-hydroxyeicosatetraenoic acid. These three compounds are potent vasoactive inflammatory mediators that can also act as chemoattractants for neutrophils. Both of these activities are consistent with the histopathology described above for the SFP monkey model.

Scheuber et al. (104) could not demonstrate an effect of prostanoid inhibitors on SEB-induced emesis but were able to correlate gastrointestinal symptoms with cysteinyl leukotriene generation. Intoxication of animals with SEB resulted in a 10-fold increase in the levels of leukotriene E4 in bile, plus an unidentified leukotriene in the urine. These investigators suggested a role for mast cells in the pathogenesis of SFP. Although induction of histamine production by SEB was responsible for some secondary nonenteric immediate hypersensitivity skin reactions, it did not correlate with emesis.

Evidence suggests that ingestion of SEs causes a stimulation of mast cells and possibly other inflammatory cells in the abdomen. Thus far, the abdominal receptor has not been identified. Experiments using anti-idiotype antibodies in binding assays and protection assays have provided circumstantial evidence for its existence on monkey mast cells (105). Komisar et al. determined that SEB stimulates rodent peritoneal mast cells as well and provided evidence for a protein receptor (106).

It is possible that SEs act directly on their receptor and circumvent the typical two-stage mast cell IgE antibody-Ag interaction (104).

Despite these observations, Alber et al. (107) were unable to directly stimulate monkey or human skin and intestinal mast cells with SEB to release inflammatory mediators. It was suggested that stimulation of mast cells *in vivo* occurs through a nonimmunological mechanism requiring the generation of neuropeptides released from peripheral terminals of primary sensory nerves. At least one putative mast cell-stimulating peptide, substance P, was implicated in SEB-induced toxicity by use of antibodies and a variety of inhibitors. This explanation is consistent with earlier predictions of a neural involvement in the pathogenesis of SFP.

A recent study using the house musk shrew (*Suncus murinus*) revealed that SEA induces 5-hydroxytryptamine (5-HT) release in the intestine (108). Pretreatment with a 5-HT synthesis inhibitor or a 5-HT_3 receptor, or with surgical vagatomy, inhibited SEA-induced emesis. Furthermore, cannabinoid receptor agonists significantly decreased the release of 5-HT and SEA-induced emesis. These results suggested that, in this model, SEA-induced emesis is mediated by the release of 5-HT and 5-HT_3 receptors on vagal afferent neurons and is downregulated by the cannabinoid receptor system by decreasing the release of 5-HT. However, results with other animal models, including kittens, piglets, house musk shrews, and ferrets, particularly those that require systemic administration rather than oral feeding, must be interpreted with caution, since they may mimic TSS rather than SFP.

Is There a Relationship Between Superantigenicity, TSS, and SFP?

The discovery of the mechanism of SAg action and the unique properties of this class of proteins provided an explanation for the multiple systemic effects observed in TSS patients. The massive cellular stimulation induced by superantigenic PTs explained the long-recognized fact that TSS patients had elevated levels of serum cytokines and other factors which mediate many symptoms of the disease. The realization that at least some of the pathogenesis of TSS could be attributed to immune cell stimulation led to the prediction by some investigators that SFP could also be a function of SAgs. Consistent with this prediction was the fact that TSS often has a gastrointestinal tract component characterized by vomiting and diarrhea. Also, patients with endotoxin shock have elevated cytokine levels and similarly display vomiting and diarrhea. If superantigenicity is responsible for SFP, the SEs presumably act directly on T cells and APCs in the gut. Although some SEs enter the circulation, they appear to be rapidly cleared by the kidneys, so that significant systemic concentrations are unlikely to be achieved (100). This, plus the fact that TSS-associated symptoms (i.e., shock and fever) are not observed in SFP patients, suggests that SEs do not mediate SFP through systemic cytokines.

Despite their similarities and the evidence cited above, several lines of evidence suggest that the partial overlap between SFP and TSS symptoms is likely coincidental and that superantigenicity is not directly responsible for SFP. First, as discussed above, nonimmunological mast cell stimulation has been linked to the release of the inflammatory mediator affecting nerve interactions. The second line of evidence was obtained via mutagenesis of several SEs. Studies have revealed that T-cell stimulation and induction of emesis are separable functions and are determined by distinct portions of the SE molecules (54, 94, 109). It has been possible to construct SEA, SEB, or SEC1 mutants which are deficient in T-cell-stimulatory activity but which retain the ability to induce emesis and vice versa. Finally, although all PTs have been reported to have SAg function, only the SEs are emetic when ingested. The lack of emesis-inducing ability of some nonenterotoxic PTs has been attributed to instability in the gastrointestinal tract. However, at least one nonemetic PT, streptococcal pyrogenic exotoxin A, is very stable in gastric fluid (110).

If superantigenicity does not explain SFP, how do PTs cause vomiting and diarrhea in TSS? Several possibilities could explain how SEs and other PTs act on the gastrointestinal tract if they are not consumed by the oral route. First, the toxins' ability to induce TSS symptoms may be limited to the systemic circulation. If so, cytokines would need to enter the abdomen or gain access to the central nervous system from the circulatory system and mediate gastroenteritis pathogenesis. Alternatively, the PTs could enter the gut from the circulation and act directly at the local level as SAgs or through

other mechanisms, as proposed for SFP. The latter possibility is less likely, since even nonenterotoxic PTs, including those that are susceptible to degradation in the gut, are known to cause gastrointestinal symptoms when they are associated with TSS.

These complex and unresolved issues are relevant to interpreting models for studying the enterotoxic activity of SEs. Oral administration of SEs, culture filtrates, or suspected food to monkeys is the preferred method for detecting enterotoxic properties of the SEs. However, intravenous administration of SEs to primates and other animals such as cats has been used as an alternative to feeding (89, 111). Considering that systemic exposure to SEs mimics the situation leading to TSS symptoms, intravenous administration may not accurately reflect the pathogenesis and toxin properties required to induce emesis in SFP. Recently, other models that demonstrate emesis following administration of toxins to shrews and piglets have been proposed as alternatives to the gold standard, the monkey feeding assay (86, 112). Additional studies will be required to determine whether these alternatives can replace the primate feeding assay.

SE Antigenic Epitopes

The need for reagents that can detect SEs in food and clinical samples, plus a desire to differentiate antigenically different toxins in the toxin family, has been the impetus for considerable effort directed toward epitope characterization and mapping. Although the increased number of identified SEs and putative SEs is beginning to make immunological detection of SEs obsolete, several commercial reagents relying on this technique are still widely used. However, detection based on antigenicity is gradually being replaced by molecular techniques, especially multiplex PCR (113, 114).

Individually, each classical SE type and subtype has a sufficient degree of antigenic distinctness to allow its differentiation from other PTs using highly specific polyclonal antisera and monoclonal antibodies. However, some degree of cross-reactivity can often occur between several SEs. The level of cross-reactivity generally correlates with shared primary sequences. The type C SE subtypes and their molecular variants exhibit a substantial amount of cross-reactivity, as do SEA and SEE, the two major serological types with the greatest sequence relatedness. For these toxins, cross-reactivity can even be demonstrated in relatively insensitive assays, such as immunodiffusion assays, in which these two toxins produce lines of partial identity with the heterologous antiserum. SEB and the SEC subtypes and molecular variants (see above) are also closely related at the amino acid level. Cross-reactivity between SEB and SEC may be demonstrated occasionally by immunodiffusion but is shown more consistently by using sensitive methods, such as radioimmunoassays or immunoblotting. Generally, it has not been possible to produce useful antibodies that cross-react among less closely related SEs. Although one investigator has produced a monoclonal antibody that cross-reacts with all five major SE antigenic types (SEA through SEE), this antibody has low affinity and cross-reacts with other staphylococcal proteins (115). The two most distantly related SEs determined to be recognized by a common epitope are SEA and SED (22).

The mapping of conserved and specific antigenic epitopes on SEs and their differentiation from potentially toxic regions have potential applications in the rational development of nontoxic vaccines. Considering the array of SE antigenic types, the most efficient toxoid would presumably contain one or more epitopes that are shared by multiple toxins. Several approaches have been used to partially localize antigenic epitopes on the SEs and differentiate them from toxic regions. One of the earlier methods used for this purpose was to identify protease-generated toxin fragments from several SEs that bind to cross-reactive antibodies (116). These studies revealed that both N- and C-terminal toxin fragments contain cross-reactive epitopes, but they were unable to define shorter stretches of residues.

There is some evidence that immunization with short, highly conserved peptides could have merit. For example, the use of synthetic peptides from highly conserved stretches of primary sequence has resulted in the production of neutralizing antibody to several of the SEs. Immunization with synthetic peptides corresponding to residues 130 to 160 of SEB or the same region of SEC1 (residues 148 to 162) induced antibodies that neutralized both native toxins (35, 103). The highly conserved SE sequence K-K-X-V-T-X-Q-E-L-D (Fig. 21.1), encompassed by both peptides, may represent part of an epitope that

could be useful for protective immunity. It is yet to be determined if major epitopes identified on other toxins, such as SEA, also show promise (117).

FOODBORNE ILLNESS OUTBREAKS

Incidence of SFP

SFP occurs as either isolated cases or outbreaks affecting many people. Since SFP is usually self-limiting, the incentive to report cases is not as great as for other foodborne illnesses. Although there is a national surveillance system for SFP, SFP is not an officially reportable disease. It has been estimated that only 1 to 5% of all SFP cases are reported in the United States, usually at the state health department level. Most of these occur during highly publicized outbreaks. Isolated cases occurring in the home are not usually reported. Staphylococci account for an estimated 14% of the total of foodborne illness outbreaks within the United States (118). According to a study conducted by the Economic Research Service of the U.S. Department of Agriculture, more than an estimated 1.5 million cases of SFP occurred in a single year (1993), resulting in 1,210 deaths and a cost of $1.2 billion (119).

According to the Centers for Disease Control and Prevention (CDC), an average of approximately 20 major outbreaks of SFP are reported annually within the United States (https://www.cdc.gov/foodsafety/outbreaks/index.html). The occurrence of SFP is cyclical, with the highest incidence typically occurring in the late summer, when temperatures are warm and food is more likely to be stored improperly (54). A second peak occurs in November and December. Approximately one-third of these are associated with leftover holiday food.

SFP may be a leading cause of foodborne illness worldwide, although reporting in other countries is even less complete than in the United States. In one study, 40% of outbreaks of foodborne gastroenteritis in Hungary were due to SFP (1). In Brazil, *S. aureus* was the second most common cause of foodborne disease between 1999 and 2011 (120). The percentage is slightly lower in Japan (approximately 20 to 25%), where contamination of rice balls during preparation is a potential problem (1). Outbreaks due to improper manufacturing of canned corned beef have been reported in England, Argentina, Malta, northern Europe, and Australia. Cases in Great Britain have been attributed to contaminated milk and cheese produced from milk from mastitic sheep (121). In some countries, ice cream has been a major vehicle of SFP. Despite increased knowledge of the epidemiology of SFP and attempts to educate the public, large outbreaks continue to represent a significant health hazard. For example, in 1998, approximately 4,000 individuals acquired SFP at a church event in Brazil (122). More recently, a massive outbreak in Osaka, Japan, in 2000 resulted in more than 10,000 cases from SEA and SEH in reconstituted milk (123).

Characteristics of a Recent Large Typical SFP Outbreak

The following is a summary of an outbreak of SFP reported by the U.S. Food and Drug Administration (124). Many of the aspects of this outbreak, such as type of food involved, mechanism of contamination, inadequate safety measures in food handling, and clinical manifestations of illness, are typical. This outbreak originated from one meal that was fed to 5,824 elementary school children at 16 sites in Texas. Of all the children exposed, a total of 1,364 developed clinical signs of SFP. Investigation into the source of the illness revealed that 95% of the children who became ill had eaten chicken salad from which large numbers of *S. aureus* were cultured.

The series of events leading up to the outbreak were as follows. Preparation of the meals was performed in a centralized kitchen facility and began on the day before the food was to be eaten. Frozen chickens used for the salad were boiled for 3 hours. After cooking, the chickens were deboned, cooled to room temperature with a fan, ground into small pieces, placed in 30.5-cm-deep aluminum pans, and stored overnight in a walk-in refrigerator at 5.6 to 7.2°C. The following morning, the remaining ingredients of the salad were added and the mixture was blended with an electric mixer. The food was placed in thermal containers and transported by truck to the various schools between 9:30 and 10:30 a.m. It was held at room temperature until served between 11:30 a.m. and noon. It is believed that the

chicken became contaminated after cooking when it was deboned. Most likely, the storage of the warm chicken in the deep aluminum pans did not permit rapid cooling and provided an environment favorable for staphylococcal growth and SE production. Further growth of the bacteria probably occurred during the period when the food was kept in the warm classrooms. Prevention of the incident would have entailed more rapid cooling of the chicken and refrigeration of the salad after preparation.

CHARACTERISTICS OF DISEASE IN HUMANS

SFP is usually a self-limiting illness presenting with emesis following a short incubation period. However, vomiting is not the only symptom that is commonly observed. In fact, many patients with SFP do not vomit. Other common symptoms include nausea, abdominal cramps, diarrhea, headaches, muscular cramping, and/or prostration. In a summary of clinical symptoms involving 2,992 patients diagnosed with SFP, 82% complained of vomiting, 74% felt nauseated, 68% had diarrhea, and 64% exhibited abdominal pain (118). In all cases of diarrhea, vomiting was always present. Diarrhea is usually watery but may contain blood as well. The absence of high fever is consistent with a lack of infection or significant toxemia in this type of food poisoning, although some patients present with low-grade fever. Other symptoms can include general weakness, dizziness, chills, and perspiration.

Symptoms typically develop within 6 hours after ingestion of contaminated food. In one report, 75% of the exposed individuals exhibited symptoms of SFP within 6 to 10 hours postingestion (118). The mean incubation rate is 4.4 hours, although incubation periods as short as 1 hour have been reported. In another outbreak, symptoms lasted for 1 to 88 hours, with a mean of 26.3 hours. Death, usually from severe dehydration or electrolyte imbalance, occurs in a small percentage of patients, with the fatality rate ranging from 0.03% for the general public to 4.4% for more susceptible populations, such as children and the elderly (118). Approximately 10% of patients with confirmed SFP seek medical attention. Treatment is minimal in most cases, although administration of fluids is indicated when diarrhea and vomiting are severe.

TOXIC DOSE AND SUSCEPTIBLE POPULATIONS

Numbers of Staphylococci Required for Sufficient Toxin Production

Since many variables affect the amount of SE produced, one cannot determine with certainty the number of *S. aureus* organisms in food required to produce sufficient amounts of SE to cause SFP. Factors contributing to the amounts of toxin produced have been extensively studied and include environmental conditions (such as food composition and temperature), other physical and chemical parameters, and the presence of antimicrobial growth inhibitors. In addition, bacterial factors to be considered include potential differences in the types, amounts, and numbers of different SEs the strain involved has the physiological ability to produce. It is likely that these combined conditions are unique for each isolated case or outbreak of SFP. Despite this variability, there are several general guidelines that are useful for assessing general risk. According to the U.S. Food and Drug Administration, doses of SE causing illness result when populations greater than 10^5 *S. aureus* organisms per gram of contaminated food are present (124). In other studies, 10^5 to 10^8 *S. aureus* organisms were observed to represent the typical range, despite the fact that lower levels were sometimes implicated (118).

Toxin Dose Required for Illness

Many investigations have been conducted to assess SE potency and the amount of toxin in food required to initiate SFP symptoms. Some of the most useful information has come from analysis of food recovered from outbreaks of the illness. Although SEs are quite potent, the amount of SE required to induce symptoms is relatively large compared to that of many other exotoxins that are acquired through contaminated food. A basal level of approximately 1 ng of SE per gram of contaminated food is sufficient to cause symptoms associated with SFP. Although 1 to 5 μg of ingested toxin is typically associated with many outbreaks, the actual levels of detectable SE were considerably less (<0.01 μg) in

16 SFP outbreaks (125). One of the most useful studies for predicting the minimal oral dose of SE required to induce SFP in humans was a well-documented investigation of an outbreak associated with ingestion of contaminated chocolate milk (126). In that study, the minimum dose of SEA required to cause SFP in school children was 144 ± 50 ng. Another massive SFP outbreak in Japan caused by low-fat milk contaminated with SEA revealed that the total intake of SEA per capita was 20 to 100 ng (127).

The minimal number of *S. aureus* organisms required to produce the toxic level of SE in food has also been investigated. Noleto et al. (128) determined that an *S. aureus* strain harboring *sea*, *seb*, and *sed* produced detectable levels of SEB and SED (1 ng/ml) at a cell population of 6×10^6 CFU/ml and SEA (4 ng/ml) at a cell population of 3×10^7 CFU/ml under nutrient-enriched conditions. However, when grown in milk, this strain produced detectable levels of SEA and SED at populations as low as 10^4 and 10^7 CFU/ml, respectively. Considering the variability in strains, culture conditions, and genetic regulation of SE production, this information needs to be interpreted with caution.

Many factors contribute to the likelihood of developing SFP symptoms and to their severity. The most important include susceptibility of the individual to the toxin, the total amount of food ingested, and the overall health of the affected person. The toxin type may also influence the likelihood and severity of disease. Though SFP outbreaks attributed to ingestion of SEA are much more common, individuals exposed to SEB exhibit more severe symptoms. Of 2,291 individuals exposed to SEB, 46% experienced SFP symptoms severe enough for them to be admitted to the hospital (118). Only 5% of 1,813 individuals exposed to SEA required such treatment. It is possible that these observations reflect differences in levels of toxin present, because SEB is generally produced at higher concentrations than SEA.

Human volunteers and several species of macaque monkeys have been used to determine the minimal amount of purified SEs required to induce emesis when administered orally. Generally, monkeys are less susceptible to SE-induced enterotoxicity than humans. A study with purified SEs provided useful comparative information that has important research applications, but its direct relevance to SFP is uncertain, because potential stabilization of unpurified SEs by food is an important consideration. Based on a study in which human volunteers ingested partially purified toxin, Raj and Bergdoll estimated that 20 to 25 μg of SEB (0.4 μg/kg) is sufficient to cause vomiting in humans (129). In the rhesus monkey model, the 50% emetic dose is between 5 and 20 μg per animal (or approximately 1 μ4g/kg) when administered intragastrically. In our investigations, the minimal emetic dose of SEC1 for pigtail monkeys (*Macaca nemestrina*) is consistently between 0.1 and 1.0 μg/kg.

Regulation of Toxin Expression

S. aureus is a widely distributed bacterium. It can exist in harsh environments as well as on various animals and humans. This versatile survival ability relies on a complex network of virulence factors and adaptability. More than 50 genes involved in pathogenesis produce proteins that are either cell free or expressed on the bacterial surface. These enable the bacterium to evade host defenses, adhere to cells and the tissue matrix, propagate within the host, and destroy cells and tissues. Expression of virulence factors is temporarily controlled in response to cell density, nutrient availability, and environmental signals so that these factors are produced when required. During growth, expression of surface proteins is upregulated early, whereas that of cell-free secreted proteins is upregulated post-exponential growth phase. These changes in expression are regulated by a complex network of regulatory genes described below. In addition, several environmental signals also affect the production of extracellular proteins, such as salt concentration, pH, and subinhibitory concentrations of antibiotics or glycerol monolaurate (130).

Regulation of SE Production by a Global Regulatory System

SEs are produced in extremely low quantities throughout most of the exponential growth phase (131, 132). There is generally a large increase in expression during the late exponential or early stationary phase of growth, with SEA and SED accumulating somewhat earlier than other SEs (30). The ability of *S. aureus* to respond to changes in the growth phase involves the density-sensing *agr* system (133),

which has some properties typical of bacterial two-component sensors and regulators (134). *agr* expression coincides temporally with expression of most SEs during the bacterial growth cycle. All are maximally expressed during late-exponential-phase and postexponential-phase growth. SEA, which is not regulated by *agr*, is produced earlier (30). The *agr* locus maps at approximately 4 o'clock on the staphylococcal genome, near *purA*, *bla*, and *sea*, on the standard map of *S. aureus*. It contains two divergent operons separated by approximately 120 bp (Fig. 21.6).

Transcription can be initiated from three promoters (P1, P2, and P3). P1 is weakly constitutive and transcribes *agrA*. P2 and P3 are induced strongly during late exponential and early stationary phase but are only weakly expressed earlier. The P2 transcript, RNA II, encodes four proteins, designated AgrA, AgrB, AgrC, and AgrD.

A model for signal transduction through the *agr* system is shown in Fig. 21.4. Together, these four proteins form a quorum-sensing system, enabling the bacterium to respond to its environment. A unique cyclic thiolactone pheromone peptide (autoinducing peptide [AIP]) derived from AgrD residues 46 to 53 is the activating molecule for the *agr* system. AIP is processed from AgrD by AgrB (135). Increased levels of AIP are recognized by the membrane-bound receptor AgrC, which in turn initiates a signal transduction pathway (135). Its homology to conserved domains of histidine protein kinases, in particular, a conserved histidine autophosphorylation site (134), further suggests that the 423-residue *agrC* gene product is likely to be the sensor. Autophosphorylation of the *agrC* product at the end of the exponential phase is thought to allow it to phosphorylate AgrA, the response regulator, by phosphorylation of aspartic acid residue, thereby activating expression of both RNA II and RNA III (136). AgrA has homology to activator proteins in other systems, such as OmpR. AgrA and other typical response regulators exhibit a highly conserved N terminus approximately 120 residues in length. Three amino acids, two aspartic acids and a lysine, are conserved among all response regulators.

Mutations in the *agr* locus result in substantially reduced transcription of the *seb*, *sec*, and *sed* genes (25, 137, 138), whereas transcription of *sea* is not affected (139). Regulation of gene expression by *agr* can be transcriptional or translational. It regulates α-hemolysin at both the transcriptional and translational levels, whereas SEB and SEC are regulated at the transcriptional level. Transcriptional regulation of the *seb* and *sed* genes is dependent on the presence of Rot (repressor of toxin), which is a member of the Sar family of transcription factors. Rot negatively regulates expression of SEB by binding to the promoter of the *seb* gene (140). Even though the transcription of *rot* is not affected by *agr*, its activity is negatively regulated by *agr*. During the postexponential growth phase, the Agr system induces transcription of RNA III, which is base-paired with the *rot* mRNA and subsequently causes mRNA degradation, resulting in transcriptional activation of the *seb* gene (141). It is not clear whether transcription of *sec* is also regulated by Rot-RNA III interference (Fig. 21.6).

The SarA protein family is a family of proteins homologous to SarA. Included in this family are SarA homologs (SarA, -R, -S, -T, -U, and -V), MgrA (a global regulator of autolysis and virulence), and Rot (the repressor of toxin) (142–144). Sequence alignment and crystal structure studies revealed that SarA, -R, and -S are typical winged-helix DNA-binding proteins with helix-turn-helix motifs (145–147). SarA binds to several target promoters, including *agr*, *hla*, *spa*, and *fnbA*. SarA binds to an intergenic region between *agr* promoters P2 and P3, which upregulates expression of RNA III, hence influencing the expression of *agr*-regulated genes (148) (Fig. 21.6). Transcriptional gene fusion analysis revealed that *sarA* upregulates both *tst* and *seb* (149). Mutation of the *sar* locus causes increased expression of α toxin and decreased expression of δ and β toxins (150).

The two-component regulatory system SrrAB is involved in anaerobic growth and inhibits transcription of RNA III by binding to the P2 and P3 promoters (Fig. 21.6). The two-component regulatory system SaeRS is a positive effector of cell-free β- and α-hemolysins, coagulase, nuclease, and protein A but apparently does not affect SEA, protease, lipase, staphylokinase, or cell-bound protein A. A recent study revealed that mutations affecting SaeRS completely abolished expression of TSST-1, whereas there was no effect on the production of RNA III (133, 151). Furthermore, SaeR, a transcriptional regulator, was able to directly bind to a region of the *tst* promoter containing two SaeR binding sites (151) (Fig. 21.6).

These results suggest that the *sae* locus is distinct from other previously identified regulatory loci and that the presence of SaeR binding consensus sequence is important for its regulation.

Expression of the alternative sigma factor (σ^B) is the major response of *S. aureus* to environmental stimuli. The *sigB* operon is constitutively transcribed under the control of σ^A and encodes SigB (σ^B) and the anti-*sigB* factor RsbW, the antirepressor RsbV, and RsbU. RsbW usually binds to σ^B, which phosphorylates RsbV (152). Under environmental stresses such as high temperature, alkaline pH, high levels of NaCl, and the presence of certain catabolites (e.g., glucose, galactose, sucrose, glycerol, and maltose), phosphorylated RsbV is dephosphorylated by RsbU or RsbP and then binds RsbW, subsequently releasing and activating σ^B (30, 153). σ^B recognizes a unique promoter ($GTTTN_{14-17}GGGTAT$), which has been identified for 23 different *S. aureus* genes, including the *sar* locus (154). This suggests that environmental signals activate σ^B, which, in turn, activates global virulence regulation. SEB expression is negatively regulated by σ^B. When σ^B is activated by stress-induced conditions, expression of SEB is reduced (155). In contrast, expression of SEB is increased in a *sigB* mutant; however, the promoter of *seb* differs from the unique promoter for σ^B. This suggests that expression of SEB is not directly regulated by σ^B.

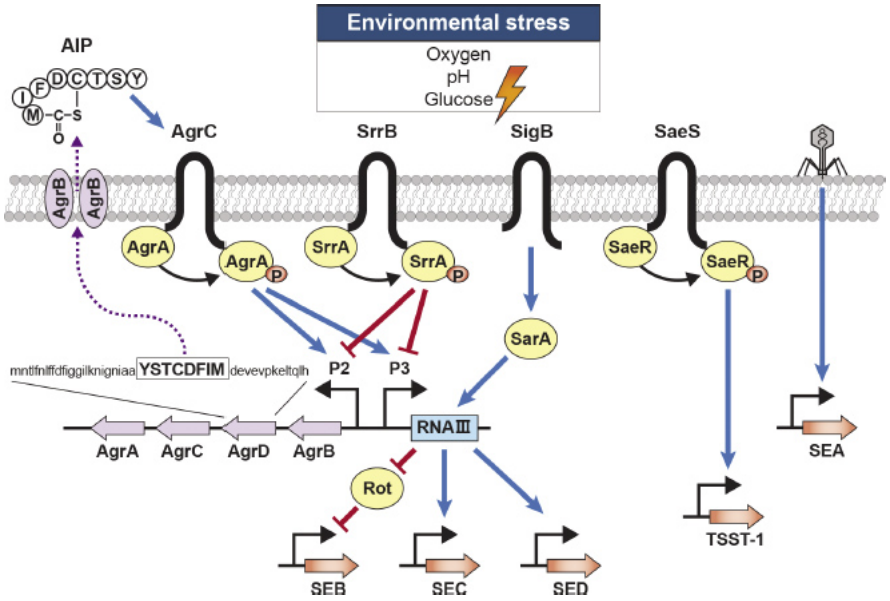


Figure 21.6 Regulation of SE expression by the *agr* locus and other interacting regulatory genes and gene products (not drawn to scale) in *S. aureus*. The blue arrow indicates activation, and the red line indicates inhibition. A letter "P" in a red dot indicates phosphorylation.

Regulation of SE Production by Environmental Factors

SFP is often associated with the growth of *S. aureus* in protein-rich foods, such as meats and dairy products, in which nutrients, salt, pH, oxygen availability, and temperature conditions are different from laboratory medium culture conditions.

Glucose affects expression of SEs through at least two different mechanisms. First, metabolism of glucose lowers pH, which negatively affects transcription of *agr*, because *agr* is maximally transcribed at neutral pH (138). Consequently, expression of *seb*, *sec*, and *sed* and other *agr* target genes is affected correspondingly (138). Second, low pH can cause induction of prophages which activate transcription of SEs associated with prophages. A recent study revealed that expression of SEA is linked to the life cycle of temperate phages (156). Sequence analysis of the *sea* gene revealed two promoters, one immediately upstream of the *sea* gene and the other at the phage-related latent promoter (Fig. 21.6). During stressinduced phage induction, both promoters are activated, resulting in the production of large amounts of SEA.

The regulatory effect of glucose described above cannot be attributed entirely to lower pH levels in cultures grown on glucose, because cultures containing glucose produce less SE, even when the pH is stably maintained. Although this effect has some attributes of catabolite repression, there are major differences between the glucose inhibitory effect in *S. aureus* and catabolite repression in *E. coli*. For example, inhibition by glucose cannot be reversed by adding cyclic AMP to staphylococcal cultures. The significance of these differences is still unclear. Even the catabolite-repressible staphylococcal *lac* operon has features that are significantly different from those of the analogous operon in *E. coli* and is apparently unresponsive to cyclic AMP.

While *S. aureus* can survive and grow in environments with low water activity, production of some SEs, especially SEB and SEC, is reduced when the bacteria are grown under osmotic stress. In experiments performed with SEC-producing strains, levels of *sec* mRNA and SEC protein are both reduced in response to high NaCl concentrations. However, addition of osmoprotectants reverses the effect. This reduced expression was also observed in *agr*$^{-}$strains (strains lacking *agr*), indicating that the signal transduction pathway used in this mechanism occurs by an alternative pathway.

Low concentrations of the commonly used emulsifier glycerol monolaurate (GML) inhibit transcription of many exoprotein genes, including *sea*, without inhibiting *S. aureus* growth (157). Inhibition of SE production is not associated with a simultaneous effect on *agr* transcription and occurs in *agr*$^{-}$strains as well as wild-type strains. These results, plus the finding that constitutive expression of some genes is not affected, suggest that GML interferes with non-*agr*-mediated signal transduction. It has been proposed that GML and a variety of related food additives exert this effect by insertion into the staphylococcal membrane, altering the membrane protein conformation and thereby interfering with signal transduction.

METHODS FOR DETECTING TOXINS

As *S. aureus* is a natural colonizer in both humans and animals, the incidence of SFP is typically associated with meats and dairy products. Approximately 2.1% of SFP reported between 1998 and 2012 in the United States was associated with dairy products (158). Surveys from 15 European countries revealed that 19% of SFP was linked to milk and dairy products (159, 160). In contrast, there was only one report of SFP associated with dairy products in Japan during the past 10 years. The most frequently detected SEs in SFP outbreaks are SEA, SED, SEH, SEE, and SEC. In the United States, 77.8% of all SFP outbreaks were linked to SEA, followed by SED (37.5%) and SEB (10%) (17, 161). Similarly, 56.9% of SFP outbreaks in the United Kingdom were attributed to SEA alone or in combination with SED (15.4%), SEB (3.4%), SEC (2.5%), or both SEB and SED (1.1%) (16). SEA was the most common SE implicated in SFP outbreaks in Japan (69.7%), France (90%), and Korea (68%) (162–164).

Bioassays

Bioassays are based on clinical symptoms such as vomiting and gastroenteritis in monkey and kitten models. Historically, the kitten was used for the detection of SEs in staphylococcal filtrates, but this model has been retired due to low sensitivity and specificity (165). Recently, the house musk shrew model has been used to characterize newly discovered SEs; however, with this animal model, the vomiting response requires considerably greater amounts of toxins than those involved in human SFP (112). Thus, this model is not truly representative of human implications. As mentioned above, the monkey feeding assay is the gold standard for confirming enterotoxigenicity.

Immunological Assays

Antienterotoxin antibodies are commonly used to detect SEs in food samples. Currently, commercial kits are available to detect the classical SEs (SEA to SEE) using enzyme-linked immunoassays (ELISAs) and enzymelinked fluorescent assays such as SET (Denka Seiken), VIA (Tecra), BTA (Alexeter Technologies), Transia tube (Diffchamb AB), and Ridascreen (rBiopharma). ELISA detection of SEA using ABTS [2,2′-azino-di(3-ethyl-benzthiazoline-6-sulfonate)] was able to detect 0.4 ng/ml

(with a 20-hour incubation period) and 3 ng/ml (with a 3-hour incubation period) (166). Other ELISA systems using a streptavidin-biotin interaction were able to detect SEB at concentrations ranging from 0.5 to 1 ng/ml (167).

The fluorescent immunoassay has become more commonly used to detect SEs in food samples. Using anti-SEB as a capture antibody and anti-SEB antibody conjugated with the cyanine dye Cy5 for detection, investigators were able to detect SEB at 0.1 ng/ml (168). However, many organic fluorochromes are susceptible to photodegradation, which limits their use. To overcome this limitation, luminescent nanoparticles such as quantum dots (QDs) and lanthanide ion chelate-doped nanoparticles have been used. Anti-SEB antibody bioconjugated with cadmium telluride QDs (QD523 and QD601) and used in a fluorescence resonance energy transfer-based immunoassay was able to detect 8.15 ng and 1 to 0.05 ng of SEB from samples (169, 170). Timeresolved fluorescence from lanthanide ion chelate-doped nanoparticles had good stability against photobleaching, low cytotoxicity, and biocompatibility. By using these new technologies, a method to simultaneously detect SEA, SEB, SEC, SED, SEE, SEG, and SEI applying hydrogelbased microarrays (biochips) has been developed and successfully used to detect these SEs at 0.1 to 0.5 ng/ml (171).

A chemiluminescence-based assay has also become popular because of its high sensitivity, low background, and simple instrumentation needs. The use of a luminol–H_2O_2–HRP (horseradish peroxidase)–*p*-iodophenolenhanced chemiluminescence system greatly improved the detection limit of SEB to as little as 0.01 ng/ml from roast beef, peanut butter, cured ham, milk, and orange juice (172). The sensitivity was further enhanced by the use of poly-HRP40, which comprises five identical covalent HRP homopolymer blocks covalently coupled to streptavidin molecules, resulting in a large number of signal-generating enzyme molecules per one bound analyte molecule (173).

The surface plasmon resonance (SPR) immunoassay offers several unique benefits, such as surface-sensitive response, label-free detection, and real-time monitoring capacity. With the SPR immunoassay, the sensor chip is coated with the appropriate antibodies and binding of the target to antibodies changes the reflection intensity which is detected through thin metal layers at the sensor chip. A sandwich SPR immunoassay detected as little as 10 to 100 ng/g of SEA in milk, hot dogs, mushrooms, and potato salad (174).

Aptamer-Based Bioassays

Aptamers are short single-stranded DNA or RNA molecules with high affinity and selectivity for target molecules. Aptamers can be selected through *in vitro* selection or the systematic evolution of ligands by exponential enrichment. Aptamers can be easily synthesized to higher purity and are amenable to modification for various detection modules, which makes them ideal flat forms for biosensors (175). A screening of aptamer sequences using SEB conjugated to tosyl-activated magnetic beads identified a panel of aptamers capable of selectively binding to SEB and other SEs. The gold nanoparticlebased colorimetric assay successfully detected SEB at concentrations as low as 10 ng/ml (175). The sensitivity of aptamers was greatly improved by fluorescence resonance energy transfer technology in which an aptamer affinity method was coupled with upconversion nanoparticle (UCNP) sensors composed of fluorescence donor probes (cDNA 1-UCNPs) and fluorescence quencher probes (cDNA 2-Black Hole Quencher 3). The formation of an aptamer-SEB complex causes the dissociation of the aptamer from the duplex of fluorescence donor probes and fluorescence quencher probes, which restores UCNP fluorescence. The addition of exonuclease I selectively degrades the single-stranded DNA in the aptamer-SEB complex, which further amplifies the fluorescence of UCNPs. This approach improves the detection limit to 0.3 pg/ml of SEB. Using multicolor lanthanide-doped time-resolved fluorescence-labeled aptamers and graphene oxide as a resonance energy acceptor, a method to simultaneously detect multiplex SEs (SEA, SEB, and SEC1) was developed and successfully detected these SEs at concentrations below 1 ng/ml in milk (176).

CONCLUDING REMARKS

Advances in genome sequencing have expanded the list of identified SEs expressed by *S. aureus*. Recent monkey feeding assays have confirmed the emetic activity of these newly discovered SEs. However, the methods used for toxin preparation and the species of test animals applied need to be considered to properly evaluate the enterotoxigenic potential of SEs. The implementation of Hazard Analysis and Critical Control Points systems and active foodborne illness outbreak surveillance programs have greatly reduced the incidence of SFP, but SFP still causes an estimated 5% of all foodborne illnesses. Recent progress in developing better detection methods using biosensors will greatly contribute to the effective control of SFP. Additional knowledge of the molecular aspects of staphylococcal survival, growth, and SE production in food will enable further development of effective ways to prevent SFP.

One issue that continues to puzzle the scientific community is the questionable benefit to staphylococci of producing an emetic toxin. Unlike the case of enteric pathogens, which inhabit the gut, the ability to induce emesis and diarrhea as a mechanism to promote exit from the host and dissemination does not appear to be important for staphylococci. Also, the lack of knowledge about the receptors for SEs in enterocytes is a significant bottleneck for developing effective therapeutics.

Based on what is now known regarding the superantigenic properties and proposed host-specific molecular adaptation of the SEs, one might suggest that SEs are produced to modulate host immune responses. Exposure of animals and peripheral blood mononuclear cell cultures to SEs and other SAgs has repeatedly been shown to induce at least a transient immunosuppression. Similarly, TSS patients often fail to produce a significant immune response to causative superantigenic PTs, and they remain susceptible to subsequent toxigenic illnesses. Hence, one could speculate that the harmful effects on the host (SFP and TSS) are merely secondary effects of the staphylococci's attempts to affect immune cell function, thereby allowing the bacteria to survive and persistently colonize their many human and animal hosts.

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IV
Nonbacterial Pathogens and Toxins

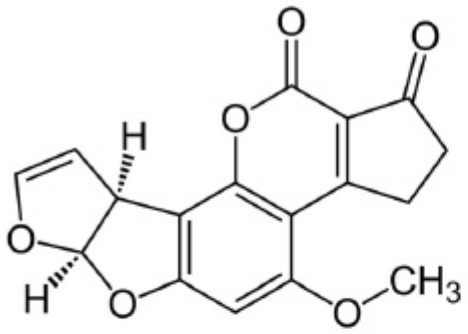
Marta H. Taniwaki
John I. Pitt

22
Mycotoxins

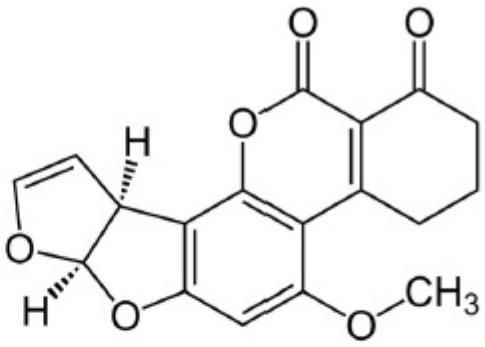
The term “mycotoxin” is derived from the Greek word “*mykes*,” which means fungi, and from the Latin word “*toxican*,” meaning toxin. Mycotoxins have been defined as “fungal metabolites which when ingested, inhaled or absorbed through the skin, can cause disease or death in man and domestic animals, including birds” (1). By general agreement, this definition excludes the toxins produced by macrofungi (mushrooms) and compounds that cause disease only in lower animals or plants. Fungi produce a vast number of secondary metabolites. Only a small number of these compounds are classified as mycotoxins; i.e., they have been demonstrated to cause illness in humans or domestic animals. Specific mycotoxins are produced only by specific fungi, usually by only a few species. A particular species of fungus may produce more than one mycotoxin, though very rarely more than one of the major compounds described here.

Mycotoxin production occurs only as a result of fungal growth: the presence of spores of a particular fungus on a foodstuff is not a good guide to mycotoxin production. However, if environmental conditions, particularly temperature and water activity (a_w), are conducive to fungal growth, toxin production may occur at any period during growing, harvesting, drying, or storage of food commodities. Mycotoxins may occur in processed foods but are much less important in such foods than when they occur in commodities such as grains or nuts. Mycotoxins are typically chemically stable once formed and persist in food even after the destruction of the fungi that produced them.

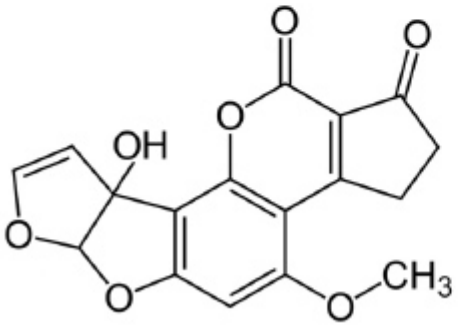
Molecular structures of mycotoxins vary widely (Fig. 22.1), so their effects on human and animal health also vary widely: they may be neurotoxins, teratogens, nephrotoxins, hepatotoxins, immunosuppressive agents, or carcinogens. The International Agency for Research on Cancer (IARC) (2, 3) has evaluated important mycotoxins according to the risk of carcinogenicity to humans (Table 22.1). Perhaps the most important point is that toxicity due to mycotoxins is almost always insidious, with no overt indication of effects on health in the short term. For this reason, the health effects of mycotoxins are among the more neglected areas of medical science.



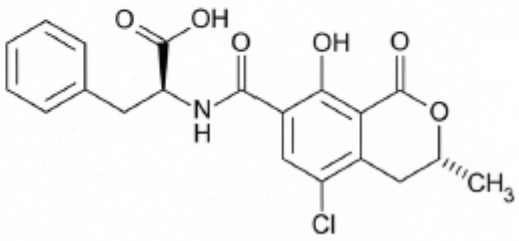
Aflatoxin B_1



Aflatoxin G_1



Aflatoxin M_1



Ochratoxin A

Figure 22.1 Structures of aflatoxin B_1, aflatoxin G_1, aflatoxin M_1, and OTA.

Table 22.1 Evaluation of mycotoxins in humans[a]

Mycotoxin	IARC classification
Aflatoxins B and G	Group 1, carcinogenic to humans
Aflatoxin M_1	Group 2B, possibly carcinogenic to humans
OTA	Group 2B, possibly carcinogenic to humans
Fumonisins	Group 2B, possibly carcinogenic to humans
DON	Group 3, not classifiable as to carcinogenicity
ZEA	Group 3, not classifiable as to carcinogenicity

[a] Data from references 2 and 3.

Many fungal secondary metabolites are toxic, but only a few are significant in relation to food safety. Most are of little concern, either because their toxicity is limited or because they are produced by species that are uncommon in foods. The most important mycotoxins are aflatoxins, ochratoxin A (OTA), fumonisins, deoxynivalenol (DON), and zearalenone (ZEA) (4). Each of these is discussed below.

AFLATOXINS

Chemical Characterization

Aflatoxins are highly substituted coumarins, with a fused dihydrofurofuran moiety. Four naturally occurring aflatoxins are important: aflatoxins B_1 and B_2, named because of their blue fluorescence under UV light, and aflatoxins G_1 and G_2, which fluoresce greenish yellow (Fig. 22.1). When aflatoxins B_1 and G_1 are ingested by lactating animals, small proportions (1 to 2%) are excreted in milk as aflatoxins M_1 and M_2, which are hydroxylated derivatives of the parent compounds (5).

Fungal Sources

Aflatoxins are produced in foods primarily by *Aspergillus flavus* and the closely related species *Aspergillus parasiticus*. On standard identification media, i.e., Czapek yeast extract agar (CYA) (6) and malt extract agar (MEA) (6), both species produce rapidly growing colonies characterized by green conidia (asexual spores) borne on typical *Aspergillus* fruiting structures (Fig. 22.2). The two species differ in small details: *A. flavus* usually bears conidia on two sets of supporting cells (metulae and phialides), while *A. parasiticus* spores are borne on phialides alone. Conidia of *A. parasiticus* exhibit rougher walls than those of *A. flavus*.

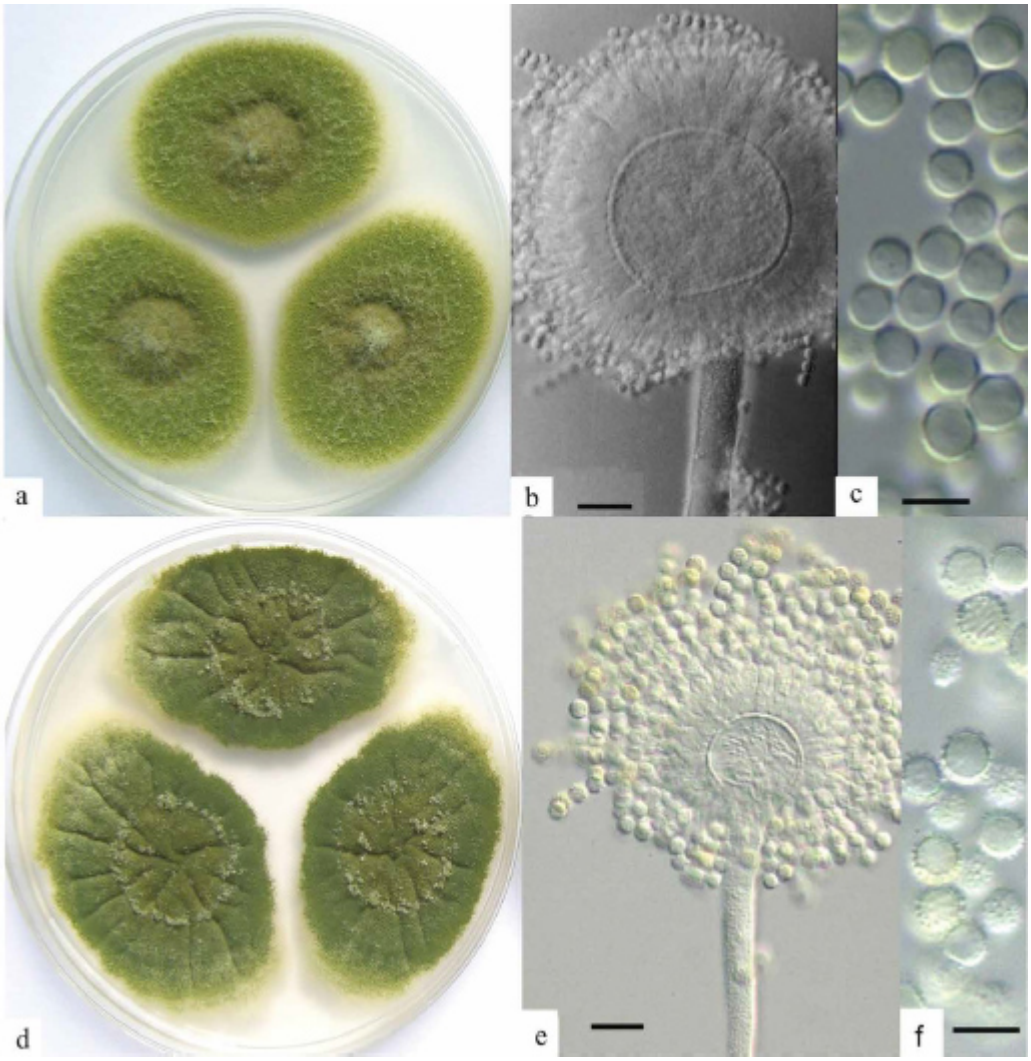


Figure 22.2 (a) *A. flavus*, CYA, 7 days at 25°C; (b) *A. flavus* fruiting structure (bar, 20 mm); (c) *A. flavus* conidia (bar, 5 mm); (d) *A. parasiticus*, CYA, 7 days at 25°C; (e) *A. parasiticus* fruiting structure (bar, 10 mm); (f) *A. parasiticus* conidia (bar, 5 mm).

A. flavus isolates produce B aflatoxins, and some also produce cyclopiazonic acid. On a worldwide basis, about 40% of *A. flavus* isolates produce aflatoxins (7), though percentages of toxin-producing isolates may vary with land use (7). Isolates of *A. parasiticus* produce both B and G aflatoxins but not cyclopiazonic acid, and almost all isolates are toxigenic.

At least 14 *Aspergillus* species are known to produce aflatoxins (8–10). Besides *A. flavus* and *A. parasiticus*, two others are of some

importance in foods: *Aspergillus nomius* and *Aspergillus minisclerotigenes*. Both resemble *A. flavus* in culture, but *A. nomius* produces bullet-shaped sclerotia, in contrast to the large spherical sclerotia produced by many *A. flavus* isolates, while *A. minisclerotigenes* produces small spherical sclerotia. Both of these species produce B and G aflatoxins.

Detection of these species from foods or soils is facilitated by the use of *Aspergillus flavus* and *A. parasiticus* agar (11). After 42 to 48 h of incubation on this agar at 30°C, colonies of *A. flavus* and *A. parasiticus* exhibit a brilliant orange-yellow reverse coloration.

A. flavus grows at temperatures from a minimum of 10 to 12°C to a maximum of 43 to 48°C, and optimally at about 33°C. It has been shown to grow at minimum a_w of 0.82 at 25°C, 0.81 at 30°C, and 0.80 at 37°C (12). Growth of *A. flavus* occurs over the pH range 2.1 to 11.2 at 25, 30, and 37°C, with optimal growth over a broad range from pH 3.4 to 10 (6). Atmospheres of 20% CO_2 and <0.5% O_2 or 80% CO_2 with 20% O_2 did not inhibit *A. flavus* growth on CYA (6, 13). However, CO_2 levels of >40% and O_2 levels of <0.5% inhibited *A. flavus* (14).

The most reliable figures for heat resistance of *A. flavus* conidia indicate a D_{45} value of >160 h, a D_{50} of 16 h, a D_{52} of 40 to 45 min, and a D_{60} of 1 min, at neutral pH and high a_w, with z values from 3.3 to 4.1°C (6). These values indicate ready destruction at pasteurization temperatures. The physiology of *A. parasiticus*, *A. nomius*, and *A. minisclerotigenes* is similar to that of *A. flavus*.

Genetics

The aflatoxin-biosynthetic pathway in *A. flavus* has been studied extensively and is now quite well understood. The pathway is encoded by a single unit on a chromosome within linkage group VII and includes more than 20 enzymes and gene products. Many of these have now been isolated and characterized (15). Two regulatory genes, *AflR* and *AflJ*, are known. The gene products of *AflR* regulate

the biosynthetic pathway at the transcription level. The function of *AflJ* is not known, but its disruption prevents aflatoxin accumulation (15). The production of aflatoxin by toxigenic *Aspergillus* species is affected by environmental and nutritional factors, including temperature, a_w, pH, carbon and nitrogen sources, stress factors, lipids, and certain metal salts (16, 17).

Ecology

A. flavus occurs universally in food crops in the tropical and warm temperate zones of the world. It is associated with peanuts, maize, and cottonseed and frequently produces aflatoxins in these commodities. It also occurs in tree nuts, especially pistachios (18) and Brazil nuts (19, 20) and less commonly in hazelnuts, walnuts, cashew nuts, coconut, copra, and pecans (6, 18, 21–23). Evidence indicates that *A. parasiticus* has a more limited ecological and geographical range than *A. flavus*. It is common in peanuts wherever they are grown but uncommon in most other foodstuffs. It occurs in the same climatic regions as *A. flavus* except that it is uncommon in Southeast Asia (24). *A. nomius* has been shown to be a common cause of aflatoxin production in Brazil nuts (19, 20). *A. minisclerotigenes* was originally detected in peanuts (25) but has been found to be widespread (26, 27). The occurrence of *A. flavus* and related species in foods has been reviewed extensively elsewhere (6, 23).

Toxicity of Aflatoxins

Aflatoxins have likely involvement in five toxic effects: acute toxicity, liver carcinogenicity, liver cirrhosis, immunosuppression, and growth retardation in children.

Acute Toxicity—Aflatoxicosis

An outbreak of hepatitis due to aflatoxin ingestion from maize occurred in India in 1974: almost 400 cases were identified, and 106 deaths were reported. Clinical features were jaundice preceded by

fever, vomiting, and anorexia, with ascites and edema in the lower limbs in extreme cases. Levels in analyzed foods were often extremely high, with estimated daily ingestion of up to 6 mg of aflatoxins (28). Two outbreaks of aflatoxicosis were documented in Kenya in 1981 and 2004. In the latter outbreak, 317 cases and 125 deaths were reported (8, 29, 30). A smaller outbreak occurred in Malaysia in 1988, where 13 children died from acute hepatic encephalopathy from consumption of commercially prepared noodles contaminated with aflatoxin (31).

With the exception of the Malaysian incident, aflatoxicosis occurs only when drought or famine causes exceptionally high levels of aflatoxins in the diet or forces the eating of substandard food.

Liver Carcinogenicity

More insidious and more frequent is the development of liver cancer from consumption of much lower levels of aflatoxin over longer periods. IARC recognizes aflatoxin B_1 and naturally occurring mixtures of aflatoxins as group 1 carcinogens; i.e., they are recognized as carcinogenic to humans (2). Studies in the past 20 years have determined that hepatitis B virus also causes human liver cancer; the two agents are causal and synergistic. In its risk assessment of aflatoxins, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) has derived two potency factors for cancer formation by aflatoxins: for aflatoxin alone, 0.01 case per 100,000 people per annum per ng per kg of body weight per day and for individuals carrying hepatitis B infection, 0.30 case. Thus, the two agents together are about 30 times as potent as aflatoxin alone (3). It has been estimated that aflatoxin is responsible for between 25,000 and 155,000 of the approximately 600,000 deaths from liver cancer in the world each year (32, 33).

In the liver, aflatoxin B_1 is converted by cytochrome P450 enzymes to the 8,9-epoxide. This is capable of binding to liver proteins, leading to liver failure and potentially to aflatoxicosis. This epoxide

is also able to bind to DNA, a precursor to the development of liver cancer.

Stunting in Children

A number of studies have now provided evidence that aflatoxin exposure before birth and in early childhood is associated with stunted growth, defined by the World Health Organization (WHO) as height for age being more than two standard deviations below average height for age in a given population. It is apparent, from the high numbers of people believed to be consuming uncontrolled levels of aflatoxin, that stunting is an important disease burden that has only recently been recognized (34, 35). Growth suppression has also been observed in a number of mammals and avian species.

Immunosuppression

Aflatoxins have been shown to suppress the cell-mediated immune response in both cell lines and domestic animals. Effects include the impairment of delayed-type hypersensitivity, decreases in the phagocytic activity of macrophages, increased susceptibility to infection, and reduced response to vaccines (3, 23). Few studies in humans have been reported, but it is apparent that if the effects in humans mirror those in animals even approximately, then the immunosuppressive effects of aflatoxins probably have very broad implications for human health.

Liver Cirrhosis

Direct evidence that aflatoxins are involved in liver cirrhosis is limited, but as death from cirrhosis of the liver is an important cause of mortality, any enhancement by aflatoxin of other factors in causing cirrhosis will have important consequences.

Analysis of Aflatoxins

Aflatoxins are usually very unevenly distributed in commodities, especially peanuts, where few nuts are contaminated but

contamination levels in individual nuts are often high, up to 1 mg kg^{-1}. For that reason, sampling is considered the largest source of error in aflatoxin assays (36). Sampling plans have been developed for continuous lines, for 10-metric-ton lots, and for bag stacks (37–39). The Food and Agriculture Organization (FAO) recently released an online system for calculating the efficacy of sampling plans over a range of mycotoxins and commodities (40). All of the recommended methods use samples sizes of 8 kg or more. Entire samples should be comminuted in a vertical chopper or similar mill. Subsamples, ideally of 500 g or more, may be extracted by a variety of mixed polar and nonpolar solvents, depending on the food matrix being analyzed. Methanol-water (80:20) is recommended for commodities such as maize, peanuts, and cottonseed (41).

The traditional method for aflatoxin assays is thinlayer chromatography (TLC), and as this is inexpensive and reliable, it is recommended for less developed economies. For "acceptable/not-acceptable" testing, immunochemical methods are most frequently used. For advanced users, such as high-volume analytical laboratories or regulatory authorities, high-pressure liquid chromatography (HPLC), preferably coupled with tandem mass spectrometry (HPLC-MS/MS), for determination of aflatoxins alone or in combination with other mycotoxins has been widely used. Limits of detection are now well below 0.1 μg/kg (42, 43).

Occurrence and Regulation of Aflatoxins in Foods

Levels of aflatoxins in foods are highly variable. Only a few commodities are at serious risk if good agricultural and manufacturing practices are observed. Peanuts, maize, and cottonseed frequently contain unacceptable levels of aflatoxins, and assays are usually carried out at wholesale intakes and frequently in finished products as well. In developed countries, regulations are generally stringently applied to human foods and animal feeds that contain appreciable amounts of these commodities. Other closely watched commodities are tree nuts, especially pistachios and Brazil

nuts, figs, and spices. Although levels in these commodities may exceed regulatory limits from time to time, such levels seldom pose a long-term risk to human health.

Under inadequate storage conditions, other grains including sorghum and rice may also permit growth of *A. flavus* and aflatoxin production. These commodities have very high consumption levels in many communities, so careful storage is of great importance.

Regulation of aflatoxin levels in foods commenced about 1970, using what was then the limit of detection, 5 μg kg^{-1}, as the permitted limit. However, as peanut producers could not reach that limit, higher limits were set in peanut-exporting countries, including the United States and Australia. As analytical techniques improved, with the introduction of liquid chromatography, lower limits were frequently set by importing countries, especially in Europe. The understanding of aflatoxin toxicology has produced a compromise safe limit for human consumption of 15 μg kg^{-1} in peanuts. Levels in most foodstuffs, where aflatoxin is less likely to occur, remain lower, usually 5 μg kg^{-1} or less. Although safe levels of aflatoxins have now been established and aflatoxin levels are closely controlled in developed countries, it has been estimated that up to 5 billion people worldwide are at risk from exposure to uncontrolled levels of aflatoxins in their diets (44).

Control of Aflatoxins in Foods

Control of aflatoxins has proved very difficult, as invasion by *A. flavus* (and *A. parasiticus* in peanuts) occurs before harvest in all of the major crops affected (6). In peanuts, it appears likely that infection by *A. flavus* while nuts are still in the ground is a prerequisite for high levels of aflatoxins to be formed after harvest (45). In the absence of high preharvest infection levels, and with rapid and effective drying, peanuts can be produced free of any appreciable level of aflatoxin. The major causes of preharvest infection are high spore numbers in soil and plant stress induced by drought and/or high soil temperatures (23, 46). *A. parasiticus* and

A. flavus are commensals in peanuts, as both fungi are able to grow in peanut plants and developing peanuts (47).

Partial control of spore numbers can be achieved by crop rotation, as numbers of *A. flavus* in soil decrease under small-grain cultivation or pasture. Irrigation, which eliminates drought stress, is regarded as the most effective method for reducing aflatoxin formation in peanuts (48). However, peanuts throughout the world are recognized as a drought-resistant crop and are mostly grown under dry culture, with irrigation being reserved for crops that are more sensitive to drought, such as rice or vegetables. In many areas where peanuts are grown, irrigation is not an option. Under these circumstances, reduction in drought stress by good agricultural practices can be a beneficial approach; for example, by weed control and wider spacing between rows. Harvesting early when drought stress occurs can help reduce contamination. Rapid drying of peanuts using mechanical dryers as soon as possible after pulling has a major effect in reducing the levels of aflatoxins in peanuts. Maintaining dryness during storage is also essential (49).

A. flavus is also a commensal in maize and cottonseed (50, 51), so preharvest infection is common in these crops. In maize, the infection route is usually through insect damage and, in cotton, through the nectaries. For maize, irrigation and improved farm management practices have a beneficial effect on aflatoxin formation (52). However, as maize is usually dried in the field, rapid drying techniques are not commonly practiced. Control of insect damage in maize (53) and a number of practices including good storage conditions (54) and genetically modified crops (55) are used to control aflatoxin levels. Cottonseed is a by-product of cotton production, and seed often accumulates in heaps at cotton factories before drying. Breeding of cotton without nectaries has been proposed as one means of limiting *A. flavus* access to cotton bolls (51).

In tree nuts, cultivar selection, harvest timing, and irrigation rate are important factors in efforts to reduce aflatoxin formation. Insect

control is also of great importance (56). Entry of *A. flavus* into pistachio nuts depends on the time of splitting of hulls. Nuts in which hull splitting occurs early are much more susceptible to *A. flavus* invasion (57). Some cultivars are more prone to early splitting than others, so where nuts are harvested from the ground, cultivar selection is important.

Figs are sometimes infected by *A. flavus*, both because of their unique structure developed for insect fertilization and because they are harvested from the ground in some countries. Immature figs are not colonized by *A. flavus*, but once they are ripe, infection occurs readily and fungal growth continues during drying (58, 59). The proportion of figs infected is usually very low (60).

Good agricultural practice has been the standard method in developed countries for attempting to control aflatoxin levels in commodities, particularly maize and peanuts. Good agricultural practice involves good farm management, including weed control, optimal row and plant spacing, insect control, adequate water supplies, rapid and complete drying, removal of defects, and effective control of storage conditions. By themselves, these approaches are often inadequate; too often, drought stress and/or insect damage results in aflatoxin formation before harvest, out of farmer control, even in developed countries. The only effective hazard analysis and critical control point plan for aflatoxins remains end product testing.

Reducing Aflatoxin Levels in Foods

In peanuts, reduction in aflatoxin levels is accomplished by color sorting of individual kernels after shelling. The process was developed originally to reject commercially unacceptable discolored nuts, regardless of cause, but as fungal growth is a prime cause of discoloration, the process is also an effective nondestructive means of removing most nuts containing aflatoxins. In crops under severe drought stress, peanuts begin to dry in the ground, and under these conditions, luxuriant growth of *A. flavus* can occur, with high

aflatoxin production. In this case, blanching to remove skins and roasting to increase discoloration permits effective color sorting to be carried out(61). Manual or mechanical sorting of Brazil nuts, and drying at 60°C for 30 to 36 h, eliminated more than 98% of aflatoxins (20).

Maize and fig samples are screened for the presence of aflatoxin by the examination of cracked kernels or fruit by UV light. No effective nonchemical testing techniques exist for cottonseed or pistachios, and, as with other commodities, nondestructive chemical assays are not available.

In developed producing countries, it is standard practice to assay aflatoxin levels, often repeatedly, in consignments of peanuts and maize from intake to shellers to final product. Such controls rarely exist in less developed countries. Other commodities are similarly screened according to needs and markets.

The extent to which aflatoxins are destroyed during heating is largely dependent on the process used. Dry roasting of peanuts at 160, 180, and 200°C resulted in aflatoxin reduction of 62, 84, and 90%, respectively (62). Microwave roasting at 140°C for 4.5 min and at 185°C for 7 min reduced aflatoxins by 21% and 100%, respectively, but both temperature and time conditions resulted in excessively dark nuts (63).

Nixtamalization, the processing of corn for making tortillas, reduces aflatoxin in maize by >90% (64). The alkali process used to produce refined table oil completely removes aflatoxin (65).

Interventions to Reduce Aflatoxins

Several approaches have been put forward to reduce aflatoxin levels in foods and feeds in recent years. The most advanced preharvest approach for limiting aflatoxin formation in peanut, maize, and cotton crops is biocontrol by competitive exclusion. This technique relies on the fact that only about 40% of *A. flavus* strains produce aflatoxins. Spores of selected nontoxigenic strains that are both

competitive in the field and unlikely to revert to toxicity are added to carrier seed (rice or sterilized sorghum) and introduced into soils during late stages of crop growth, where spores multiply rapidly (47, 66–68). The nontoxigenic spores compete with the existing toxin-producing spores in the soil for infection sites on developing nuts, kernels, or seeds. If the fungus grows to sufficiently high numbers, control can be very effective. This process was developed independently in Australia (47, 66) and in the United States (67), where it is used commercially for peanut and cotton crops (68, 69). Studies on biocontrol for maize in Africa have reported the method to be very successful (68, 70), and biocontrol is now in use on a large scale in several African countries (71). Experiments in Thailand have been less successful (72).

Other reported experimental studies have aimed to improve farm efficiency by providing advice and improved management for farmers in less developed countries (73). Some success has been achieved. Another approach has been to use weather and soil data to forecast when drought stress and aflatoxin production are about to occur. Such forecasting has been very well developed and successful in Queensland, Australia, for both peanut (74) and maize crops (75).

The discovery that certain montmorillonite clays adsorb aflatoxins very strongly has led to experiments where such clay is added at concentrations of a few percent to animal feeds, where it has been very effective. Trials with clay added to human food have also showed promise, but this approach is likely to be reserved for emergency remedial use (23, 76).

OCHRATOXIN A

Chemical Characterization

Ochratoxins are mycotoxins containing a dihydroisocoumarin ring linked to phenylalanine through a 7-carboxy group (Fig. 22.1). Several ochratoxins are known: OTA; its ethyl ester, ochratoxin C; and dechlorinated analogues, i.e., ochratoxin B and its methyl and

ethyl esters. Ochratoxins A and B are the only ones so far detected in naturally contaminated foods or feeds. OTA is the most toxic compound and the only one discussed here.

Fungal Sources

OTA is produced by three well-defined groups of fungi: (i) the ochre-colored aspergilli, including *Aspergillus ochraceus*, *A. westerdijkiae*, *A. steynii*, and a few other much less important, closely related species; (ii) the black aspergilli, including *A. carbonarius* and *A. niger* (which produces OTA only infrequently); and (iii) *Penicillium* species, including *Penicillium verrucosum* plus the closely related species *Penicillium nordicum* (6, 77, 78).

A. ochraceus and Related Species

After 1 week at 25°C on the standard growth medium CYA, *A. ochraceus* and closely related species produce colonies 40 to 55 mm in diameter with ochre (light yellow brown) conidia, characteristics distinctive for this group of species. *A. westerdijkiae* and *A. steynii* are distinguished from *A. ochraceus* by their inability to grow on CYA at 37°C. *A. westerdijkiae* produces finely roughened, spherical conidia, and *A. steynii* produces smoothwalled, ellipsoidal conidia, while those of *A. ochraceus* are smooth-walled and spherical (6) (Fig. 22.3a to c).

These three species have similar growth requirements so far as is known apart from the difference in maximum growth temperature, ca. 40°C for *A. ochraceus* and about 5°C lower for the other species.

Although *A. ochraceus* was the species first recognized as producing OTA, advances in molecular and fungal metabolite techniques have resulted in the separation of *A. westerdijkiae* and *A. steynii* from *A. ochraceus* (79). Studies reporting *A. ochraceus* before 2005 were likely to include all three species under the name *A. ochraceus* (80–82). *A. westerdijkiae* is now recognized as the main source of OTA in coffee (79, 83). These three species are xerophilic, growing at an

a_w of 0.8 or below, and so have been isolated from stored dry foods on occasion.

A. carbonarius and *A. niger*

A. carbonarius and *A. niger* produce rapidly growing, black or deep reddish-brown colonies on CYA, usually 60 mm or more after 7 days at 25°C. *A. carbonarius* produces much larger conidia (6 to 7 μm in diameter) than *A. niger* (4 to 5 μm). *A. carbonarius* grows at from 10 to 40°C and at an a_w as low as 0.85 (6) (Fig. 22.3d to f).

A. carbonarius has a major habitat in vineyards and grape-drying yards, where its ability to tolerate high temperatures and strong sunlight (as a result of its dark hyphae and spores) provides a competitive advantage. It has been recognized as the primary source of OTA contamination in grapes and grape products, including wines, throughout the world (84–86). Grapes are usually sun dried without preservatives, so raisins and sultanas sometimes contain unacceptable levels of OTA (87–89). OTA can also result from growth of *A. carbonarius* in coffee beans (82, 90, 91) and cocoa beans (92, 93). Other sources, including figs (59, 94), peanuts and maize (95, 96), and paprika (97), are less important.

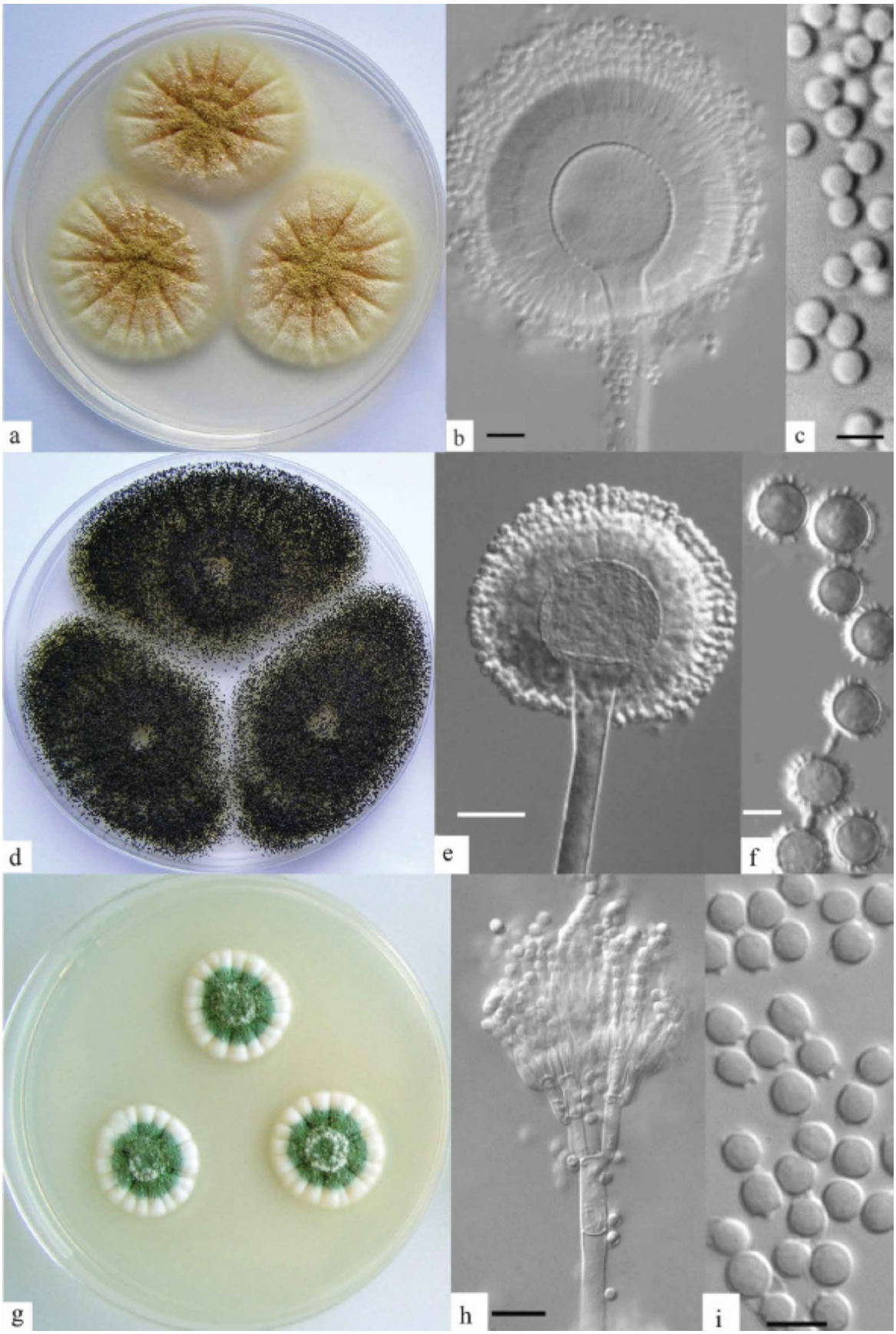

a
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c
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Figure 22.3 (a) *A. ochraceus*, CYA, 7 days at 25°C; (b) *A. ochraceus* fruiting structure (bar, 20 mm); (c) *A. ochraceus* conidia (bar, 5 mm); (d) *A. carbonarius*, CYA, 7 days at 25°C; (e) *A. carbonarius* fruiting structure (bar, 40 mm); (f) *A. carbonarius* conidia (bar, 5 mm); (g) *P. verrucosum*, CYA, 7 days at 25°C; (h) *P. verrucosum* fruiting structure (bar, 10 mm); (i) *P. verrucosum* conidia (bar, 5 mm).

A. niger has usually been regarded as a benign fungus and has been widely used in enzyme production and ingredients for food processing. It holds GRAS (generally regarded as safe) status from the U.S. Food and Drug Administration. Usually, only a low percentage of *A. niger* isolates are able to produce OTA. Although *A. niger* is very frequently isolated, it is not a significant source of OTA as a rule.

P. verrucosum and *P. nordicum*

P. verrucosum and *P. nordicum* are very similar, slowly growing species, with colonies reaching only 15 to 25 mm in diameter on CYA after 7 days at 25°C and being colored dull green to dark green. Both grow from about 0 to 30°C and at an a_w as low as 0.80 (6) (Fig. 22.3g to i).

P. verrucosum is the major OTA producer in cereals grown in cool temperate climates, ranging across northern and central Europe, Canada, and northern Asia (98). In these regions, OTA is frequently present in cereal products, especially bread and flour-based foods, and in the meat of animals which eat cereals as a major dietary component (99). *P. verrucosum* is not found in warmer climates, so small grains from the tropics and warm temperate zones do not contain OTA (6). *P. nordicum* is closely related to *P. verrucosum* (100) but occurs in manufactured meat products such as salami and ham, not in cereals. This species does not appear to be a major problem.

Toxicity of OTA

OTA is a chronic nephrotoxin, affecting kidney function in all animal species tested. OTA is readily absorbed through the intestines and, once it enters the bloodstream, has a long half-life, up to 3 weeks in monkeys. As a result, the blood of healthy humans regularly contains detectable amounts of OTA in areas where it is frequently part of the diet, i.e., northern Europe, northern North America, and northern Asia (101). Recent indications of OTA in human blood from other parts of the world are almost certainly due to the presence of *Aspergillus* species producing OTA in foods.

OTA also has carcinogenic properties, but the mechanism of carcinogenicity remains unclear. The carcinogenic effects in animals are considered to be of less importance than the nephrotoxicity (101, 102). Although OTA is demonstrably toxic to animals of all kinds, its effects in humans remain unclear and the subject of debate. Both genotoxic and nongenotoxic modes of action have been proposed (102, 103). IARC has classified OTA as a possible human carcinogen (group 2B), based on sufficient evidence of carcinogenicity in experimental animal studies and inadequate evidence in humans (2). The target organ of toxicity in all mammalian species tested is the kidneys, in which lesions can be produced by both acute and chronic exposure (103).

Although the mechanism of action of OTA is unclear, its structural similarity to phenylalanine and the fact that it inhibits many enzymes and processes that are dependent on phenylalanine strongly suggest that it acts by disrupting phenylalanine metabolism (104). As it is a phenylalanine analogue, OTA toxicity in animals can be reduced by treatment with phenylalanine (105).

Exposure Assessment

Assessment of OTA exposure has proved to be very difficult. People in Europe and northern North America are exposed to OTA in barley and wheat and their products, especially bread, and also to that in meat, especially pork, from animals fed contaminated feed (23). Low levels also occur in beer, wines, coffee, cocoa, chocolate, and

dried vine fruits. However, as wheat and barley crops from warmer climates are not infected by *P. verrucosum*, OTA intake is much lower in tropical and subtropical regions (106).

Risk Characterization

JECFA set the provisional tolerable weekly intake (PTWI) of OTA at 100 ng/kg of body weight in 2001 (101) and confirmed that value more recently (103). This PTWI is based on the lowest observed level of acute toxicity in pigs, as risk assessments have indicated that the acute toxicity occurs at lower intake levels than any carcinogenic effect (101). However, a Canadian reevaluation of the risk associated with OTA has reduced the PTWI to 21 ng/kg of body weight, by applying a larger uncertainty factor to available toxicity data (102). Despite this evaluated high toxicity, the fact remains that although OTA is commonly found in blood from persons in some regions, OTA has not been correlated with any known human syndrome.

Dietary exposure to OTA is based primarily on data from Europe, where processed cereal foods sometimes contain high OTA levels (102, 103). JECFA (103) has assessed the dietary exposure to OTA from processed cereals in Europe as 8 to 17 ng/kg of body weight per week, well below the PTWI. However, in a previous evaluation, JECFA had determined that the 95th percentile for OTA consumption was about 90 ng/kg body weight, approaching the PTWI (101). Even with some additional intake from wines and coffee, the hazard from intake of OTA in Europe appears low. Intake in other parts of the world is likely to be much lower, in the absence of appreciable levels in cereals.

Analysis of OTA

The introduction of monoclonal immunoaffinity columns as a cleanup step for the analysis of OTA was a major improvement in assessment of OTA contamination in foods (43). These columns selectively separate OTA from interfering compounds, and this

effective separation is usually followed by analysis by HPLC with fluorescence detection (43). Other methods for quantification of OTA in foods include TLC and immunoassays such as enzymelinked immunosorbent assays. LC-MS/MS allows accurate determination of OTA without any need for cleanup in several types of matrices (43).

Occurrence and Control of OTA in Crops

None of the fungi producing OTA are known to be systemic invaders or pathogens of food crops, so the occurrence of OTA in foods is usually due to postharvest contamination (107). In consequence, control of OTA relies on good practice. For cereal grains, coffee cherries (dry process), and grapes, rapid drying ensures low production of OTA. However, climatic conditions often make that difficult to achieve.

A. carbonarius occurs in vineyards and can infect grapes before harvest, as the result of damage by plant-pathogenic fungi, including powdery mildews and *Rhizopus* and *Botrytis* species, or skin splitting due to rain damage. The resulting *Aspergillus* bunch rots generally occur near harvest, when the sugar content of berries is highest (86, 108), resulting in the prevalence of OTA in grape juice and wines. OTA mostly occurs in wines from the warmer growing areas throughout the world, but levels are usually low (109). If grapes for wine production are substantially sound, i.e., free from rotting fruit, and are crushed quickly after harvest, leading rapidly to anaerobic conditions, OTA can be held to low levels (110), as the fermentation process stops growth of the fungus.

When grapes are dried, mechanical damage during harvesting and the time in the drying yard increase the probability of OTA formation. The population of *A. carbonarius* in vineyards can be reduced by good management practices, including irrigation, pruning to improve air flow through vines, use of cover crops between rows, and appropriate fungicide applications (108, 111, 112).

Fungi capable of producing OTA are rarely present on coffee cherries (mature fruit) at harvest (82). Harvested coffee cherries are processed via two basic systems: (i) the dry system, where the whole fruit remains intact, producing what is called natural coffee or dried coffee, and (ii) the wet processing system, where the seed is enclosed only in the inner integument or endocarp, producing what is called parchment coffee (113). Slow drying in either system can potentially lead to OTA formation. Effective sun drying or a combination of sun drying and mechanical dehydration provides effective control (82).

If grains are stored under safe conditions, OTA formation will also be minimal (46, 114). However, in cool temperate climates, *P. verrucosum* has the potential to produce OTA during slow drying or in storage (98).

Effect of Processing on OTA

OTA is largely removed during the winemaking process, as it is bound to solid fractions and sediment. Use of some fining agents can also reduce OTA levels in wine. Red wines retain slightly more OTA than white wines, but overall, the carryover from grapes into finished wine is <10% (110, 115, 116).

When the kinetics of OTA destruction during coffee roasting was studied at four temperatures (180, 200, 220, and 240°C) over three time periods, a reduction from 8% to 98% was observed, with reduction depending directly on the degree of roasting (117). Although thorough roasting of coffee beans may destroy OTA effectively, the use of highly contaminated beans in coffee blends is not recommended, as beverage quality may be affected. The effects of processes such as roasting, drink preparation, and instant coffee production on OTA destruction have been reviewed elsewhere (118, 119).

Bran and germ removal reduces OTA levels in flour and flour-based products, with the degree of loss being influenced by the process used (120). Milling hard wheat to produce white flour produces an

approximately 65% reduction in OTA, and a further 10% decrease occurs during baking. Whole-meal flour and bread showed much less reduction in OTA during processing, as might be expected, because less of the grain is discarded (120).

Dry and moistened samples of wheat contaminated with OTA were heated at several temperatures and times, and the times to 50% destruction of OTA under various heating conditions were calculated. A 50% inactivation of OTA at 100, 153, 200, and 250°C required 707, 201, 12, and 6 min, respectively, for dry wheat. For moist wheat, the corresponding inactivation times at 100, 150, and 200°C were 145, 64, and 19 min, respectively. Complete destruction did not occur even at 250°C (121).

FUSARIUM MYCOTOXINS

The most important general observation to be made about the production of mycotoxins by *Fusarium* species is that they all grow only at high a_ws (>0.9) (6), so toxin production in crops occurs only before harvest or during early stages of drying (107). Production of mycotoxins ceases long before the crops are fully dried and occurs only during storage under catastrophic conditions, such as flooding. Production of *Fusarium* mycotoxins occurs as the result of growth of the causal fungus in the living plant and seed.

FUMONISINS

Chemical Characterization

Fumonisins are a family of compounds comprising longchain aliphatic acids with esterified carboxylic acid side chains. Fumonisins are large molecules: the most important, fumonisin B_1 (CAS [Chemical Abstract Service] registry no. 116355-30-0), has the molecular formula $C_{34}H_{59}NO_{15}$, with a molecular weight of 721. Fumonisins are structurally similar to the sphingoid base backbone

of sphingolipids, which are important membrane constituents (122) (Fig. 22.4).

Fungal Sources

Fumonisins are produced by *Fusarium verticillioides* (referred to in older literature as *Fusarium moniliforme)* and some closely related species, in particular, *Fusarium proliferatum*. These species are systemic in maize worldwide, being always present in the plants, even in healthy kernels (123). Surprisingly, fumonisins are also produced by *A. niger* (124, 125).

F. verticillioides grows rapidly at 25°C on any standard mycological medium, including CYA, MEA, and potato dextrose agar (PDA) (6, 126). Colonies are white to pale salmon, with low and often ropy mycelium and a powdery texture due to production of chains of microconidia. The reverse color on PDA is variable and can be pale salmon, grayish violet, brownish violet, or deep violet. Slow growth occurs at 37°C on these media.

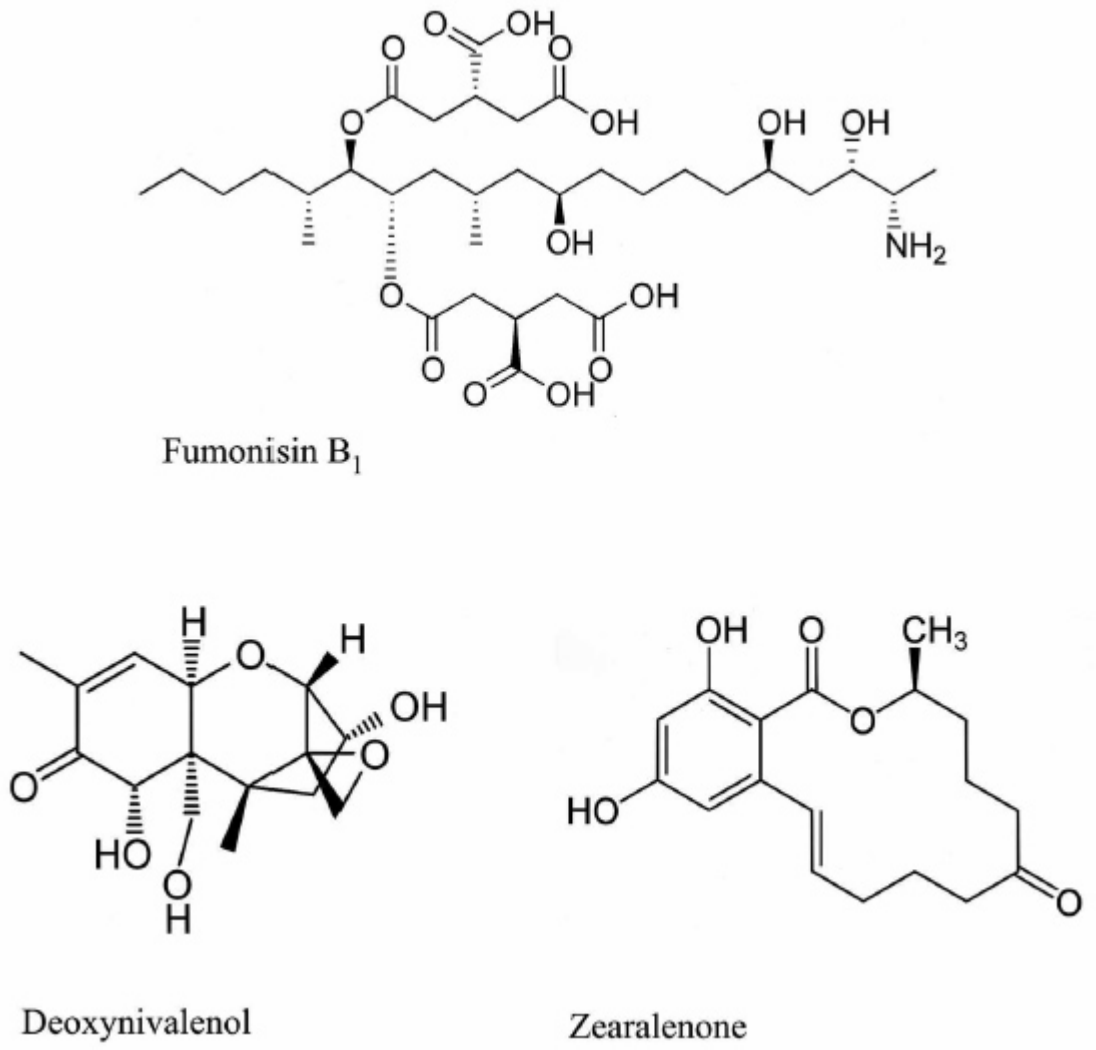


Figure 22.4 Structures of fumonisin B_1, DON, and ZEA.

Characteristic *Fusarium* spores, macroconidia, are produced on dichloran chloramphenicol peptone agar (6) or other specialist media. Macroconidia are long (40 to 50 µm) and slender, almost straight, and thin walled, with foot-shaped basal cells. Microconidia are pointed at both ends or club shaped, are 7 to 10 µm long, and are produced in chains from long single phialides (fruiting cells) (6, 126) (Fig. 22.5a and b).

F. proliferatum is very similar, but colonies on PDA lack purple colors in the reverse, and microconidia are produced from phialides with more than one fertile neck (126).

Fumonisin production in maize is favored by relatively high temperatures (127). Fumonisins are found primarily in maize and sorghum, as *F. verticillioides* and *F. proliferatum* rarely infect other crops. Levels are mainly influenced by local climatic conditions near harvest. Under plant stress, the normally symptomless endophytic relationship may change to a disease- and/or mycotoxin-producing interaction (128–130). It is likely that water stress and insect predation, factors related to the onset of this change in the fungus-plant interaction, may be involved in this conversion (131). Biological interactions between the maize plant and the fungus are complex (132). Endophytic growth of *F. verticillioides* within the maize plant may be of benefit to plant growth, as observed in other members of the *Gramineae* (133). *F. verticillioides* has been reported to suppress the growth of *Fusarium* species which cause *Gibberella* ear rot (134).

The production of fumonisin B_2 by *A. niger* was reported quite recently (124). Previously, production of fumonisins was believed to be exclusive to species of *Fusarium*. *A. niger* has been shown to produce fumonisin B_2 and fumonisin B_4 on grapes and raisins (135), while only fumonisin B_2 has been found in coffee (136). *A. niger* is among the fungi most commonly detected in foods, raising the possibility of co-occurrence of OTA and fumonisin B_2 in foods.

Besides *A. niger*, the very closely related species *Aspergillus welwitschiae* (also known as *Aspergillus awamori*) has been shown to produce OTA and fumonisin B_2 (136, 137). Of 175 Brazilian isolates collected from dried fruits, Brazil nuts, coffee beans, grapes, cocoa, and onions and previously identified as *A. niger*, about one-half have now been reidentified as *A. welwitschiae*. Among *A. niger* isolates, approximately 74% were fumonisin B_2 producers, compared to 34% of *A. welwitschiae* isolates (137).

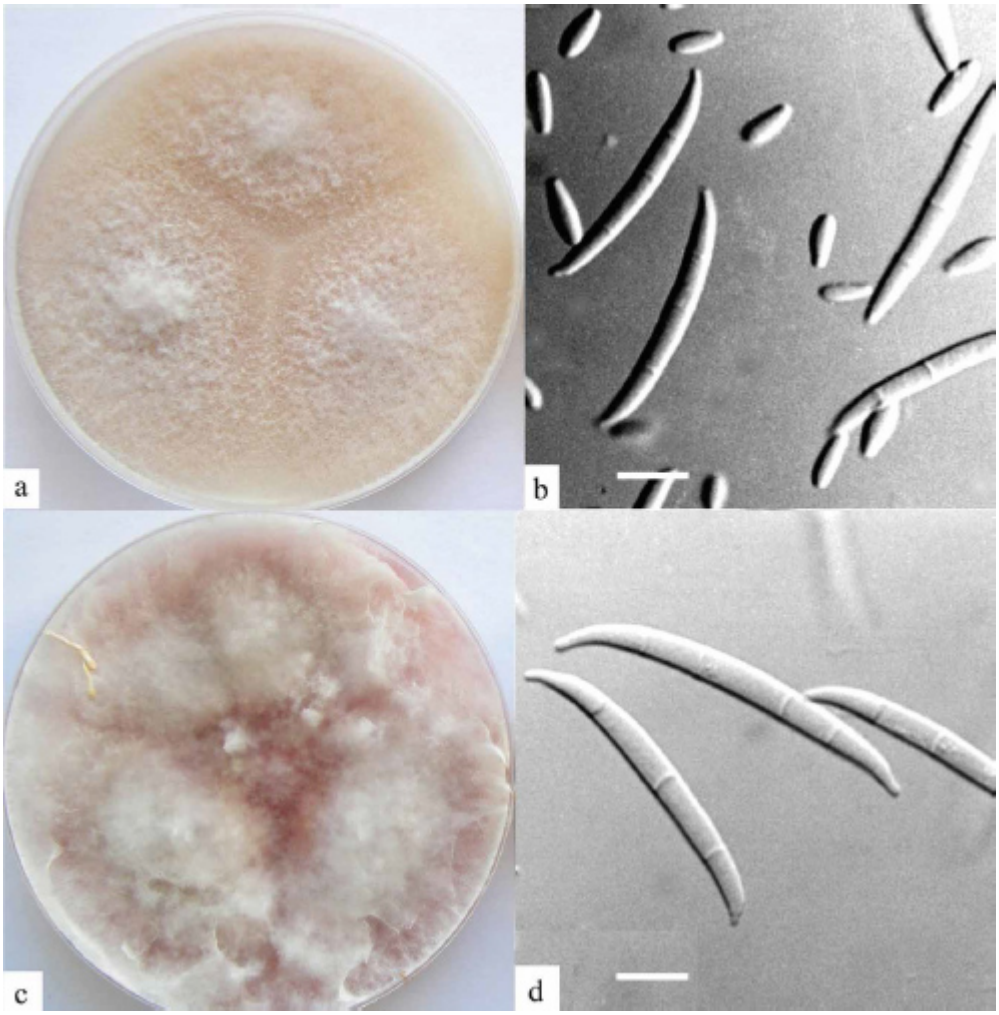


Figure 22.5 (a) *F. verticillioides*, PDA, 7 days at 25°C; (b) *F. verticillioides* macroconidia (bar, 10 mm); (c) *F. graminearum*, PDA, 7 days at 25°C; (d) *F. graminearum* macroconidia (bar, 10 mm).

Toxicology

Fumonisins are remarkable for their wide range of effects in animals and humans. Fumonisins act by inhibiting ceramide synthase, causing accumulation of intermediates of sphingolipid metabolism and also causing depletion of complex sphingolipids. These effects interfere with the functions of some membrane proteins, including folate binding. The most dramatic effect occurs in horses, in which it causes equine leukoencephalomalacia. This is a rapidly progressing disease that turns equine brains to mush. For

horses, consumption of feed containing >10 mg/kg fumonisin B_1 in the diet (equivalent to 0.2 mg/kg of body weight per day) was associated with increased risk of developing this disease (103). In pigs, fumonisins cause pulmonary edema due to left ventricle heart failure (103). In rats, the primary effect is liver cancer (138), but fumonisins also cause programmed cell death (apoptosis) (139). It is unusual for a single toxin to have such diverse effects in different animal species, and the reasons for that remain unknown (140).

In humans, fumonisins and *F. verticillioides* are associated with esophageal cancer. Extensive studies in areas of low and high maize consumption in South Africa have established this connection (103, 141). This disease is also prevalent in areas of China (142) and also occurs at significantly higher levels than background in parts of Iran, northern Italy, Kenya, and a small area of the southern United States (103). In all those areas, consumption of maize and maize products is very high.

Some evidence exists that a high intake of fumonisins from maize may be associated with neural tube defects such as spinal bifida in areas of Guatemala, South Africa, and China and a population along the Texas-Mexico border (143). In animal models, folate supplementation has been shown to reduce the incidence of neural tube defects (144). Numerous studies have shown that antioxidants can prevent some of the cytotoxic effects of fumonisins and other mycotoxins (103, 145).

Analysis of Fumonisins

The most effective solvent for extraction of fumonisins is methanol-water (146). The most satisfactory cleanup method is the use of a solid-phase extraction cartridge (146).

The traditional TLC method is still in use and is recommended for less developed economies, as it is inexpensive and reliable (146). Enzyme-linked immunosorbent assay techniques are also effective for fumonisin analysis. Although antibodies are raised against fumonisin B_1, crossreactivity usually occurs with fumonisins B_2 and

B_3 as well. Commercial kits are available from several manufacturers, and a range of methods has been described (147).

Detection of fumonisins by HPLC continues to be widely used, although ultra-high-pressure liquid chromatography (UHPLC) is in increasing use, as it has the potential to reduce separation times. As the fumonisins lack a good chromophore, chromatographic methods generally rely on MS or derivatization and detection by fluorescence or UV absorbance. A large number of such methods for fumonisins have been published, either singly or in combination with other mycotoxins (43).

Occurrence and Regulation in Foods

The major source of fumonisins in foods is maize, though other small grains may have low levels at times. With the discovery that *A. niger* may produce fumonisins, the range of foodstuffs where fumonisins may be found has become much wider. The toxicological implications of this discovery have not yet been assessed, but it is unlikely to have a major impact on food safety.

The United States has established guidelines for fumonisin levels: dry milled grain products should contain no more than 2 mg/kg of total fumonisins (148). In the European Union, the limits are 1 mg/kg for maize flour, semolina, germ, and oil and 0.4 mg/kg for products that are ready for consumption, except that for babies and children the limit is 0.2 mg/kg (149).

Control

Fumonisins occur in maize preharvest. It is known that drought stress and insect damage are major factors inducing production, so good agricultural practices, rain, irrigation, and the cultivation of cultivars carrying genes from *Bacillus thuringiensis* (Bt maize genotypes) are all important in limiting fumonisin formation. Some progress has been made in breeding cultivars resistant to *Fusarium* ear rot (107, 123, 127).

As insect damage and fumonisin levels correlate, transgenic Bt maize genotypes can lower levels of *F. verticillioides* infection and fumonisin formation (127).

Freshly harvested maize should be rapidly dried to a stable moisture level as soon as possible (150, 151), but this is important only in the initial stages of drying because *Fusarium* species do not grow below an a_w of about 0.9, so once the kernel moisture content has been reduced below that figure, fumonisin accumulation ceases (107). Moreover, fumonisins are not produced in storage. Even if very high moisture occurs due to water ingress, competition with other microorganisms at such high water activities will prevent any significant increase in fumonisin levels (107).

In most geographical areas, the main methods for controlling fumonisin levels are visual inspection of lots for fungal damage, followed by fumonisin analyses and rejection of lots that do not meet specifications. Visible sorting of maize grain as a technique to reduce fumonisin levels by subsistence farmers has been proposed (152) and optimized (153, 154).

In Central America, the process of nixtamalization removes almost all fumonisins, so tortillas and other maizebased foods are substantially free of these mycotoxins in that region (155).

Effect of Processing on Fumonisins

Wet milling of maize produces maize starch, germ, and fiber, while dry milling produces bran, germ, and fractions of decreasing particle size—grits, corn meal, and corn flour (156). Fumonisins are not destroyed during these processes and are found in all fractions, with higher concentrations in bran and germ (157–159).

Fumonisin levels are reduced by processing at temperatures above 150°C, including frying (160), baking, roasting, and extrusion (158, 161, 162). Extrusion is used extensively in the production of breakfast cereal, snack foods, and textured foods. The highest

reduction in fumonisin levels occurs at extrusion temperatures of 160°C or higher and in the presence of glucose (163, 164).

DEOXYNIVALENOL

Chemical Characterization

DON belongs to the family of chemicals known as the trichothecenes, sesquiterpenoid compounds that are characterized by a 12,13-epoxy ring. At least 100 trichothecene molecules are known, differentiated by hydroxyl or acetyl groups and side chains. DON, still sometimes known as vomitoxin in the United States, is 12,13-epoxy-3,7,15-trihydroxy-trichothec-9-en-8-one, CAS registry no. 51481-10-8 (165) (Fig. 22.4). It is the most commonly produced trichothecene. Nivalenol differs from DON by the substitution of a hydroxyl group for the hydrogen atom at the 4 position. The most toxic of these molecules is known as T-2 toxin, but it is not commonly produced in foodstuffs.

Fungal Sources

DON is produced by *Fusarium graminearum* (often listed as *Gibberella zeae*, its sexual stage), *Fusarium culmorum*, and, less commonly, some related species.

F. graminearum grows rapidly on any standard mycological medium, including CYA, MEA, and PDA (6). Colonies on CYA and MEA are grayish rose, grayish yellow, or paler, with reverses being orange red to yellowish brown. On PDA, colonies are yellowish brown to reddish brown, sometimes with a central mass of red-brown to orange areas bearing macroconidia, with the reverse being dark red.

Characteristic *Fusarium* conidia, macroconidia, are produced on dichloran chloramphenicol peptone agar or other specialist media, usually with five septa, and are thick walled and straight to

moderately curved, with a distinctly foot-shaped basal cell; microconidia are not produced (6, 126) (Fig. 22.5c and d).

Colonies of *F. culmorum* are similar to those of *F. graminearum* but are pale red to pastel red on CYA, MEA, and PDA; the reverse on CYA and PDA is pastel red to deep red, and that on MEA is brown to reddish brown. Macroconidia are relatively short, wide, and only slightly curved, with four or five septa; they are 30 to 45 μm long, with basal cells having a slight to definite notch. Microconidia are not produced (126).

F. graminearum occurs in maize, and both *F. graminearum* and *F. culmorum* occur in small grains, especially wheat and barley. These species are rank pathogens, invading plants and grains by causing diseases, known as *Gibberella* ear rot in maize and *Fusarium* head blight in wheat, barley, and triticale. Epidemics of *Gibberella* ear rot require the congruence of three factors: airborne or insect-borne spores, inoculation at the time of susceptibility, and appropriate moisture and temperature (123, 127). This disease is prevalent in north temperate climates, especially in wet years, and much less common in the tropics.

Fusarium head blight affects all commercial cultivars of wheat and barley. The formation of DON or nivalenol by *F. graminearum* depends on the geographical origin of the fungal strain (165, 166), while *F. culmorum* always produces DON (166, 167).

Toxicology

Like all trichothecenes, DON and nivalenol are inhibitors of protein synthesis (168). Nivalenol is much more toxic than DON but is produced in much lower quantities in grains and is not considered a significant mycotoxin (168). Trichothecene toxicoses in humans appear to be rare (169), but DON has been known to cause gastrointestinal problems and immunotoxicity in humans (170). DON causes feed refusal by pigs at quite low concentrations, resulting in retarded growth and economic losses (171, 172). Apart

from a general effect on protein synthesis, the mechanism of action of DON remains unclear (103).

Analysis of DON

Analysis for DON requires solvent extraction followed by cleanup by filtering through a C_{18} or silica gel column. HPLC with UV or UV plus diode array detection is the most common assay method; the more sophisticated UHPLC-MS/MS is also used (43). A multimycotoxin immunoaffinity column for the simultaneous determination of DON, ZEA, and T-2 and HT-2 trichothecenes is available commercially (173). The authors claim that the advantages of combining an immunoaffinity column with HPLC-MS/MS include efficient removal of matrix interferences, clean chromatograms, high selectivity, low limit of detection, and separation of a wide range of molecules with different physicochemical properties in a single run (173). A number of publications have reported methods to detect the so-called "masked" mycotoxins, such as DON-3-glucoside (43, 174). Such compounds are not assayed by the usual techniques.

Occurrence and Regulations

DON is found in maize and small grains in all areas where these crops are grown. It is especially prevalent in cooler areas where rainfall is high, such as Canada (175, 176) and Argentina (177), and less common in drier, hotter areas, such as Australia (178).

A number of studies have shown that fungal infection rates are higher in crops planted in fields previously planted with maize, particularly when residues from those crops are left in the field (123).

Favorable weather conditions are critical for infection by *F. graminearum* to occur in wheat heads. Researchbased field observations have confirmed that temperature and moist conditions during heading and anthesis are the major factors of importance (179).

The U.S. Food and Drug Administration has issued guidelines for DON in foods and feeds as follows: for finished wheat products that may be consumed by humans, 1 mg kg^{-1}; for grains and by-products for feedlot and dairy cattle, 10 mg kg^{-1}, except that for dairy cattle the total DON content in feed should not exceed 5 mg kg^{-1}; in feed for pigs, 5 mg kg^{-1}, with the added recommendation that these ingredients not exceed 20% of their diet; and for all other animals, 5 mg kg^{-1}, with the same recommendation that these ingredients not exceed 40% of the total diet (180).

Control

Some success has been achieved in controlling DON formation in wheat by the use of azole fungicides at anthesis (181, 182). Forecasting systems to advise farmers of the likelihood of DON formation have been developed in Canada (183) and Europe (184, 185). Otherwise, control relies on reducing levels of *Fusarium* species in the field by good management and crop rotation (103, 107).

ZEARALENONE

Chemical Characterization

ZEA is an estrogenic mycotoxin, described as belonging to the resorcylic acid lactone group. Its molecular formula is $C_{18}H_{22}O_5$, and its CAS registry number is 17924-92-4 (5) (Fig. 22.4).

Fungal Sources

ZEA is produced by the *Fusarium* species that produce DON and nivalenol, and generally speaking, it is produced under the same conditions, the main sources being maize and small grains (186).

Toxicology

ZEA has a low acute toxicity, but it and its metabolites, the zearalenols, possess estrogenic activity in pigs, cattle, and sheep. The most obvious problems are seen in pigs: doses of ZEA as low as 1 mg/kg can induce vulvovaginitis and vaginal and rectal prolapse in young female pigs (171). ZEA is also considered to be hepatotoxic, hematotoxic, immunotoxic, and genotoxic, but no studies of human carcinogenicity have been reported. The nature of its toxicity is not completely understood (186). IARC has classified ZEA as group 3, noncarcinogenic for humans (2). Estimates of human exposure from dietary sources are generally in the range of 1 to 30 ng/kg of body weight per day (183).

The metabolism of ZEA involves reduction of the 6-keto group, resulting in the formation of α- and β-zearalenol. α-Zearalenol has greater estrogenic activity than ZEA (187). ZEA has been implicated in early puberty and advanced growth of girls in Hungary and Italy (188). Its importance in human health is still poorly understood.

Analysis

The use of the immunoaffinity column method for the simultaneous detection of major trichothecenes in cereals is also effective for analysis of ZEA (173). Methods coupling MS to HPLC or UHPLC are also in use (43). UHPLC-MS/MS methods have also been developed for the quantification of ZEA and its derivatives α-zearalenol and β-zearalenol (43). A novel immunoassay using a magnetic bead electrochemical immunoassay on microfluidic chips for the determination of ZEA has also been reported (43).

Occurrence and Regulation

ZEA occurs in the same grains as DON, i.e., maize and small grains, and it occurs in all regions of the world (186).

No internationally recognized limits for ZEA exist, and specific regulations vary from 20 to 1,000 μg/kg or more (186). JECFA has established a provisional maximum tolerable daily intake of ZEA and its metabolites of 0.5 μg/kg of body weight per day (101).

Control

Control of ZEA in crops is similar to that of DON.

GENOMICS

Genomics, the study of entire genomes, provides basic information to build the knowledge base of gene functions, which will assist in understanding mycotoxin formation and reduction in crops. Understanding the molecular basis of plant resistance, where it exists, will hopefully lead to identification of biochemical factors for use in plant breeding or genetic engineering (56). Studies on economically important fungi at the genomic level will assist in understanding mycotoxin biosynthesis and also in understanding the biology, evolution, biochemical function, and genetic regulation of the genes in these fungal systems. "-Omics" studies, including genomics, transcriptomics, metagenomics, proteomics, and metabolomics, have been carried out on several toxigenic fungi, including *A. flavus*, *A. niger*, *F. graminearum*, and *F. verticillioides* (189, 190). Genome sequences have been determined for some toxigenic fungi, notably *A. niger*, *F. graminearum*, *F. verticillioides*, and *Fusarium solani*. The availability of large amounts of genomic and expressed sequence tag data has provided tools for large-scale functional analysis of gene expression in *F. graminearum* and *F. verticillioides*.

The sequencing of the *A. niger* genome led to a most unexpected result. Searches for biochemically active genes showed the presence of the genes for fumonisin (191), which was independently verified (192). It was soon confirmed that this gene cluster (consisting of at least 15 genes) was complete and active, and the ability of at least some strains of *A. niger* to produce fumonisins was confirmed (124). Indeed, recent information indicates that fumonisin production by *A. niger* is common. In one study on *A. niger* strains from a sample of Californian raisins, 50 of 66 strains (77%) produced fumonisins (135). In a second study, where isolates were

taken from 13 samples of dried vine fruits from several countries, 20 of 30 (67%) were producers (193).

Research on *Fusarium* head blight has used fungal genomics to determine the role of DON in pathogenicity and the effect of accumulation of this toxin on crop production. The information may be useful in developing resistant germplasm (56).

The availability of modern "-omics" techniques has facilitated a rapid expansion of data on the biology of toxigenic fungi, increasing significant knowledge of the biological, biochemical, and biophysical molecular processes regulating the production of mycotoxins and the adaptation of these fungi to environmental stresses (189).

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23
Foodborne Viral Pathogens

Human enteric viruses are responsible for substantial morbidity worldwide. Transmitted predominantly by the fecal-oral route and exclusively in association with human feces and/or vomitus, these viruses come into contact with humans by a variety of routes, including the consumption of contaminated foods. A functional rather than taxonomic group, the human enteric viruses are represented by many different virus families and genera (Table 23.1). From an epidemiologic perspective, the most significant of these are human noroviruses (hNoVs), which are the most common cause of acute gastroenteritis worldwide and are now recognized as the leading causes of foodborne illness (1). For example, in the United States, hNoVs are estimated to be responsible for about 58% of all domestically acquired foodborne illnesses of known etiology, causing more than 5 million estimated foodborne cases annually (2). Additionally, epidemiologic surveillance demonstrates that they were the cause of 48% of all foodborne disease outbreaks of confi rmed etiology reported to the U.S. Centers for Disease Control and Prevention (CDC) via the National Outbreak Reporting System (NORS) in 2015 (3). Furthermore, hNoVs were estimated to be responsible for ~ 15,000 (range, 8,097 to 23,323) foodborne diseaserelated hospitalizations every year, second only to *Salmonella enterica* as the leading cause of hospitalizations due to foodborne disease. Human noroviruses also cause an estimated 150 foodborne-disease-related deaths annually in the United States (2). Enteric viruses in general may also be responsible for a large proportion of foodborne diseases of unknown etiology, the burden of which is substantial (4).

Enteric viruses can be transmitted directly by personto-person contact or indirectly by consumption of contaminated food or water or contact with fomites. The usual source of enteric virus contamination is human fecal matter, which can easily harbor up to 10_{10} genomic RNA copies per gram when shed by infected individuals—both symptomatic and asymptomatic (5). However, the role of vomitus cannot be overlooked, particularly for hNoVs, as virus particles can be shed during a vomiting episode (e.g., up to 10_5 viruses per ml), and this is likely a significant factor contributing to transmissibility (6). Aerosolization of vomitus can result in infection of exposed subjects who inhale and subsequently swallow the aerosolized virus (7); it can also provide a source of virus to be deposited on and contaminate nearby surfaces.

Table 23.1 Human enteric viruses

Family	Genus (type species)	Disease syndrome(s)	Role of foodborne transmission
Adenoviridae	*Mastadenovirus* (human adenovirus C)	Six subgroups (A–F); serotypes 40 and 41 in group F are the major causes of gastroenteritis in young children; second in prevalence to rotavirus	Credible but not documented
Astroviridae	*Mamastrovirus* (human astrovirus)	Two genogroups (A and B); human astroviruses cause acute gastroenteritis in children, elderly, and the immunocompromised	Credible but not well documented
Caliciviridae	*Norovirus* (Norwalk virus)	Seven genogroups (I–VII); I, II, and IV cause disease in humans with II being the most common; hNoVs cause acute gastroenteritis that is usually self-limiting	Well documented
	Sapovirus (Sapporo virus)	Five genogroups (I–V), four infect humans; causes sporadic outbreaks and cases of gastroenteritis that is usually self-limiting	Documented but rarely causes Outbreaks

Family	Genus (type species)	Disease syndrome(s)	Role of foodborne transmission
Hepeviridae	*Orthohepevirus* (hepatitis E virus Burma)	Causes outbreaks and cases of enterically transmitted acute hepatitis that are usually self-limiting; pregnant women are at increased risk for mortality	Travel-related transmission via waterborne routes; zoonotic transmission documented in developed countries
Picornaviridae	*Enterovirus* (poliovirus)	Cause asymptomatic or mild forms of gastroenteritis, meningitis, encephalitis, myelitis, myocarditis, and conjunctivitis	Credible but not well documented
	Hepatovirus (hepatitis A virus)	Causes relatively mild form of acute hepatitis, usually self-limiting; symptoms and severity increase with age	Well documented
	Parechovirus (human parechovirus)	Respiratory and gastrointestinal symptoms in young children; occasionally infection of the central nervous system	Credible but not well documented

Family	Genus (type species)	Disease syndrome(s)	Role of foodborne transmission
	Kobuvirus (Aichi virus)	Three species (A–C); species A type 1 causes gastroenteritis in humans	Documented
Reoviridae	*Rotavirus* (rotavirus A)	Five groups (A–E); type A causes gastroenteritis and dehydration in children <24 mo, milder disease in older children; major cause of child mortality worldwide	Documented but rare

Human enteric viruses have properties that are distinct from those of bacterial foodborne pathogens. Viruses are usually species specific and tissue tropic, meaning that the human enteric viruses are believed to infect only humans. Since they must resist the enzymatic conditions and pH extremes encountered in the gastrointestinal tract, enteric viruses are also resistant to a wide range of commonly used food-processing, preservation, and storage treatments (8). In addition, human enteric viruses are notably persistent in foods and the environment, frequently surviving for days to weeks and even months without significant loss in infectivity. Although frequently present in low numbers in contaminated foods, their infectious doses are also low (9), meaning that very low levels of contamination may pose a human health risk.

HUMAN ENTERIC VIRUSES OF EPIDEMIOLOGIC SIGNIFICANCE AND THEIR DISEASES

Virus groups that can be transmitted by foodborne routes include the human enteroviruses (poliovirus, coxsackievirus, and echovirus), enteric adenoviruses, astroviruses, hepatitis A and E viruses, rotavirus, hNoVs, and sapoviruses, among others. From an epidemiologic perspective, hNoV and hepatitis A virus (HAV) are the two most important foodborne viruses. The former is most significant by virtue of the sheer numbers of cases, the latter because it causes a more severe disease.

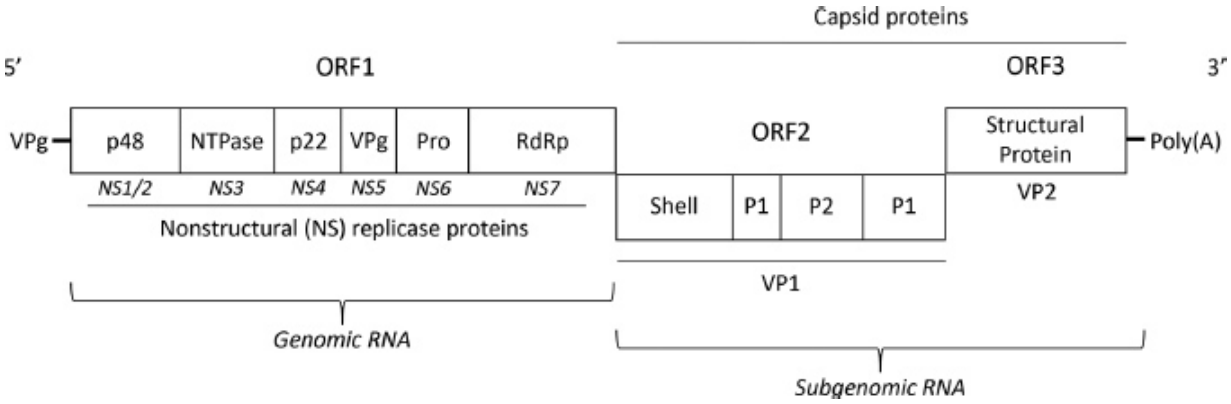


Figure 23.1 Norwalk virus genome organization (adapted from reference 10). VPg, genomelinked virion protein. ORF1 encodes a polyprotein that is posttranslationally cleaved into the nonstructural proteins helicase (with a predicted nucleotide triphosphate-binding domain [NTPase]), proteinase (Pro), and RNA-dependent RNA polymerase (Pol). ORF2 encodes capsid protein, which is translated into major and minor structural proteins consisting of shell and protruding domains. N, NH_2-terminal arm. ORF3 encodes a basic protein of unknown function.

Norovirus

The Norwalk virus was first reported in 1972 by Kapikian and colleagues, who identifi ed a small, roundstructured virus 27 nm in diameter by electron microscopy when viewing fecal material obtained from a 1968 gastroenteritis outbreak (10). Since that time, many similar so-called small, round-structured viruses have been reported. Virtually all of these viruses are now considered members of the family *Caliciviridae* (from the Greek word *calyx*, which means “cup”). Research over the past 4 decades has resulted in significant advances in

Caliciviridae taxonomy, and current classifications include five genera: (i) *Vesivirus*; (ii) *Lagovirus*; (iii) *Norovirus*, with Norwalk virus as the prototype strain; (iv) *Sapovirus*, represented by the Sapporo virus; and (v) *Nebovirus* (11). There are additionally six unclassified caliciviruses that may represent new genera; these are tentatively named *Bavovirus*, *Nacovirus*, *Recovirus*, *Salovirus*, *Sanovirus*, and *Valovirus* (11). Members of the genera *Nebovirus*, *Vesivirus*, and *Lagovirus* infect animals, posing no known human disease risk, whereas the genera *Sapovirus* and *Norovirus* are responsible for epidemic gastroenteritis in humans.

The genomes of viruses within the family *Caliciviridae* consist of a single strand of positive-sense RNA ranging in size from 7.4 to 8.3 kb. For example, the Norwalk virus genome sequence is 7,642 base pairs (bp) in length, excluding the 3′ poly(A) tail, and has a base composition of 48% guanine plus cytosine (G+C). The genome contains three open reading frames (ORFs): ORF1 (~5 kb) is the largest (corresponding to ~1,700 amino acids) and encodes a nonstructural polyprotein that is posttranslationally cleaved into p48, NTPase, p22, VPg (genome-linked virion protein), protease, and RNA polymerase; ORF2 (1.8 kb) encodes the primary viral capsid protein (VP1; 550 amino acids; molecular weight, ~56,600); and ORF3 (~0.6 kb) encodes a minor structural capsid protein (VP2) that is associated with the interior surface of the capsid formed by the shell (S) domain of VP1 (12) (Fig. 23.1).

The genus *Norovirus* consists of more than 40 genotypes, which are subdivided into seven genogroups (GI to GVII) based on RNA genome sequence analysis (13). There is only 51 to 56% nucleic acid sequence similarity across genogroups. Each genogroup can be further subdivided into several genotypes. Strains within a genotype share 69 to 97% nucleic acid sequence homology; there are 9 distinct genotypes of GI NoVs and 22 genotypes of GII NoVs (13). Genogroups I, II, and occasionally IV can cause human disease, with GII strains being responsible for the vast majority of human illness (13). Over the last 2 decades, the epidemic variants of the GII.4 genotype have been of particular interest, with multiple successive pandemic strains causing the majority of hNoV outbreaks worldwide (14, 15). Interestingly, GII.4 strains tend to be transmitted more commonly by person-to-person

spread rather than by food or waterborne mechanisms, although this observation may be confounded by their propensity to cause outbreaks in health care settings (16).

Hepatitis A Virus

First visualized by immune electron microscopy in 1972, HAV is classifi ed as a member of the genus *Hepatovirus* within the family *Picornaviridae* (17). The virus is a nonenveloped, round particle with a diameter of 27 to 32 nm. It has a linear, single-stranded, 7.5-kb, positive-sense RNA genome with a single ORF that is enclosed in an icosahedral capsid composed of three structural proteins, designated VP1-px, VP2, and VP3, as well as the viral capsid protein (VP4). The genome can be subdivided into three regions: a long 5′-terminal untranslated region (5′UTR) of about 735 bp, a large ORF (~7 kb) encoding a polyprotein of 2,227 amino acids, and a short 3′UTR with a poly(A) tail (Fig. 23.2). The single ORF is divided into three regions, designated P1, P2, and P3. P1 codes for the capsid proteins and is co- and posttranslationally cleaved into the four smaller capsid proteins (VP1-px, VP2, VP3, and VP4). P3 encodes the viral protease 3C, which cleaves the structural proteins of the capsid. Additional nonstructural proteins (2A, 2B, 2C, 3A, 3B [viral genome-linked protein], and 3D) are processed from the P2 and P3 regions of the ORF, and these are required for RNA synthesis and virus particle assembly (17). The approximate genome locations of these various proteins have been defined (17).

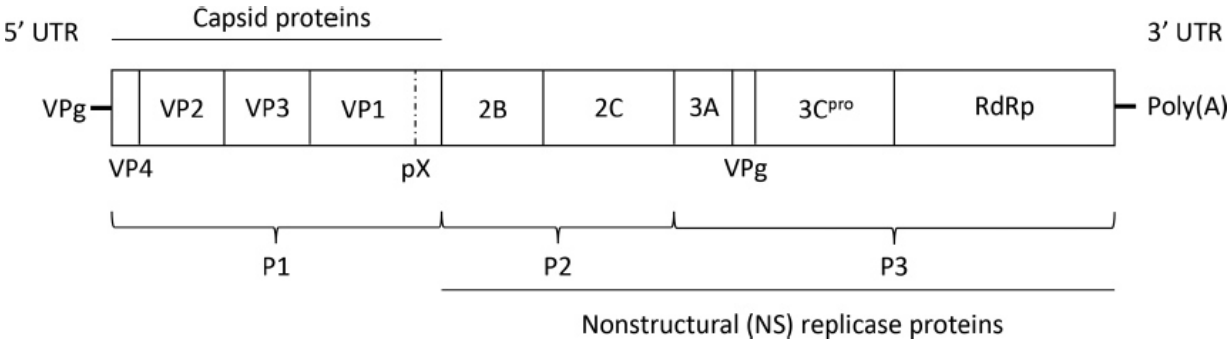


Figure 23.2 HAV genome organization (adapted from reference 121; see also reference 114). The 5′UTR is 624 to 1,199 nt long. VPg, genome-linked virion protein, 22 to 24 amino acids. The single ORF encodes a polyprotein that posttranslationally cleaved into VP1, VP2, and VP3, which form the capsid; VP4 is the inner surface capsid protein. 2A, unknown function; 2B, RNA synthesis and cell membrane permeability; 2C, RNA replication; 3A and 3B, RNA replication proteins, cofactor for 3D; 3C, viral proteinases; 3D, RNA-dependent RNA polymerase. The poly(A) tail is about 35 to 100 nt long; the 3′UTR is 47 to 125 nt long.

Human isolates of HAV constitute a single serotype, and monoclonal antibodies targeting different isolates fail to distinguish them from one another. Nonetheless, there is substantial sequence heterogeneity within certain genome regions that encode the putative VP1-P2A junction of HAV. When this region is used as the basis for typing, HAV isolates can be differentiated into six unique genotypes such that a genotype is defined as a group of viruses that differ from each other in sequence homology by no more than 15% (18). Of these six genotypes (designated I to VI), only genotypes I, II, and III are associated with human disease, and these are additionally subdivided into subtypes A and B, whereas genotypes IV, V, and VI have been isolated from non-human primates (17).

Other Foodborne Human Enteric Viruses

There are other human enteric viruses that can be transmitted by contaminated foods, although their epidemiologic significance is not as well understood. The human enteroviruses, for example, are smooth, round, nonenveloped particles 27 nm in diameter with singlestranded,

positive-sense RNA. These viruses are members of the family *Picornaviridae* (poliovirus is the type species) and historically have been transmitted through the consumption of contaminated water and unpasteurized milk (19). Although the disease can occasionally be problematic in developing countries, outbreaks of foodborne poliomyelitis usually do not occur in developed countries due to effective vaccination. Other human enteroviruses, such as the coxsackie- and echoviruses, have also been associated occasionally with foodborne disease (20, 21). The symptoms of enteroviral infection are diverse and virus specific but may include gastrointestinal, respiratory, neurological, and skin manifestations.

Rotaviruses usually are transmitted by the person-to-person route, but occasionally foodborne outbreaks occur (19). This virus group is the leading cause of infantile diarrhea worldwide (22) and is responsible for significant morbidity and an estimated 197,000 deaths per year; the vast majority of these deaths occur in developing countries. Rotaviruses are 70 to 75 nm in diameter and appear roughly spherical in electron micrographs. They consist of 11 segmented, double-stranded RNA molecules encased in a double-layered protein coat. The 5′ and 3′ ends of the double-stranded RNA are highly conserved, with sizes ranging from 667 bp (segment 11) to 3,302 bp (segment 1) and totaling 6,120 kDa, or 18,500 bp (23). All rotavirus genes are monocistronic (i.e., coding for only one polypeptide) except segments 9 and 11. There are six structural viral proteins (termed VP1, VP2, VP3, VP4, VP6, and VP7) and five nonstructural proteins (termed NSP1 through NSP5). Based on VP6 reactivity with monoclonal antibodies, there are at least seven different rotavirus groups (A through G). Second-generation rotavirus vaccines are now recommended for global use by the World Health Organization; however, there is a small recognized risk of intussusception, at around 1 in every 60,000 vaccinated infants (24).

Other significant human enteric viruses include the astroviruses, sapoviruses, and HEV. Astroviruses are spherical nonenveloped viruses that are star shaped and have single-stranded, positive-sense RNA that is 6.8 to 7.2 kb in length (19). These viruses have been occasionally associated with outbreaks, causing a diarrheal disease with an

incubation period of 1 to 3 days and symptoms lasting for 1 to 4 days. HEV is a small (~30-nm) virus with single-stranded, positive-sense RNA and three ORFs. Recently classifi ed in the species *Orthohepevirus A* and genus *Orthohepevirus* in the family *Hepeviridae*, HEV appears to have at least four major genotypes shown to infect humans (25). HEV causes a disease similar to that caused by HAV but with severe manifestations in pregnant women. It is much more prevalent in developing countries, although there is recent evidence of autochthonous infections in the developed world (26). HEV is transmitted predominantly through sewage-contaminated water and consumption of raw or undercooked meats from infected animals (27, 28).

The last human enteric viruses of note are the sapoviruses. These were thoroughly covered by Lopman et al. (29) and continue to be the cause of both sporadic gastroenteritis and outbreaks related to consumption of contaminated food. Sapoviruses are part of the genus *Sapovirus* in the family *Caliciviridae*. This group of viruses shares many characteristics with the closely related noroviruses. These similarities include clinical presentation of disease, broad age range of impacted populations, outbreak settings (e.g., long-term-care facilities and hospitals), and implicated food products (e.g., oysters). With respect to microbiological aspects of sapoviruses, they are small (~30-nm) viruses with singlestranded, positive-sense RNA and two ORFs. Similar to noroviruses, sapoviruses are divided into seven genogroups, with four (GI, GII, GIV, and GV) infecting humans and the remaining three (GIII, GVI, and GVII) infecting pigs. The actual burden of disease due to sapovirus infection is difficult to quantify given the limited foodborne outbreaks reported and the lack of routine diagnostics for suspected clinical cases.

FOODBORNE TRANSMISSION OF HUMAN ENTERIC VIRUSES

From a foodborne disease standpoint, three types of commodities are commonly associated with viral disease outbreaks: (i) molluscan shellfish contaminated by feces-impacted growing waters; (ii) fresh

produce items contaminated by human feces during production or packing, usually through workers' hands or contact with contaminated water; and (iii) ready-to-eat (RTE) and prepared foods contaminated by infected food handlers (Fig. 23.3).

Contamination of marine waters with human sewage is the critical factor with respect to virus contamination of molluscan shellfi sh (e.g., mussels, clams, cockles, and oysters). Sources of contamination in shellfish harvest waters include illegal dumping of human waste, failing septic systems along shorelines, sewage treatment plants overloaded with storm water, and discharges of treated and untreated municipal wastewater and sludge. Molluscan shellfi sh are of particular concern because they are filter feeders, meaning that they filter and even concentrate microorganisms in their gut during the process of feeding. Interestingly, hNoVs can specifically bind carbohydrate ligands within the oyster digestive tract (30). This is further complicated by the fact that viruses tend to be environmentally persistent and the shellfish are usually consumed either raw or only lightly cooked. Furthermore, there is no significant relationship between the presence of virus contamination and the levels of coliform and fecal coliform indicator bacteria, which are used in most countries to monitor and regulate molluscan shellfi sh harvesting waters. This means that there is no reliable means of screening by which to preventvirally contaminated shellfish from reaching the marketplace (19).

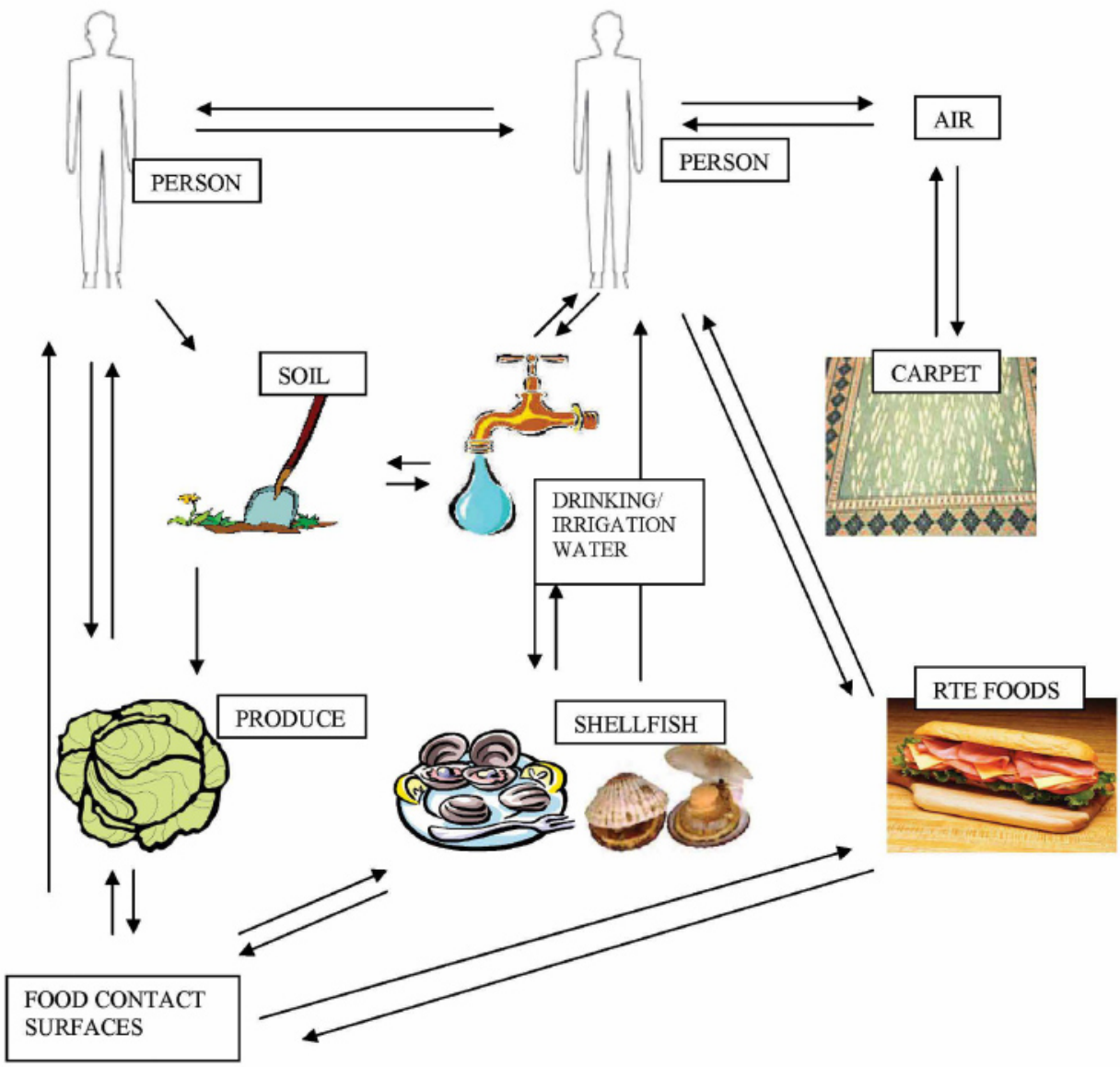


Figure 23.3 Transmission routes of foodborne viruses.

Although viral gastroenteritis outbreaks due to contaminated fresh produce occur regularly, there are many unanswered questions with respect to how fresh produce items actually become contaminated with enteric viruses. Certainly, produce may become contaminated when grown in fields irrigated with wastewater or fertilized and conditioned with improperly decontaminated sewage effluent. In fact, common sewage sludge treatments, such as drying, pasteurization, anaerobic digestion, and composting, reduce but do not necessarily eliminate enteric viruses (31). Viruses can survive in contaminated soil for long periods of time, depending upon factors such as growing season, soil

composition, temperature, sunlight, moisture, rainfall, resident microflora, and virus type (32, 33). Despite the potential for preharvest contamination, the role of infected farm workers who pick, wash, and pack produce cannot be overlooked, especially since simple produce items that are often associated with outbreaks (e.g., leafy greens, strawberries, raspberries, and green onions) are most often harvested by hand (34). While washing of produce may reduce contamination loads, it will not eliminate enteric viruses if they are present (35).

Poor personal hygienic practices of infected food handlers are one of the most important contributors to the spread of viral foodborne disease, more so for hNoVs than HAV (36). The CDC indicates that about 70% of foodborne hNoV outbreaks are linked to ill food service workers (3). There is strong evidence suggesting that contaminated hands frequently play a role in enteric virus spread, acting as either virus donors or recipients. Hands usually become contaminated by direct contact with any virus-containing fluid from oneself or others; they may also become contaminated by contact with virus-contaminated surfaces or objects. The extent of such contamination will vary depending on a variety of factors, including the virus load, the handwashing habits of the infected person, and the efficiency with which the virus is transferred and persists (37). Studies of hNoV persistence have revealed that viral RNA can be detected on the hands of volunteers for at least 2 hours after deliberate contamination (38). Food handlers may transmit viruses to foods from contaminated hands, a contaminated surface, or other food items. Technically, any food handled by a person infected with the virus—symptomatic or asymptomatic—and then consumed without a subsequent heating step can be a potential vehicle for transmission of enteric viruses. Common food items falling into this category include hand-sliced deli meats and cheeses; sandwiches; meat, vegetable, and fruit salads; baked goods; and various desserts.

EPIDEMIOLOGY OF FOODBORNE VIRAL DISEASE

Although many different types of enteric viruses can contaminate bivalve mollusks, only a few (HAV, hNoVs, sapoviruses, and astroviruses) have been epidemiologically linked to shellfi sh-associated viral disease, particularly in oysters (39). The first documented epidemiologic linkage between hNoVs and shellfi sh-associated gastroenteritis occurred in the mid-1970s, and many more outbreaks have been reported since then. For example, three large outbreaks associated with improper discharge of untreated human waste material occurred in Louisiana in the 1990s alone (40, 41). Between 2003 and 2004, three distinct hNoV outbreaks in Australia were associated with the consumption of imported oyster meat whose harvest was traced back to a single Japanese estuary system (42). In 2010, 334 hNoV cases associated with shellfish consumption were reported in five European countries (43). Interestingly, a 1988 outbreak of HAV disease in Shanghai, China, which was linked to the consumption of shellfi sh harvested from a feces-impacted site, ranks among the largest foodborne disease outbreaks ever reported, with a total of about 300,000 cases (44).

Enteric virus illness outbreaks have been associated with fresh and frozen produce as well. Among “simple” food products (i.e., those consisting of a single commodity type), berries and green onions have been the most common culprits. For example, between 2005 and 2009, berry-associated hNoV illness outbreaks were reported in France, Denmark, Sweden, and Finland (45). More recently in 2012, a large multistate outbreak of hNoV due to contaminated frozen strawberries was reported in Germany, with nearly 11,000 cases of gastroenteritis (46). A similar outbreak of hNoV illness linked to individually quickfrozen raspberries resulted in 615 confi rmed cases in Quebec and 15 cases in the United States from August 2016 to July 2017 (47). In most cases, these products were imported and thought to be contaminated during production or processing in their country of origin rather than through subsequent handling. Recent HAV illness outbreaks linked to imported frozen pomegranate arils (Turkey) and frozen strawberries (Egypt) were reported in 2013 and 2016, respectively, and were linked to 165 and 143 cases of viral hepatitis (48, 49).

An interesting group of HAV illness outbreaks occurred in 2003 in the United States. The largest of these occurred among patrons of a single Pennsylvania restaurant over a 3-day period, with a total of 601 confi rmed illnesses, 3 deaths, and at least 124 hospitalizations. A case-control study implicated foods containing green onions, particularly mild salsa. Although some of the restaurant workers became symptomatic, all became ill at the same time as the patrons, ruling them out as the source of the outbreak (50). A series of smaller outbreaks occurred among restaurant patrons in three other states in the months preceding this one, also implicating green onions (51). In both cases, the origin of the green onions could be traced back to northern Mexico. Comparative analysis of the HAV genome sequences associated with these 2003 outbreaks revealed substantial sequence similarity to endemic Mexican HAV strains, suggesting that the green onions were likely contaminated before arrival at the restaurants (50).

Although the ultimate source of viral contamination (e.g., water or harvester) in the case of the green onion and berry outbreaks was never identifi ed, it should be noted that these two products are almost always harvested by hand and hence subject to substantial human handling. The potential importance of human hands is also supported in a recent study by León-Félix et al. (34), who determined that between one-quarter and one-third of northern Mexican field workers' hands had evidence of hNoV contamination, with 30 to 45% of the green peppers they harvested having contamination as well. There is a clear need to better characterize the source of viral contamination in fresh produce items.

The contamination of RTE and prepared foods most frequently results from poor handwashing practices of infected food handlers after toilet use, as fecal material can be left on hands or even under nails and then can come in contact with food products. Consequently, handling cooked products with bare hands has been identified as a major factor for pathogen transfer to RTE foods (52). HAV outbreaks can also occur as the result of a single infected food handler at a single food establishment and have been reported in association with lettuce, salads, sandwiches, hamburgers, spaghetti, and bakery products (53). However, the numbers of these types of outbreaks have declined since

the implementation of vaccination recommendations for high-risk groups and infants (54).

Human NoVs have been associated with many outbreaks caused by poor hygiene of food handlers, including outbreaks involving the consumption of contaminated salads and sandwiches, delicatessen meats, and bakery products (3). In recent outbreak investigations, scientists have increasingly used molecular sequencing to support epidemiologic evidence indicating a common food source for hNoV transmission (55). These methods have enabled investigators to link multiple clusters of illnesses as well as implicate the contaminated food items. For example, Friedman et al. (56) described an hNoV disease outbreak among 2,700 guests attending 46 different weddings on a single weekend. All of the wedding cakes were baked at the same bakery, where there were at least two ill employees. The same hNoV strain was detected in stool specimens from two wedding guests, one wedding hall employee, and one ill bakery employee. Similarly, using molecular epidemiologic evidence, Malek et al. (57) were able to link 137 cases of hNoV illness occurring on 13 Colorado River rafting trips to one batch of delicatessen meat purchased from a single processing plant. It was determined that the implicated meat was sliced by an employee who did not wear gloves and had just returned to work one day after an episode of gastroenteritis. This outbreak was an eye-opener to facilities inspected by the U.S. Department of Agriculture Food Safety and Inspection Service, which frequently do not consider enteric viruses as hazards in their hazard analysis and critical control point (HACCP) plans.

Secondary transmission after a primary foodborne outbreak of hNoV illness can also occur. For instance, a GI hNoV that initially infected athletes on a single college football team who consumed sandwiches made by an infected food handler was later passed on to the rival team after contact during a football game in which the first team's members were actively ill (58). Alfano-Sobsey et al. (59) reported on a foodborne outbreak of hNoV associated with undercooked oysters and determined a secondary attack rate of 14% among susceptible household contacts. Isakbaeva et al. (60) investigated a hNoV gastroenteritis outbreak on a cruise ship that affected six consecutive cruises despite extensive

sanitation measures. Using genetic sequence analysis, the investigators documented strain persistence with likely foodborne, environmental, and person-to-person transmission. In general, foodborne hNoV illness outbreaks have higher primary attack rates than outbreaks spread through other routes, but as illustrated above, a single contamination event can affect many more people that those simply consuming the contaminated food.

DISEASE, PATHOGENESIS, AND TREATMENT

Human Noroviruses

Symptoms of Disease

The disease caused by hNoVs is usually self-limiting and characterized by nausea, vomiting, diarrhea, and abdominal pain, with occasional headache and low-grade fever. Data from outbreak investigations and human challenge studies indicate that the incubation period ranges from 12 to 51 hours, with a mean of 24 hours, and illness usually lasts 48 to 72 hours (61). In a community-based cohort study of hNoV infection in The Netherlands, Rockx et al. (62) reported a median duration of symptoms of 5 days and found that diarrhea was more prevalent in hNoV-infected children of <1 year of age, whereas infected children of ≥5 years of age were more likely to vomit. Some outbreak investigations have revealed less frequent vomiting in older individuals (63). Severe illness or hospitalization is uncommon except in children, the elderly, and the immunocompromised, for whom rehydration therapy may be necessary. Some deaths, particularly in elderly patients, occur in association with norovirus infection (64). Outbreaks associated with hNoV were historically characterized by the Kaplan criteria defined as vomiting in >50% of cases, a mean incubation period of 24 to 48 hours, an average duration of symptoms of 12 to 60 hours, and stool specimens that are negative for bacterial pathogens. However, Lively et al. (65) reported greater sensitivity and specificity as well as broader application in reported acute gastroenteritis outbreaks with the following CART (classification and regression tree)-derived profile: ratio of proportion of cases with fever to the proportion of

cases with vomiting, <1; proportion of cases with bloody stool, <0.1; proportion of cases with vomiting, ≥0.26. Finally, median attack rates are usually 20 to 53% (16).

Asymptomatic hNoV infections have been documented in outbreaks (66), human challenge studies (61, 67), and community- and hospital-based studies of sporadic hNoV infection (68, 69). Virus can be shed in the stool both before symptoms occur and for at least 60 days after the onset of infection, although peak shedding occurs during the acute phase of illness and for the first few days thereafter (5, 61). Atmar et al. (61) reported that median peak virus concentration in stool specimens from volunteers infected with Norwalk virus was approximately 10^{11} genomic copies per gram of feces. Meanwhile, Teunis et al. (5) estimated average peak values ranging from 10^5 to 10^9 genomic copies per gram of feces. As stated previously, virus can also be shed in high numbers (10^8 genomic copies per vomiting episode) in vomitus (6).

Pathogenesis

Host susceptibility to infection appears to be due to both genetic determinants and acquired immunity. Human challenge studies with Norwalk virus, a GI hNoV, and various GII strains have revealed that some volunteers remained uninfected even after exposure to high doses (6, 67). Further investigation revealed that these subjects lacked the H type 1 antigen, a histo-blood group antigen (HBGA) present in saliva and also on the surfaces of epithelial cells that likely serves as a receptor or coreceptor for Norwalk virus binding. *In vitro* assays provide additional evidence that recombinant Norwalk virus capsid protein binds to H type 1 antigen; specifically, the P domain of the capsid protein is now recognized as the exact binding domain (70). Synthesis of the H type 1 antigen depends on the α-1,2-fucosyltransferase enzyme, which is encoded by the *FUT2* gene and determines the secretor status of an individual. Individuals who are homozygous recessive for the *FUT2* gene are secretor negative and appear to have innate genetic resistance to infection with Norwalk virus while maintaining susceptibility to other norovirus strains (71). Overall, *FUT2* varies with ancestry, as confi rmed by Currier et al. (72). About 20% of Caucasians are secretor negative, and this proportion is

variable in Asian and African ethnic groups (73). Along with secretor status, the role of individual blood type in Norwalk virus infection has also been investigated. For instance, blood group O individuals in Norwalk virus human challenge studies were more susceptible to Norwalk virus infection than group A or B individuals (67, 74). In contrast to Norwalk virus, a human challenge study with Snow Mountain virus, a GII.2 hNoV, revealed no relationship between infection with the virus and blood group secretor status (75).

There have been mixed reports about whether there is a relationship between infections with other hNoV strains and HBGAs in outbreaks. Hennessy et al. (76), in their investigation of an outbreak in a military field hospital in Afghanistan, reported that individuals with blood group B were less susceptible to symptomatic hNoV infection than people of other blood types. Tan et al. (77) reported that secretor status and HBGA were associated with risk of infection in two GII outbreaks in China. Positive secretor status was strongly associated with risk of symptomatic infection in both outbreaks ($P = 0.0007$), and in the GII.4 outbreak, individuals with blood type A had a significantly increased risk of infection, whereas type O individuals had a decreased infection risk. In contrast, Halperin et al. (78) did not find a relationship between infection with GII hNoV and ABO histo-blood group in their investigation of two outbreaks in military units in Israel. The GII.4 hNoVs appear to bind to all HBGAs (not just type 1) (79) and, hence, are likely to have a greater pool of susceptible individuals in the population. This characteristic may explain the greater frequency of outbreaks due to GII.4 than that of other hNoV strains. The relationship between hNoV infection and HBGAs was recently reviewed (80). Through a meta-analysis, the authors concluded that recognition of and binding to carbohydrate receptors constitute an important feature of hNoV infection and may also play a role in virus evolution.

Immunity

Immunity to hNoV was recently reviewed by Newman and Leon (81). There is some evidence of acquired immunity, although the mechanisms for this have not been elucidated. Some human challenge

studies have revealed that volunteers who became ill after a primary hNoV challenge did not become ill when rechallenged with the same virus 6 to 14 weeks later; however, the volunteers did become ill when rechallenged with same virus 24 to 42 months after the primary challenge (82, 83). This suggests the development of short-term protective immunity but perhaps not long-term immunity. The high attack rates observed in hNoV outbreaks also suggest that most of the population do not have protective immunity against hNoV infection, either because the protective immunity is so short-lived or because hNoV strains are very diverse, and protective immunity is not cross-reactive.

The role of serum antibodies in hNoV infection is also not well understood. Sera collected in human challenge studies and hNoV outbreak investigations indicate that titers of hNoV-specific humoral antibody (immunoglobulin G [IgG], IgM, and IgA) increase in response to infection. Serum IgG produced in response to infection with one hNoV strain often cross-reacts with antigens from other hNoV strains, and common epitopes within and between genogroups have been identifi ed (84). IgA and IgM responses appear to be more type specific than that of IgG. However, it is not clear that serum antibodies provide any protection against subsequent hNoV infection, and these antibodies may only be markers of susceptibility to hNoV infection. *In vitro* experiments suggest that serum IgG produced in response to Norwalk virus infection is capable of blocking Norwalk virus attachment to synthetic H type 1 antigen (85). However, volunteers with anti-Norwalk virus IgG in their prechallenge sera were more likely to become infected after Norwalk virus challenge than those who did not have anti-Norwalk virus IgG (67). There are a small number of studies on the cellular immune response to hNoV infection (75, 86, 87). Overall, these studies have revealed that peripheral blood mononuclear cells from subjects in hNoV challenge studies respond to hNoV antigen *in vitro* and secrete anti-inflammatory and inflammatory cytokines, but gamma interferon secretion responses differ by individual and are probably confounded by the effect of individual prechallenge exposure history.

One Norwalk virus human challenge study from 2003 revealed that 62% of secretor-positive volunteers became infected, suggesting that some mechanism exists to protect genetically susceptible individuals from Norwalk virus infection (67). Examination of Norwalk virus-specific salivary IgA levels revealed that the secretor-positive volunteers who did not become infected mounted an early (before 5 days postchallenge) Norwalk virus-specific salivary IgA response to the virus. In contrast, infected secretor-positive volunteers did not mount a Norwalk virus-specific salivary IgA response until after 5 days postchallenge. Until recently, the role of the mucosal response in protective immunity to Norwalk virus infection was not understood. Ramani et al. (87) reported that a lower risk of gastroenteritis and decreased severity of gastroenteritis were associated with preexisting hNoV-specific salivary IgA. hNoV-specific fecal IgA was also evaluated and showed no correlation with protection from infection or disease; however, the authors did observe that prechallenge levels of fecal IgA did correlate with reduction of viral load and that fecal IgA induction seemed important in decreasing the longevity of virus shedding.

Histopathology

Patients who suffer from hNoV illness have broadened and blunted villi of the proximal small intestine (duodenum), resulting in a 47% reduction in villus surface area (88). Intraepithelial infiltration of cytotoxic lymphocytes along with cytoplasmic vacuolization has also been visualized. In addition, duodenal biopsies in subjects with hNoV infections had reduced expression of tight junctional proteins and an increase in epithelial apoptosis (88). Biopsy specimens collected in 1973 during one of the first human challenge studies reveal that histopathologic changes appear within 24 hours of virus challenge and usually persist for up to 2 weeks (89). Histopathologic testing revealed that the intestinal mucosa appear intact, and viruses were not detected in the epithelial cells of the mucosa by electron microscopy. Also, transient malabsorption of fat, d-xylose, and lactose was observed, along with decreased levels of small intestine brush border enzymes (trehalase and alkaline phosphatase). However, adenylate cyclase activities of the jejunum were not elevated. Reduced gastric motility is likely responsible for the nausea and vomiting caused by hNoV (90).

Vaccine Development

Vaccine development efforts are under way and have been reviewed by Herbst-Kralovetz (91) and most recently by Melhem (92) and Lucero et al. (93). Researchers are exploring various vaccination strategies, which have most recently focused on the use of recombinant hNoV capsid protein virus-like particles (VLPs) made in insect cells and plants to stimulate immunity in oral and parenteral vaccination strategies (91, 94). Additional approaches to hNoV vaccines include Venezuelan equine encephalitis virus replicon-based vaccines (95), hNoV P particles (96), and an adenovirus-vectored vaccine that uses an oral pill formulation for which the phase I human trial was completed in 2018 (NCT02868073). Nearly 2 decades ago, phase 1 clinical trials in humans found that Norwalk virus VLPs are safe and immunogenic, stimulating the production of systemic and mucosal anti-Norwalk virus antibodies and antibody-secreting cells (97, 98). Additional hNoV vaccine trials in humans have evaluated the efficacy of intranasal administration of GI.1 VLPs with and without an adjuvant to elicit greater immune response (99, 100). Phase 1 clinical trials have also been performed using GI.1 and GII.4 VLPs administered intramuscularly, and strong immune responses were observed (101–104).

Because hNoV outbreaks continue to occur in countries with high standards of sanitation and hygiene, vaccination may be an effective way to control epidemic hNoV infection and reduce the burden of hNoV disease. Moreover, there are a variety of potential vaccination strategies (e.g., age based, occupational, and risk based) which could all potentially help in preventing epidemic disease. However, at this stage there are many questions that need to be addressed about whether the current vaccine approaches stimulate a cross-protective immune response, whether it is possible to develop long-term immunity, and how to measure immune correlates of protection (92). Furthermore, these viruses are diverse and evolve rapidly. The number of antigens that would be needed for an effective vaccine and the degree of cross-protection provided against other strains is not known. The development of a chimpanzee model for hNoV infection was recently reported and used to test GI (Norwalk virus) and GII (MD145) VLP

vaccines (105). This landmark study revealed that the Norwalk virus VLP vaccine induced protective homologous immunity in chimpanzees that were challenged with Norwalk virus 18 months later, but the chimpanzees that received the MD145 VLP vaccine or placebo were not protected from Norwalk virus infection.

Treatment

hNoV infection is self-limiting, and if necessary, oral fluid and electrolyte replacement therapy is usually adequate for replenishing fluid loss. The symptoms of the disease can also be reduced by the oral administration of bismuth subsalicylate (106). In cases of severe vomiting and diarrhea, intravenous administration of fluids may be necessary. Hospitalization, particularly for severe dehydration, is necessary and occurs most often in young children and older adults. Norovirus-associated deaths can also occur across the age spectrum, but over 90% are estimated to occur among elderly patients (107, 108). Repeated diarrheal episodes may promote intestinal mucosal damage, resulting in eventual malnutrition (106). The development of antiviral drugs that block hNoV binding to host cells may be valuable as a prophylactic approach in settings where hNoV outbreaks are common. For instance, Chang and George (109) have reported that interferon and ribavirin inhibit Norwalk virus replication in replicon-bearing cells and may have therapeutic value. More recently, antiviral strategies targeting viral entry and inhibition of viral protein production have been reviewed, as well as additional approaches to inhibiting viral replication (110). One such approach includes the use of small inhibitory molecules that target host cell factors (e.g., La protein and DDX3) thought to be involved in viral RNA replication and genome circularization. Another option could be the use of nonnucleoside analogs that target and inhibit the hNoV RNA-dependent RNA polymerase, such as suramin (111).

Hepatitis A Virus

Symptoms of Disease

The incubation period for HAV infection is 14 to 50 days, with an average of 28 days. The virus is more readily transmitted during the

latter half of the incubation period and can be attributed to inadequate hand hygiene, but not as frequently as hNoV. Symptomatic HAV infection generally presents in four phases. The first phase is characterized by viral replication but a lack of symptoms. In the second, the so-called preicteric, or prodromal, phase, which usually lasts 5 to 7 days, patients may experience anorexia, nausea, vomiting, alterations in taste, arthralgias, malaise, fatigue, urticaria, and pruritus. When seen by a health care provider during this phase, patients can be misdiagnosed. The third, or icteric, phase presents as darkening of the urine, pale stools, jaundice, and right-upper-quadrant pain with hepatomegaly (112). Fecal shedding and viremia are maximal at the onset of the icteric phase and subside during its 7- to 28-day duration. During the last, or convalescent, phase, symptoms resolve, and liver enzymes return to normal. Although icteric disease occurs in fewer than 5% of children younger than 3 years of age and in 10% of 4- to 6-year-olds, it occurs in 40 to 50% of older children and in 70 to 80% of adults. In most cases, infection is mild and self-limiting; however, in older patients, more severe disease is possible. Complications such as acute liver failure, cholestatic hepatitis, and relapsing hepatitis occur but are rare (e.g., <1% of cases result in acute liver failure). A variety of extrahepatic manifestations occasionally occur in patients with acute hepatitis A, including hemolysis, skin involvement, vasculitis, acute renal failure (i.e., glomerulonephritis), lung inflammation, aplastic anemia, and pancreatitis; on occasion, neurological syndromes have also been reported (112). The overall mortality rate for HAV infection is approximately 0.01%. Immunity after infection is complete and considered to be lifelong (112).

Pathogenesis

HAV infection begins with ingestion of the virus, which penetrates the mucosal barrier of the gastrointestinal tract and reaches the liver via the portal circulation to begin replication in hepatocytes.

Approximately 10^2 to 10^5 HAV copies/ml of blood serum can be detected during the clinical illness phase of the disease (113). Studies are still under way to elucidate the nature of the host cell receptor that determines the tissue tropism of HAV for the hepatocyte. Hypotheses for viral cell entry have indicated a mucin-like glycoprotein receptor or

possibly an asialoglycoprotein receptor through recognition of a virus-IgA complex (112). However, a more recent review by Lemon et al. (114) indicates that the previously assumed HAV receptor, TIM1 (T cell immunoglobulin and mucincontaining domain protein 1, also known as HAVCR1), is not an essential factor for HAV cell entry based on cell culture and mouse models. Interestingly, the study authors determined that TIM1 may play a role during the early stages of infection by binding quasi-enveloped HAV virions—another infectious form of HAV resulting from nonlytic release from infected cells (115). Possible "enterohepatic" cycling of HAV may occur via ingestion of infected material, absorption from the small intestine, replication in the liver, secretion into bile following delivery to the intestines, and excretion in stool or reabsorption (112). Enlargement of Kupffer cells (liver macrophages) is the first evidence of infection by HAV. In experimental studies with owl monkeys, viral antigens were first detected in Kupffer cells of the liver at 14 days and in hepatocytes at 21 days after oral administration of the HM-175 strain of HAV (116).

Histopathology

Cardinal pathologic features of acute hepatitis include the presence of hepatocellular degeneration, characterized as either ballooning or acidophilic (apoptotic) change, together with varying degrees of portal and lobular inflammation and hepatocyte regeneration (117). Interestingly, although HAV targets liver cells, it usually does not kill those cells. Rather, immune-mediated lysis, particularly involving natural killer and lymphokine-activated killer cells, is recognized as the most probable cause of hepatic inflammation even before the initiation of cytotoxic-T-cell injury (118). In addition, human gamma interferon produced by HAV-specific T cells may participate in pathogenesis and probably promotes clearance of HAVinfected hepatocytes, as increased levels of interferon have been detected in the serum of infected patients (119, 120). Biochemical changes associated with active HAV infection include bilirubin excretion in the urine and elevated alkaline phosphatase, alanine aminotransferase, and aspartate aminotransferase/serum glutamicoxaloacetic transaminase levels, all of which persist until recovery from illness (121).

Immunity

The immune response against HAV infection occurs on two levels, humoral and cellular. In the humoral response, IgM, IgG, and IgA antibodies are directed against conformational epitopes, mostly the surface proteins VP1 and VP3 and precursor protein (VP2+VP4) of the HAV particle. The IgA and IgM responses precede the IgG response with IgM and IgG detectable within 5 to 10 days before symptoms and at the onset of symptoms, respectively, but the latter is long-lived and provides immunity (117). Because immune-mediated injury causes hepatic inflammation, cytotoxic T cells also play a significant role in the cellular immune response. Since detection of virus particles, viral antigen, or RNA is complicated, the method of choice for diagnosis of infection is immunoassay, which detects the presence of IgM anti-HAV antibody in the blood (113).

Treatment

Historically, administration of Igs at doses as low as 0.01 to 0.04 ml/kg of body weight was used for HAV postexposure prophylaxis. However, in the United States, due to the decreased levels of HAV in the overall population, the dose has been increased over 10-fold to 0.1 ml/kg of body weight (122). This can be effective in controlling both the incidence and severity of disease as long as the Igs are administered within 2 weeks of exposure (123). The prophylactic administration of Ig is recommended for patrons and food handlers provided that all of the following criteria are met: (i) the infected worker was responsible for handling RTE foods and was not wearing gloves; (ii) the infected worker demonstrated poor hygienic practices or already had diarrheal symptoms; and (iii) the patrons can be identifi ed and treated within 2 weeks of exposure (124). As Ig provides only short-term protection against HAV infection, its administration is not recommended for pre-exposure prophylaxis, though some exceptions exist for travelers. For that sort of protection, various FDA-licensed formalinkilled whole-virus vaccines are available, generally administered as a single primary immunization followed by a booster dose after 6 to 12 months (114). The efficacy of the vaccine is 87 to 100%, and protection should last for about 20 years (125). Routine vaccination of all food handlers and

restaurant employees is usually cost prohibitive, even during a hepatitis A epidemic (126). In 2006, hepatitis A vaccination was recommended for children up to 2 years of age (124, 127).

BARRIERS TO THE STUDY OF HUMAN ENTERIC VIRUSES

Most naturally occurring human enteric viruses cannot be easily cultivated *in vitro*, nor are there relevant animal models to facilitate their study. This has been the single most important barrier to understanding these important agents of foodborne disease. Human NoVs are the best example of this phenomenon. For instance, the only way to work with these viruses in their natural form is to obtain fecal material from infected individuals. Hence, there are only limited supplies of this material, usually obtained from federal, state, or local health departments, or from the few investigators engaged in human challenge studies. In the early days following their identification, hNoVs could be detected only by electron-microscopic methods, which lack sensitivity. Antibodies were later raised against some of these viruses, but it quickly became apparent that hNoVs are antigenically diverse, with antibodies raised to one strain having minimal crossreactivity with other strains. Likewise, these viruses are genetically diverse.

With the advent of the molecular biology revolution, the first hNoV genome was cloned and sequenced (128), and investigators began making VLPs, i.e., intact virus capsids lacking only the viral RNA (129). These have become important reagents for binding studies and antibody production. Molecular techniques also provided an opportunity to detect hNoVs and other enteric viruses using nucleic acid amplification methods, most notably reverse transcription-PCR (RT-PCR). While it is a vast improvement over electron microscopy, RT-PCR is not perfect: it relies on the availability of broadly reactive primers and probes, it is prone to matrix-associated inhibition, and interpretation of positive results must be approached cautiously, as detection of viral RNA does not necessarily correlate with the presence of infectious virus.

In the absence of an easily and reproducibly cultivable hNoV strain, surrogate viruses are often used as proxies. In the early years, bacteriophages such as MS2 were commonly used surrogates, but these viruses have significant structural and functional differences from mammalian viruses. Over the last 20 years, two cultivable mammalian viruses have emerged as the most widely used surrogates for hNoVs, i.e., feline calicivirus (FCV), which can be readily propagated in feline kidney cells (130); and murine norovirus (MNV), which is slightly more difficult to propagate but shares more biochemical and genetic features with hNoVs (131). An additional, more recently discovered surrogate, Tulane virus (TuV) (132), is a member of the genus *Recovirus* of the family *Caliciviridae*. TuV was isolated from the stools of rhesus macaques and shows genetic similarities and relatedness to GII NoVs. Moreover, TuV binds to HBGAs, unlike the more closely related MNVs, which recognize only sialic acids and glycoproteins (133). Overall, the availability of these surrogates has enabled investigators to extrapolate the behavior of hNoV strains with respect to persistence, resistance, and inactivation kinetics. However, there is growing evidence that the behavior of the cultivable surrogates does not always mimic that of human strains. For example, FCV is more sensitive to extremes of pH than is MNV or hNoVs, and MNV is more sensitive to ethanol and to desiccation (134, 135). Comparison of TuV and MNV also reveals different characteristics between surrogates and lack of alignment with hNoV strains (134, 136). Hence, surrogate data must be interpreted with caution. Ideally, comparative studies using both hNoVs and cultivable surrogates would provide a more comprehensive picture of the behavior of these viruses, but only a few such studies have been reported to date (137).

Fortunately, the ability to actually evaluate the physicochemical characteristics of hNoVs *in vitro* is now a possibility with the recently described replication of hNoVs in stem cell-derived human enteroids (138). A team of researchers reported on the cultivation of multiple GII.4 variants as well as GII.3, GII.17, and GI.1 hNoVs, with the latter group requiring the presence of bile. These strain-specific requirements for replication of hNoV are noteworthy, and further investigation revealed that it was the exposure of the human intestinal enteroids to bile and not the virus itself which increased hNoV

replication. The identification of this combination of human intestinal enteroids and bile as the replication niche for certain hNoV genotypes was groundbreaking and led to further identification of enterocytes as the primary targets for infection and replication in the small intestine.

PERSISTENCE AND RESISTANCE OF ENTERIC VIRUSES

As a general trend, the persistence, resistance, and stability of enteric viruses depend on the initial load, temperature of the environment, relative humidity, degree of inoculum drying, suspension medium (organic load or fecal material), type of virus, and type of material (surface or food matrix) being contaminated, as previously reviewed (139). Most laboratory studies on the persistence and stability of hNoVs have been based on cultivable surrogates or molecular detection of hNoV RNA. The following sections briefly summarize and highlight studies on viral persistence in foods and water, on fomite surfaces, and under various treatment conditions.

Persistence in Foods and Water

Studies of virus persistence on food commodities have revealed that hNoV GII.4 and GI.4 remained detectable after a 7-day storage at 10°C when inoculated on the surfaces of lettuce, turkey, strawberries, and raspberries, with decreased survival when stored at 21°C for 3 days (140). Enteric viruses (hNoVs, HAV, and rotavirus) and their surrogates (FCV) can survive on berries and herbs when kept frozen at −20°C or colder for 3 months (141). Also, MNV persisted, with minimal reduction (approximately 1.6 log), in bottled water over 42 days at room temperature (142). Fermented food products have also been investigated with respect to hNoV survival. Lee et al. (143) reported on the persistence of hNoV GII.4 (ca. 3 log genomic copies) in cabbage kimchi stored at 4°C and 10°C for 28 days. Intact-hNoV titers of 1.67 (4°C) and 1.31(10°C) log genomic copies/20-μl reaction mixture were detected even in the presence of increasing concentrations of lactic acid and acetic acid—two compounds known to inactivate microorganisms.

While many studies on the persistence of enteric viruses, specifically hNoVs, in bivalve mollusks have been published (144, 145), Souza et al. (146) reported recently on the bioaccumulation and persistence of enteric viruses by *Anomalocardia brasiliana* clams during a 24-h period. hNoV GI was the most adsorbed virus after 24 h, with hNoV GII having the least bioaccumulation at <1% among the viruses tested (HAV, rotavirus, and adenovirus). Previous studies have revealed detection of infectious HAV for up to 3 weeks in oysters that were allowed to accumulate HAV for 16 h in experimentally contaminated water (147).

With regard to enteric viral survival and persistence in surface and groundwater, MS2 bacteriophage, FCV, and poliovirus survive better at lower temperatures of 4°C than at 25°C (148). TuV remained stable in surface water even after 28 days, with <1 log reduction, although in groundwater, TuV was reduced by ≥3.5 to 4 log by day 21 (149). To date, the only human volunteer challenge study on the long-term persistence of hNoV in groundwater revealed that the volunteer subjects became ill after drinking groundwater 61 days after inoculation with hNoV; however, hNoV GI.1 RNA could still be detected after 3 years of storage of the contaminated groundwater (150).

Along with postharvest persistence, a growing body of work has focused on the persistence of viruses on fresh produce under preharvest conditions. Hirneisen and Kniel (151) reported that hNoV could persist on the leaves of mature spinach plants during a 7-day study period. Additional studies have reported on the persistence of viral surrogates (MNV, porcine sapovirus, and TuV) on lettuce and spinach plants subjected to abiotic stressors, including physical damage, heating, and flooding (152). Overall, reductions (≥1.5 log) in infectious virus 7 days after inoculation on both lettuce and spinach plants—a trend also observed for hNoV GII.4 RNA over a 14-day period—were observed. Furthermore, these viral surrogates persisted on lettuce leaves stored at room temperature for 3 days and at 4°C for up to 14 days (153).

Persistence on Fomite Surfaces

Considerable research has been conducted on the persistence of human enteric viruses on fomite surfaces (139, 154, 155). For brevity, only selected studies are highlighted here. In 2009, Liu et al. (38) determined the persistence of two hNoV strains on food contact surfaces and reported a <1-log reduction after 42 days on stainless steel, ceramic, and Formica coupons. According to Lamhoujeb et al. (156), enteric virus persistence on surfaces is increased (49 to 56 days) at low temperatures (7°C) and elevated humidity (86%) compared to room temperature (20°C) and decreased humidity (30%), at which hNoV persisted for only 7 days on plastic. A similar study revealed 2-log and 2.3-log reductions of hNoV GI.1 and GII.2, respectively, after 42 days at room temperature (157). More recently, Colas de la Noue et al. (158) reported that virus infectivity after storage at 25°C in high and low relative humidity was increased at low (10%) as opposed to high (85 to 100%) humidity, counter to most of the previously reported data. Interestingly, the authors investigated the integrity of binding capacity of hNoV VLPs as opposed to relying on detection of viral RNA. While most available data address hard, nonporous surfaces, the importance of porous, soft surfaces in the persistence of enteric viruses was recently reviewed by Yeargin et al. (159). To summarize, the authors noted that temperature, relative humidity, organic content, and deposition method all influence viral survival on soft, porous surfaces.

Transfer of microorganisms between surfaces has also been frequently investigated. This transfer is dependent upon the application of varying levels of pressure, friction, time, temperature, and moisture, though variable rates of transfer have been observed even under the most controlled conditions (160). Sequential transfer of hNoV by contaminated fingers occurred after contact with soiled toilet paper, leading to contamination of seven clean surfaces (161). A study involving the transfer of GI hNoV from stainless steel surfaces to lettuce revealed positive hNoV transfer from the stainless steel surface to eight of nine lettuce samples (162). Additional studies by Escudero et al. (157) revealed that transfer of enteric viruses from fomite surfaces depended on the type of food: the rate of transfer of GII.2 hNoV and MNV to deli meats was 55 to 95%, whereas that to lettuce was 0 to 26%. Stals et al. (163) also determined that rates of hNoV and MNV transfer from contaminated gloves and stainless steel to

sandwich components (meat, lettuce, and bread buns) varied according to food type. Varying rates of hNoV transfer to raspberries, blueberries, and grapes after handling with contaminated hands have also been reported after direct contact and by sequential contamination, which can be encountered in kitchens and food preparation settings (164, 165).

Physical and Chemical Resistance

Along with the persistence of human enteric viruses, their resistance to common control strategies on fomite surfaces as well as on foods has also been extensively investigated, and many of these studies have been summarized by Hirneisen et al. (8), Alidjinou et al. (155), and Shukla et al. (166). One of the most recent studies was a comprehensive evaluation of virus inactivation by Cromeans et al. (134). The responses of Aichi virus, hNoV, TuV, MNV, FCV, and porcine enteric calicivirus were determined after exposures to extreme pH, heating at 56°C, alcohols, chlorine on surfaces, and high hydrostatic pressure. The results revealed variability across all viruses and treatments. For instance, FCV was completely inactivated at pH 2, whereas MNV, TuV, and Aichi viruses were resistant. In addition, all viruses were resistant when exposed for 5 minutes to 1,000 ppm chlorine on stainless steel surfaces, with the exception of FCV, which was reduced by 5 logs. Of note, viruses are more resistant to chemical inactivation on surfaces than in suspension, as determined by Tung et al. (167) and Hirneisen and Kniel (136). Additional studies have determined the inactivation rates of enteric viruses when exposed to organic acids and phenolic acids. Lacombe et al. (168) reported a 4.5-log reduction of TuV after a 5-minute exposure to 0.5:0.7% (vol/vol) for lactic acidsodium dodecyl sulfate at pH 3.5, whereas only a 2.6-log reduction was observed with 0.5:0.7% (vol/vol) acetic acid-sodium dodecyl sulfate at pH 4.0.

With respect to thermal resistance *in vitro*, Cromeans et al. (134) reported complete inactivation of all viruses (Aichi virus, hNoV, TuV, MNV, FCV, and porcine enteric calicivirus) after exposure to 56°C for 20 minutes as well as variable log reductions after treatment with alcohols, with TuV and Aichi viruses not being affected. These data on

thermal inactivation align with the conclusions from a 2012 meta-analysis on thermal treatment of enteric viruses in which the authors state that thermal inactivation was generally most successful at temperatures above 50°C (169). Another evaluation of thermal inactivation studies of enteric viruses revealed similar findings in addition to highlighting methodological discrepancies which hinder the ability to compare results across studies (170).

Thermal resistance of various hNoV genotypes in food items such as tomato sauce, minced meat, baked frozen pizza, and fresh culinary herbs (i.e., when added to dishes during the cooking process) has also been investigated, and the data typically reveal greater inactivation at higher temperatures and longer exposure times (171, 172). This resistance to heat treatment within the food matrix is of particular interest when effective control strategies in bivalve mollusks are being considered. Araud et al. (173) determined that oyster tissues inoculated with TuV required heating for nearly 2 min at 80°C to obtain a 1-log reduction in TuV, whereas <1 min at 80°C was needed for a 1-log reduction of MNV. The thermal resistance of hNoV GI and GII in naturally contaminated oysters was determined after heating at 72 and 95°C (174). Using a PMAxx pretreatment before quantitative RT-PCR (RT-qPCR), the authors observed reductions of 2.99 and 1.93 log RT-PCR units/100 µl for GI and GII hNoV, respectively. On the basis of data such as these, higher temperatures and longer treatment times are being recommended to inactivate hNoV in shellfish to prevent hNoV illness and outbreaks (175).

CONTROL OF VIRUSES IN FOODS AND THE FOOD PRODUCTION, PROCESSING, AND PREPARATION ENVIRONMENTS

Molluscan Shellfish

The role of molluscan shellfi sh in the transmission of human enteric viral disease has been recognized for decades, with accompanying efforts to develop effective pre- and postharvest controls for this food commodity. Preharvest controls continue to include prevention of

human sewage pollution of shellfish harvesting waters and the establishment of alternative microbiological indicators to monitor shellfi sh-growing areas. Prevention of sewage pollution can be difficult because of the presence of both diverse and diffuse sources and the unpredictable nature of a contamination event; however, preventing illegal dumping of human waste in marine waters is likely to be a particularly important control practice (176). Oyster beds close to sewage plants may need special consideration because of concerns about the potential for sewage overflow, particularly during times of excessive rainfall (177).

Preventing human sewage contamination of molluscan shellfi sh overlay waters is complicated by the lack of a reliable microbiological indicator whose presence and/or levels are correlated with the presence of enteric virus contamination (178). Bacteriophages, especially F-specific coliphages (FRNA phages or male-specific coliphages), somatic coliphages, and phages of *Bacteroides fragilis*, have long been proposed as alternative indicators of viral contamination of the environment. Among these, the distribution and survival of FRNA phages are considered more similar to those of human enteric viruses (179), although shellfish may contain pathogenic viruses even though they lack FRNA phages (180). Similar difficulties in establishing relationships between the presence and levels of somatic coliphages and those infecting *B. fragilis* have been reported (181). The feasibility of using human adenoviruses and polyomaviruses as indicators of human enteric viruses in environmental and shellfi sh samples has likewise been proposed; however, the evidentiary data reveal poor correlations between these viruses (178). More recently, pepper mild mottle virus (PMMoV) has been identifi ed as a suitable indicator for human enteric viruses. While PMMoV is a pathogen of peppers, it has been reported to be the most abundant virus in human stool (182), and it is detected more frequently and in greater abundance in wastewater, surface water, and marine water than previously proposed human enteric virus indicators, including human adenoviruses and polyomaviruses (183, 184). In addition, PMMoV is cited as being more persistent in surface and marine waters than human enteric viruses (183). However, a reliable

microbiological indicator for enteric virus contamination in shellfi sh and their harvesting waters has yet to be officially established (185).

Controlled purification, widely used in Europe, is an early postharvest control strategy. Depuration and relaying are based on the principle that shellfi sh can purge at least some of their microbial contaminants by extended feeding in clean water, provided that the feeding conditions (e.g., temperature, salinity, and dissolved oxygen) are favorable (186). In depuration, freshly harvested shellfish are placed for several days in a controlled environment, usually in tanks provided with a supply of clean, disinfected seawater under specific operating conditions. In relaying, shellfish are transferred from contaminated growing areas to approved or naturally unpolluted (pristine) areas (186). Unfortunately, the scientific consensus is that enteric viruses are not eliminated during controlled purification. For example, Schwab et al. (187) found only a 7% reduction in hNoV concentration after 48 hours of depuration treatment. More recently, Polo et al. (188) reported that HAV and hNoV (GI and GII) at concentrations ranging from 10^3 to 10^5 RNA copies/g digestive tissue could be detected in shellfish undergoing commercial depuration over a 7-day period.

Other postharvest controls for virus contamination of shellfish include heating and alternative food-processing technologies, including nonthermal approaches such as high-pressure processing (HPP), ionizing irradiation, and high-intensity ultrasound. Early reports documented outbreaks of both HAV and viral gastroenteritis linked to the consumption of cooked shellfi sh (189, 190). Thermal inactivation studies—as discussed above—have substantiated the resistance of enteric viruses in bivalve mollusks to heat treatment. These studies have resulted in European heat processing recommendations (internal temperature of 90°C for 1.5 min) to control HAV in shellfi sh (175). Unfortunately, such high temperatures often result in unacceptable sensory changes to the product.

In general, enteric viruses are quite resistant to ionizing radiation, and studies have determined this to be an inconsistently effective control for molluscan shellfish contaminated with viruses, at least at the relatively low doses necessary to maintain the organoleptic quality of the product (191). There has, however, been much interest in the

efficacy of HPP as a postharvest treatment to inactivate human enteric viruses in shellfish, particularly because it also works well for inactivating pathogenic *Vibrio* species under conditions that result in a product that maintains its "fresh-like" properties (192). In early work, Kingsley et al. (193) determined that HAV and FCV suspended in cell culture medium were inactivated after exposure to 450 MPa for 5 minutes and 275 MPa for 5 minutes, respectively. Grove et al. (194) reviewed the studies on HPP inactivation of enteric viruses prior to 2006, and they concluded that there is a wide range of viral susceptibility to HPP and that continued research was needed. Despite some promising preliminary results, Leon et al. (195) determined that when human subjects consumed Norwalk (hNoV GI.1) virus-contaminated oysters with and without prior HPP treatment, infection occurred among one or more volunteers consuming HPPtreated products, except those products receiving the most severe treatment (600 MPa). However, this treatment resulted in a product having a mildly cooked, whitish appearance. A subsequent study by Lou et al. (196) provided additional evidence that the hNoV capsid is highly resistant to HPP based on experiments with hNoV VLPs and HBGA receptor binding assays. An additional study was conducted to characterize the HPP susceptibility of TuV-inoculated oysters and determined that only moderate treatment levels (≤300 MPa at 4°C) were needed to inactivate TuV (197). Overall, more research is needed to determine whether HPP is a viable postprocessing technology for inactivation of enteric viruses in molluscan shellfish (192).

Fresh Produce

Preharvest measures directed at prevention of viral contamination of fresh produce are addressed by the good agricultural practices recommendations provided by federal agencies and industry trade groups (198). One important consideration in these documents is ensuring the absence of human fecal contamination in irrigation water and waters used to mix pesticides and fungicides (199) or in manure, fertilizer, and/or compost. Unfortunately, sewage spills, storm-related contamination of surface waters, illicit discharge of waste, and residential septic system failures are widely recognized as the leading sources of surface water and groundwater contamination, and these are

relatively common and frequently unavoidable occurrences (200). Since many produce items are subject to extensive human handling during harvesting, preharvest food safety strategies must also focus on individuals harvesting the food, particularly if the product is hand-picked. This might include issues such as ready access to clean, on-site toilet and handwashing facilities, restriction of children and ill workers in production fields, and adequate employee education (198).

From a postharvest perspective, it is generally recognized that current preservation and storage processes such cooling, freezing, acidification (pH ≤4.5), and moderate heat treatments (pasteurization) are usually inadequate to completely inactivate foodborne viruses such as hNoV or HAV if they are present on or in a food matrix (171). Most of the decontamination efforts applied to fresh produce have focused on washing. The water used for washing and ice used for transport and cooling must be free of human fecal contamination. For produce washing, waters are frequently supplemented with sanitizers, the most common of which is chlorine, although others, such as chlorine dioxide and organic acids, have been used. Generally, washing fresh produce can reduce but not eliminate foodborne pathogens. In some instances, simple home-use remedies such as water immersion, hand rubbing, or use of detergents or vinegar (10%) can be moderately effective, providing approximately 1-$\log_{10}$ reductions in virus concentration on the surface of strawberries (201). Alternatives to chlorine include hydrogen peroxide, peroxyacetic acid-hydrogen peroxide formulations, and trisodium phosphate (8). Use of ozonated wash water also has promise (202). Particularly promising, Predmore and Li (203) determined that supplementation of chlorinated (200 ppm) wash water with surfactants (sodium dodecyl sulfate and polysorbates) enhanced the removal of MNV from fresh produce approximately 100-fold. Since some of these compounds are approved as food additives and/or GRAS (generally recognized as safe), this may be a promising control strategy for the future.

Although novel surface sanitizers continue to be evaluated, the data on chemical disinfection for the inactivation of viruses from food surfaces remain less than desired with respect to the level of virus inactivation. Not only is efficacy relatively poor, but some of the novel agents (such

as hydrogen peroxide) cause unacceptable organoleptic changes to the product. Product-specific surface morphology and physiological characteristics make disinfection complicated; leafy vegetables can be more difficult to decontaminate because of their rough or wrinkled surfaces, and small fruits such as raspberries and blackberries have more porous and complex surfaces, including "hydrophobic pockets" that can entrap virus particles (140). Furthermore, disinfectants incorporated into wash water may not be effective in removing or inactivating viruses that have penetrated the skin of the product or those that might have entered tissues through cuts and abrasions.

There is substantial interest in several emerging foodprocessing technologies for inactivating viruses on the surface of fresh produce. For example, FCV and poliovirus are highly susceptible to inactivation by UV radiation, with 3-$\log_{10}$ reductions obtained by treating contaminated water with doses of 23 and 40 mJ/cm^2, respectively (204, 205). Fino and Kniel (206) determined that when green onions, lettuce, and strawberries were artificially contaminated with HAV, Aichi virus, or FCV and subsequently exposed to UV light at doses of ≤240 mW • s/cm^2, about a 4.6-$\log_{10}$ reduction for lettuce, a reduction of 2.5 to 5.6 $\log_{10}$ for green onions, and a reduction of 1.9 to 2.6 $\log_{10}$ for strawberries were obtained. Su et al. (207) studied the effect of high-intensity ultrasound on the inactivation of MNV and FCV in buffer and orange juice. Using a treatment of 20 kHz for 2, 5, 10, 15, 20, and 30 minutes with pulses of 30 s on/30 s off, a 15-minute treatment was required for complete inactivation (reduction of 4 $\log_{10}$) of FCV, but only a 1.6-$\log_{10}$ reduction in MNV was achieved, even after a 30-minute treatment. More recently, atmospheric cold plasma technology was investigated for inactivating viruses on fresh produce. Lacombe et al. (208) investigated the inactivation of TuV and MNV on the surfaces of blueberries by cold plasma with and without air and reported a 3.5-log reduction in the number of TuV PFU per gram after a 120-s treatment with air and a 5-log reduction of MNV after a 90-s treatment with air. Based on preliminary data, novel processing technologies have promise, but much more research is needed to validate their efficacy, and a major investment is needed to commercialize those that are most promising. As is the case for molluscan shellfish, ionizing radiation is

not a promising technology for inactivating viruses in produce, as high doses are relatively ineffective and likely to adversely affect product quality (209).

A combination of two or more controls, so-called hurdle approaches, may be necessary to completely eliminate viral contamination of fresh produce. A few studies have investigated such combinations. For example, Li et al. (210) determined that a minimal reduction in the titer of MNV (<1 $\log_{10}$) inoculated on shredded iceberg lettuce occurred with a treatment of liquid (2.1%) or vaporized (2.5%) hydrogen peroxide; however, the combination of UV light and vaporized hydrogen peroxide substantially increased the efficacy of inactivation. Butot et al. (172) determined the effect of freeze-drying, freeze-drying combined with heating, and steam blanching on inactivating HAV and FCV on the surfaces of berries and herbs. While freeze-drying itself was only minimally effective, the addition of a terminal dry heat treatment at 120°C improved the degree of inactivation.

RTE and Prepared Foods

Adherence to strict hygienic practices when handling and preparing foods is critical to controlling viral contamination in RTE food products. These issues have been reviewed extensively by others (211, 212). The first line of defense in this regard is employee education and regular supervision of employees. For instance, a 2006 study associated having a certified kitchen manager with a lower incidence of hNoV outbreaks in restaurants (213). The importance of controlling viral contamination is further illustrated by the FDA's 2017 Food Code, which includes recommendations for (i) exclusion and restriction of ill employees, (ii) emphasis on handwashing procedures, and (iii) prohibition of bare-hand contact with RTE foods (214). Because ill employees shed viruses in large numbers, managers of food manufacturing, catering, and food service industries should restrict these individuals from working directly with food or food equipment and should provide a sick leave policy that allows workers to stay home while ill. Even with such controls, virus shedding by individuals is still a concern because it can occur pre- or postsymptomatically as well as asymptomatically (5, 61). Glove use (and changing of gloves if damaged

or soiled), frequent handwashing, and prevention of cross-contamination during handling and preparation of foods are essential. One of the most important considerations in hand hygiene is the role of fingernails, as virus-containing fecal material can be harbored here and long, artificial fingernails have been linked to consistently greater hand contamination (215).

Hand disinfection remains an important issue. Reliable hand decontamination relies on three primary factors: (i) an effective disinfecting agent, (ii) adequate use instructions, and (iii) regular compliance (216). Compliance with recommended handwashing regimens is notoriously poor (217). Additionally, although handwashing helps prevent and control the transfer of viruses, handwashing agents differ in their ability to inactivate viruses (137). Consistent with the results of suspension assays described previously, finger pad assays confi rm that commonly used handwashing agents have minimal efficacy against human enteric viruses, rarely exceeding 1- to 2-$\log_{10}$ inactivation when used at manufacturer-recommended concentrations (218). Some have suggested that many of the commercial hand sanitizers are not even as effective as soap and water alone (219). Tuladhar et al. (220) confirmed this suggestion by reporting greater log reduction of hNoV and MNV after 30 s of handwashing with soap and water than with an alcohol-based hand disinfectant using finger pad assays.

Overall, there is increasing consensus on the need to establish new standards for the selection of effective formulations for handwashing agents with regard to antiviral activities, particularly with respect to labeling. Over the past decade, research has aimed to develop more effective virucidal formulations for handwashing and sanitizing agents. For example, Macinga et al. (221) described a new ethanol-based hand sanitizer containing a synergistic blend of polyquaternium polymer and citric acid that reduced the infectivity of FCV and MNV by >3 $\log_{10}$ after a 30-s exposure, as evaluated by both suspension and finger pad assays. More recently, Ionidis et al. (222) described another novel, alcohol-based hand disinfectant with added urea and citric acid that resulted in a 4-log reduction of virus after a 60-s exposure using the standardized suspension assay.

Frequently handled objects, such as faucet taps, doorknobs, and even food preparation surfaces, can serve as sources of virus contamination in the food preparation environment, and standard surface disinfection is used as a control measure (155). Not unexpectedly, the efficacy against enteric viruses of most chemical disinfectants commonly used in both institutional and domestic environments is questionable (167). Early research revealed that sodium hypochlorite was most effective for HAV and rotavirus inactivation, with improved efficacy when used at high concentrations and extended contact times (223). Nearly the same results were obtained in most of the surface decontamination studies involving hNoV and its surrogates, with variation depending on the surrogate-disinfectant combination (159). In particular, bleach used at concentrations ranging from 1,000 to as much as 5,000 ppm appears to be the most effective surface-sanitizing regimen relative to other disinfectants tested. However, the specific $\log_{10}$ inactivation achieved using sodium hypochlorite varies with virus type, surface type, and study design. In addition, the presence of fecal material not only increases virus survival but is likely to protect viruses from the antimicrobial activity of bleach (224). Based on such data, the CDC recommends a minimum of 1,000 ppm for disinfecting surfaces contaminated with hNoVs (225).

DETECTION METHODS

Despite extensive efforts to develop robust methods to not only recover viruses but also detect them in foods, this remains a challenging area of research (166, 226). Because the numbers of virus particles present in contaminated foods are usually low and there is no universal or rapid culture-based method for growing hNoVs or HAV, cultural enrichment is not an option. Therefore, the viruses must be concentrated and purifi ed from the food matrix before a detection method is applied, with molecular amplification being the detection method of choice. Sequence-based determination of amplicon identity is used for further confi rmation. The major steps for the detection of viruses in foods are as follows: (i) virus concentration and purification, (ii) nucleic acid extraction, (iii) detection of amplicons, and (iv) confi rmation of amplicon identity.

Virus Concentration and Purification

Virus concentration and purification schemes are designed to reduce sample volume and remove at least some of the matrix while simultaneously recovering most of the contaminating viruses. In order to achieve these goals, sample manipulations are undertaken that capitalize on the behavior of enteric viruses to act as proteins in solutions, to cosediment by simple centrifugation when adsorbed to larger particles, and to remain infectious at extremes of pH or in the presence of organic solvents. Many virus concentration and purification methods use manipulation of pH and/or ionic conditions to favor virus adsorption to, or elution from, the food matrix. This is then followed by relatively lowspeed centrifugation, after which the virus-containing phase (either precipitate or eluate) is recovered for further purification. Other steps in the virus concentration and purification process may include various forms of filtration (crude filtration and ultrafiltration), ultracentrifugation, precipitation (achieved through the addition of polyethylene glycol or organic flocculants or by manipulation of pH), organic solvent extraction (to remove matrix-associated lipids), ligand-bound magnetic separation (using immunobeads or cationic particles), and/or enzyme pretreatment (to break down matrixassociated organic matter, particularly complex carbohydrates). In almost all instances, virus extraction is accomplished by combining two or more of these steps in series (227).

It is generally recognized that elution of viruses from the surface of foods is relatively inefficient, mostly because of underlying hydrophobic, ionic, and van der Waals interactions that tend to favor adherence of viruses to organic matter. In addition, other forces may influence virus binding to solids, including virus association with sugar or carbohydrate moieties on food tissues, as has been observed for shellfi sh and fresh produce (30, 228). During the past decade, efforts to capitalize on these interactive forces have brought about some new methods to facilitate virus concentration and purification. For example, Papafragkou et al. (229) used cationically charged beads to facilitate HAV capture from a variety of food matrices, achieving recoveries of about 50% of input virus and detection limits of ca. 10 PFU per 25-g

sample. Cannon and Vinjé (230) took advantage of the specifi city of HBGAs by using them as potential binding ligands for hNoVs, revealing that a magnetic bead-based HBGA assay could be developed for the recovery of low numbers (30 to 200 genomic units) of hNoVs from seeded environmental waters. This approach has since been applied and expanded upon by others, who have used recirculating magnetic capture equipment to increase the sample size and volume (231, 232).

A good virus concentration and purification method (Fig. 23.4) should be relatively simple and result in sample volume reductions of 10- to 1,000-fold and recovery of >90% of the target virus. However, recovery efficiency is both virus and matrix specific (226). For example, HAV recovery tends to be low in comparison to the relatively high recovery of human enteroviruses (e.g., poliovirus); simple sample matrices such as lettuce tend to be easier to work with than more complex matrices, such as sandwiches and RTE salads. All methods have their own advantages and disadvantages. For instance, immunocapture methods tend to be simple, requiring fewer sample manipulations, which entails a lower likelihood of virus loss and favors the recovery of infectious virus; however, they may be hindered by high specifi city, since broadly reactive antisera are not readily available for hNoVs (233). To address these limitations, development of novel cross-reactive bivalent synbody (i.e., a synthetic protein constructed from two peptides) affi nity ligands are being investigated for their ability to capture a diverse group of hNoVs. Gupta et al. (234) recently reported on the development of 20 synbodies for the capture of both GI and GII hNoVs. The authors determined that four of the synbodies were able to crossreact with four diverse genotypes of hNoV (GI.1, GI.6, GII.4 Sydney, and GII.6) as well as producing results similar to those for porcine gastric mucin-coated magnetic beads—a standard method for capturing hNoV (230). The potential advantages of synthetic ligands include the ability to standardize the production of the ligands as well as the ligand-coated beads (234). Regardless, there is no universal extraction method currently available that can be applied to all foods, particularly when contamination levels are low (235, 236).

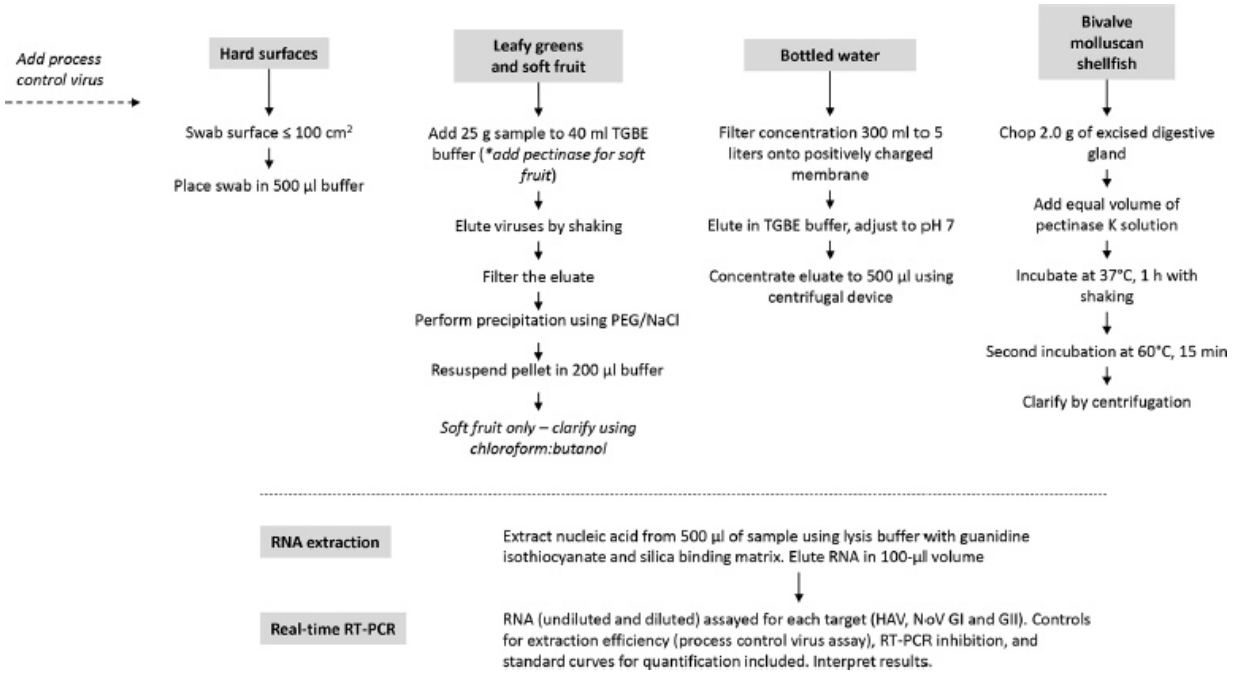


Figure 23.4 Flow diagram of the ISO method for the detection of HAV and NoV in food and bottled-water samples. Image adapted from ISO/TS 15216-1:2017, annex A (251). TGBE, Tris-glycine-beef extract; PEG, polyethylene glycol.

Nucleic Acid Extraction

Although viral RNA can be released from capsids and made available for amplification using a simple heat treatment (99°C for 5 minutes), this is rarely done because, even with the best virus concentration method, residual matrix-associated components persist and frequently interfere with, or inhibit, nucleic acid amplification. Therefore, an efficient nucleic acid extraction step is critical. The science of RNA extraction has developed rapidly over the last 2 decades, and what was once a complex procedure is now much simpler and more reliable. Over the years, there have been many comparative studies to identify the best RNA extraction method(s) for use with food systems. Many investigators are now using combination guanidinium-silica-based methods, which have actually been automated by a number of diagnostics manufacturers (203). In addition, to enhance RNA purity, investigators have also added gel filtration columns (i.e., Sephadex-based columns or similar products) to the sample preparation to

further purify extracted RNA, resulting in an increased likelihood of virus detection through the removal of RT-PCR inhibitors (237, 238).

Detection of Amplicons

RT-PCR is the most commonly used method for the detection of viral RNA. The sensitivity and specificity of RT-PCR depend upon the efficiency of the virus concentration and purification steps, as well as the nucleic acid extraction. They also depend on primer choice and amplification conditions. The use of primers with low degeneracy and high melting temperature is recommended, because this reduces the likelihood of nonspecific amplification, which is common when nucleic acids are extracted directly from a complex sample matrix. For HAV detection, broadly reactive primers with high annealing temperatures are available, usually corresponding to the VP1-2A junction or 5′UTR of the viral genome (110). The high degree of genetic diversity of hNoVs has made the development of broadly reactive primers difficult. Four regions of the hNoV genome have been used for primer design (designated regions A, B, C, and D) (13, 239), but the ORF1-ORF2 junction (just downstream of region B) is the most conserved and is frequently used for genogroup-specific detection. For strain comparison (as might be appropriate in outbreak investigations), a dual-typing scheme with primer sets corresponding to the hNoV capsid and polymerase genes was recently recommended for molecular surveillance of hNoV (55) and is now utilized by CaliciNet. It is also recommended that multiple primer pairs be used when food samples are screened for NoV contamination (240), as one pair may perform better than another, depending on virus load and the matrix from which the sample was derived. Historically, nested reactions have been used to increase the sensitivity of detection, particularly for naturally contaminated food products (240).

Beginning in the mid-2000s, real-time PCR or RT-qPCR methods replaced the traditional method. The term “real time” refers to the simultaneous detection and confirmation of amplicon identity as the amplification progresses through the use of hydrolysis probes (e.g., TaqMan), hybridizing probes (Molecular Beacons), or double-stranded-DNA intercalating dyes (e.g., SYBR green). It is said that real-time

assays can be made quantitative, but residual matrix-associated inhibitors frequently affect the reliable quantification of viral load in naturally contaminated samples. Therefore, simple presence-absence testing, rather than quantification, is generally used for virus detection in foods and environmental samples. Further, there is some debate as to whether an RNA or DNA standard is best when the standard curve for real-time quantification is designed. While using a DNA standard provides a defined assessment of the efficiency of DNA amplification, it does not take into account an inefficient reverse transcription step, which may result in an underestimate of the number of viral genome copies in the sample.

Confirmation of Amplicon Identity

When RT-PCR products are obtained from naturally contaminated foods, the amplicons must be sequenced for further confi rmation and strain typing. RT-qPCR is not very amenable to the sequencing that needs to be done for confi rmation due to the small amplicon size and target region, so presumptively positive samples are almost always rescreened with traditional RT-PCR prior to cloning and sequencing of amplicons. Sequences derived from foods can be compared to those obtained from clinical specimens in an effort to make causal associations. Although this approach has been used success successfully to link contaminated foods to outbreaks of viral gastroenteritis, it is often challenging to achieve amplification of viral RNA from implicated foods (241, 242).

Several ways to facilitate confirmation of a positive signal for target viruses in foods have been under development. For example, Schultz et al. (243) developed an RT-qPCR assay that allows the user to detect and genotype hNoV strains in a single step using a long-template approach. DNA microarrays are now used for the simultaneous detection and genotyping of hNoVs (244) and even for the identification of a broad range of foodborne viruses, including HAV, hNoVs, rotavirus, and certain human enteroviruses (245). Over the past several years, these methods have been improved upon, especially with respect to analysis of clinical samples. For instance, Chen et al. (246) developed a tiling microarray assay that can simultaneously

detect and genotype multiple foodborne viruses, including coxsackievirus, HAV, and hNoVs; however, the detection limit was cited at 10^4 viral genome copies, although this has since been improved upon (200 to 500 genome copies) by other researchers (247). A thorough description of nucleic acid-based detection methods for foodborne viruses was recently reviewed (248).

Recent Advances in Detection and Diagnostics

Initial efforts in the development of methods to detect viral contamination in foods were limited almost exclusively to molluscan shellfi sh. By the mid-2000s, relatively standard methods were developed for detecting HAV and hNoVs in shellfi sh, with the most recent method comparison study having been completed in 2015 (249). Here, the authors reported that an adsorption-twice elution-extraction method yielded high sensitivity (1.4 genome copies/g) for the detection of rotavirus in bivalve digestive shellfish tissues. Ensuing studies characterized the prevalence of contamination, particularly in European shellfish (188, 250). Since then, there has been an explosion of interest in developing methods for other foods. For example, there are now well-accepted methods for the detection of viruses in berries, and the European Committee for Standardization has established standard methods (251) for the detection of hNoVs and HAV in selected foods (soft fruits and leaf, stem, and bulb vegetables), bottled water, and food contact surfaces (EN ISO 15216-1:2017).

There has also been increased scrutiny relative to the need for inclusion of appropriate controls in attempts to detect viruses in naturally contaminated products. Not only are the standard negative and positive amplification controls needed, but there is now a strong recommendation from the international community for the inclusion of sample process (extraction) controls and internal amplification controls (IACs) as are published in the ISO (International Organization for Standardization) methods for extraction and detection of viruses in foods (251). Extraction controls are intended to evaluate the efficiency of the virus concentration and purification steps; as such, the analyst adds a relatively high concentration (10^6 to 10^8 infectious units) of a harmless virus with physical characteristics similar to those of enteric

viruses. Viruses used as process controls have included mengovirus, MNV, FCV, TuV, armored RNA, and various bacteriophages. The concept is that if the process control is recovered and detected by RT-qPCR, the sample preparation method was successful; if not, it calls into question the efficiency of that method (226).

Recent efforts have been aimed at determining the most appropriate process controls depending on the matrix and target virus. Gentry-Shields and Jaykus (252) investigated five different process control viruses for use in the recovery of hNoV from lettuce and sliced deli ham. The process control viruses included mengovirus, MNV-1, MS2 coliphage, TuV, and turnip crinkle virus. Results revealed that mengovirus most closely mimicked the behavior of hNoV throughout the sample processing steps, whereas MNV-1 had the least similar behavior. Hennechart-Collette et al. (253) also evaluated process control viruses in the recovery and detection of HAV and hNoV in bottled water, lettuce, and semidried tomatoes and determined that MNV-1 was the most appropriate virus for validating the detection of HAV and hNoV GII across all three matrices and of hNoV GI in lettuce. Interestingly, mengovirus was the most appropriate virus for validating hNoV GI detection in bottled water and semidried tomatoes. These conflicting observations will be important to consider during selection of the appropriate process control virus, and it is important to note that currently mengovirus is the selected process control for EN ISO 15216-1:2017.

Differing from the process control, IACs are sequences of nontarget RNA or DNA, usually flanked by the target-specific primer binding sites. These are added to the amplification reaction mixtures to account for reaction failure due to residual matrix-associated amplification inhibition (238). Diez-Valcarce et al. (254) constructed and applied IACs for viruses relevant to the food industry. The authors designed IAC primers to include a 16- to 18-bp fragment of the *Listeria monocytogenes prfA* gene combined with the specific primer sequence for each target virus and a minor groove-binding probe targeting the *L. monocytogenes prfA* gene. Conversely, the ISO method for detection of hNoV and HAV in food employs an external amplification control RNA

for detection of sample inhibition, requiring a reaction separate from that for detection of the target virus.

Another important area of research has focused on differentiating infectious and noninfectious viruses. Several candidate methods have been identifi ed, to be used in conjunction with RT-qPCR, to aid in this discrimination. These methods fall into two general categories, those estimating capsid integrity and those targeting genome integrity. Candidates in the former category are more popular and include the use of nucleic acid intercalating agents (e.g., propidium monoazide [PMA]), RNase pretreatment, ligand and antibody capture, and direct detection of damaged protein. The most widely used method to date is RNase pretreatment. While these methods have been reviewed on various levels (255, 256), researchers have also attempted to undertake comprehensive, sideby-side comparisons of these techniques. For example, Escudero-Abarca et al. (257) compared PMA and RNase pretreatment to determine the thermal resistance of a GII.2 hNoV called Snow Mountain virus (SMV). The authors reported slightly different decimal reduction values of SMV depending on the exposure temperature and pretreatment method applied. Overall, it was concluded the RNase pretreatment achieved more consistent results with respect to nucleic acid amplification and that the PMA treatment proved difficult to optimize unless larger amplification products were used, as reported previously by others when applying this method for the pretreatment of bacteria (258). Moreno et al. (259) also evaluated two intercalating agents—PMA and ethidium monoazide—for discrimination of viable HAV in food samples (lettuce, parsley, spinach, cockles, and clams), and reported successful viability discrimination when samples were treated with PMA plus 0.5% Triton X-100 (a nonionic surfactant). Recently, novel iterations of these intercalating dyes (e.g., PMAxx and PEMAX) have been released on the commercial market and have been evaluated by researchers focused on food virology (174). Additional viability-discriminating reagents, including platinum and palladium compounds, were recently evaluated for virus infectivity and were determined to perform more efficiently than PMA and other related intercalating dyes (260).

Advances in detection of viral nucleic acids targets have also been reported. Recently, novel detection technologies were investigated and compared to the RT-qPCR standard, including isothermal amplification (nucleic acid sequence-based amplification and loop-mediated isothermal amplification), microfluidic digital RT-PCR (RT-dPCR), and nucleic acid aptamers (or aptamer-based biosensors). While the isothermal amplification technologies have been thoroughly investigated and reviewed (13, 166), RT-dPCR is one of the newer technologies on the horizon.

Briefly, RT-dPCR is an absolute quantification approach that allows the determination of target copy numbers without the reliance on a standard curve. In this approach, the sample is partitioned into microdroplets, thus creating individual reaction mixtures with one or no copies of the nucleic acid target. Absolute quantification is achieved using binomial Poisson statistics by calculating directly from the ratio of positive to total reactions (261). Moreover, by micropartitioning the sample, inhibitors are effectively diluted out and/or eliminated, resulting in increased confidence of virus quantification (262). Investigators in the European Union have compared RT-dPCR to RT-qPCR for the quantification of both HAV and hNoV in lettuce, water, and soft berries (263, 264). Coudray-Meunier et al. (263) reported that while sensitivity of the RT-dPCR assay was comparable to or lower than that of RT-qPCR, viral recoveries determined by RT-dPCR analysis were significantly greater than those obtained with RT-qPCR for lettuce and water samples, possibly due to the removal of inhibitory substances. Similarly, Fraisse et al. (264) compared these methods for the detection of hNoV and HAV recovered from soft berries (raspberries and strawberries) and reported that RT-dPCR reduced PCR inhibition, thus increasing viral efficiencies.

With respect to the development of biosensors for the detection of foodborne viruses, researchers have been working to improve the analytical sensitivity of biosensors for the detection of pathogens in complex samples (265). Recently, Moore et al. (266) described the development of broadly reactive single-stranded DNA aptamers targeting the P domain protein of a GII.4 hNoV capsid; however, these

aptamers were merely proof of concept to show the potential for optimization into biosensors, or “aptasensors,” for detection.

Applications of Virus Detection Methods to Naturally Contaminated Samples

Regardless of their drawbacks, there has been an increase in the use of virus detection methods applied to naturally contaminated samples. For example, in their 2007 U.S. market survey, DePaola et al. (267) determined (as evaluated by RT-qPCR) a prevalence of virus contamination (hNoVs or HAV) in U.S. oysters ranging from 5.2 to 11.3%, depending upon the region from which they were harvested. Kokkinos et al. (268) determined the prevalence of viral pathogens and indicators on leafy green vegetables across three countries, including Greece, Serbia, and Poland. Positive detection of viruses on produce samples ranged from 0.82% to 9%, with hNoVs detected in about 2.5% of general samplingpoint samples. Baert et al. (269) reviewed data on the prevalence of hNoV in fresh produce from Belgium, France, and Canada and reported relatively high rates of detection of hNoV in leafy greens, soft red fruits, and other popular fresh produce items. For instance, hNoV was detected in 28.2% to 50% of leafy green samples depending on the country of origin, although none could be confi rmed by sequencing. Unfortunately, no such survey studies on the prevalence of hNoV in U.S.-grown fresh produce have been conducted to date. Detection of hNoVs in environmental swabs and hand swabs has been used for hNoV outbreak investigations of restaurants and cruise ships (270). Boxman et al. (271) successfully detected hNoV RNA in foods associated with two outbreaks, although only with the aid of nested RTPCR. Environmental contamination by hNoVs has also been detected in the absence of outbreaks, and this approach was recently reviewed by Leone et al. (272).

Despite the tremendous strides that have been made in virus detection, these protocols are still underutilized and are usually applied only in response to known or suspected foodborne disease outbreaks. The primary reasons for their limited use include (i) the inability of molecular amplification methods to consistently discriminate between infectious and inactivated virus, even with the use of sample

pretreatment; (ii) the lack of widely accepted, collaboratively tested methods, as the available ISO methods are used only to a limited extent within the United States; (iii) the requirement that most methods be product specific, meaning that universal approaches for detecting a variety of viruses in a variety of foods do not exist; and (iv) the cost and the need for highly trained personnel.

In addition, care should be taken when both negative and positive test results are interpreted. For example, while a sequence-confirmed positive test definitively indicates the presence of the viral genome in the product, it does not necessarily confi rm that the contaminant was infectious. Negative test results are fraught with interpretive challenges related to sampling, the potential for virus inactivation (particularly if an extended time occurs between sampling and testing), low levels of virus contamination, and the impact of the matrix on assay detection limits as well as sample inhibition. Hence, a negative test result cannot be relied upon to ensure the absence of virus contamination, especially given the low infectious dose (50% human infectious dose, ~1,320 to 2,800 virions) of hNoVs (9).

An additional emerging issue is how to interpret marginally positive RT-qPCR results (e.g., a quantification cycle [C_q] value of >37), especially when amplicon concentrations are too low to be used in sequencing reactions. These difficulties were recently highlighted by Li et al. (273), who reported that 7 positive samples out of over 2,000 samples of frozen berries were collected and processed for recovery and detection of foodborne viruses over a period of 7 years. More specifically, positive samples corresponded to high C_q values (36.9 to 41.1), and these high C_q values are deemed suspect, requiring further investigation and inquiry.

RISK ASSESSMENT

Interest in applying the principles of risk analysis to foodborne viruses has increased over the past decade. The Food and Agriculture Organization and the World Health Organization conducted an expert consultation in 2007 to address this topic (274). Overall, the panel believed that there were so many unanswered questions that

embarking on a formal risk assessment would be premature. However, the document produced from their meeting served as the basis for a new work charge for the Codex Alimentarius Committee on Food Hygiene. In the meantime, the academic sector has sought to apply mathematical modeling to the development of criteria for enteric pathogens in irrigation waters (275) and the transmission of hNoVs in the food-handling environment (276). Recently, Duret et al. (211) published a quantitative risk assessment of hNoV transmission in food establishments designed to evaluate the impact of intervention strategies (e.g., handwashing, glove use, and elimination of contact between hands) and the behavior (e.g., exclusion from work and limited contact with food) of food service employees on the risk of hNoV contamination of foods. The study revealed that efficient handwashing and handwashing frequency combined with glove use as well as elimination of contact between hands and high-touch-rate fomite surfaces in bathrooms would significantly reduce the average number of infected customers. A similar study quantified the effects of environmental and direct transmission of hNoV on cruise ships and again confirmed that while isolation of ill persons is beneficial, good handwashing practices are more effective for preventing outbreaks (277).

A particularly interesting study seeks to use hNoV genotype profiles for selection and differentiation of foodborne outbreaks. The first phase of this work focused on developing a predictive model that could serve prospectively in the selection of outbreaks most likely to be associated with foodborne transmission. Using prospective epidemiologic surveillance data from the European Union, the investigators developed a logistic regression model that was adapted to a Web-based tool. The odds of an outbreak being foodborne were highly influenced by venue (home versus institution versus restaurant) and genotype (most foodborne outbreaks are associated with GI strains) (278). A follow-up project was initiated to determine whether hNoV genotype frequency distributions could be used to enhance detection of the sources of foodborne outbreaks (279). Both studies revealed how epidemiologic data might eventually be used to support better source attribution of foodborne virus illnesses and perhaps a move toward eventual risk-based decision-making for management of foodborne

viral diseases. Matthews et al. (16) conducted a similar study examining global trends of hNoV outbreaks based on a systematic literature review; however, this study considered only published outbreaks, and foodborne outbreaks are disproportionately published relative to nonfoodborne outbreaks. In contrast to the report by Verhoef et al. (278), the investigators reported that multiple strains (GI and GII) were associated with foodborne and waterborne outbreaks, whereas GI strains were significantly associated with waterborne outbreaks and GII strains were primarily associated with health care-related and seasonal outbreaks.

CONCLUSIONS

Epidemiologic evidence reveals that foods play an important role in the transmission of human enteric viruses. This can in large part be attributed to improvements in the ability to detect viral pathogens in clinical specimens, mostly related to recent advances in molecular detection techniques, as well as improvements in surveillance. Because both HAV and hNoVs can be transmitted by a variety of nonfoodborne routes, it is necessary to conduct comprehensive surveillance for outbreaks transmitted through all potential routes to determine the relative importance of foods as a vehicle of transmission (280). The advent of the NORS provides such comprehensive outbreak surveillance in the United States and an opportunity to better characterize foodborne attribution for enteric viruses. Nonetheless, widespread surveillance for individual cases of viral gastroenteritis does not occur, and most people with such illness do not seek health care or undergo diagnostic testing. In addition, tests for the detection of hNoVs and HAV in stool specimens are not routinely applied, although the recent development of commercial multipathogen panels may change these practices (281). When outbreaks are investigated, the implicated food is rarely tested for viral contamination, and when it is, detection limits minimize the usefulness of the results. Improved and more widespread reporting and investigation of foodborne viral disease outbreaks, as well as targeted epidemiologic studies to identify the risk factors for acquiring viral gastroenteritis, would improve our understanding of source attribution. The implementation and

continued enhancement of outbreak surveillance systems, such as NORS and CaliciNet, may help to provide this information.

Even if better detection of viral disease is implemented, the availability of effective controls remains a stumbling block. For molluscan shellfi sh, the absence of a reliable indicator of viral contamination of harvesting waters, along with the weaknesses in efficacy of conventional control strategies (depuration, relaying, and mild heat), suggests that occasional viral contamination of shellfi sh will continue to occur. However, there may be promise on the horizon with the application of PMMoV as an adequate and conservative enteric virus indicator to monitor shellfi sh growing areas. Fresh produce can become contaminated with viruses through a variety of routes, but our understanding of how, why, and how often such contamination events occur is quite limited. Water contamination and subsequent produce contamination are likely to occur sporadically, and implementing effective measures to improve personal hygiene at the farm level is challenging. The lack of truly effective antiviral disinfectant agents for both hands and surfaces is a further complication. Multiple control strategies will likely be needed, requiring significant resources, including a comprehensive education campaign. As more people understand why and how enteric viruses are spread, day-today changes, such as routine handwashing and hand and surface sanitation, will become more of a way of life and lead to better control of foodborne virus transmission. Finally, further development of candidate norovirus vaccines may one day provide another tool for reducing the incidence of disease and preventing outbreaks.

Our understanding of the importance of enteric viruses for the overall burden of foodborne disease has improved dramatically over the past 30 years. However, many unanswered questions remain, and much research is still needed. The field has been limited in the past by the small number of scientists working with foodborne viruses as well as the absence of easily reproducible *in vitro* culture methods, which limits studies of infectious virus strains. However, this is changing as more food microbiologists begin to tackle this fascinating and challenging subject. It is likely that the next decade will bring exciting

new developments as more is done to understand and control these important causes of human foodborne disease.

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24
Helminths in Meat

Foodborne parasites pose a risk to human health in virtually allregions of the world. In addition to the direct effect that these parasites have on human health, zoonotic parasites found in food animals often serve as trade barriers for countries where these parasites occur. A considerable body of legislation has been developed for the purpose of preventing and controlling zoonotic parasites in food animals, including very costly meat inspection programs. There are four meat-borne helminths of medical significance: *Trichinella* spp., *Taenia solium*, and *Taenia asiatica*, which occur primarily in pork, and *Taenia saginata*, which is found in beef.

Despite the availability of sensitive, specific diagnostic tests, veterinary public health programs (meat inspection), and effective chemotherapeutic agents for human tapeworm carriers, these parasites continue to be a threat to public health in many parts of the world. There are a variety of reasons for this, including animal management systems that perpetuate infection, inadequate or poorly enforced inspection requirements for slaughtered animals, new sources of infection, and demographic changes in human populations that introduce new culinary practices of preparing meats. Current control and preventive procedures are often inadequate, and more effective control measures are needed to ensure safe meat for human consumption.

TRICHINELLOSIS

The history of trichinellosis is fascinating, going back many centuries. Whether the commandment in the Bible (Leviticus 11 and Deuteronomy 14) to not eat the flesh of cloven-footed animals (swine) was due in part to the potential danger of contracting trichinellosis is only speculative. In the 7th century CE, Mohammed prohibited the eating of pork. Hence, to this day, trichinellosis is rare among Jews and Muslims. The earliest known case of human trichinellosis may be evidenced by the mummy of a person who lived near the Nile River ca. 1200 BCE, an observation made only in 1980 when *Trichinella* larvae were presumptively identifi ed in the mummy's intercostal muscle (1).

Trichinella spiralis was first observed by Paget in 1835 in the muscles of a man during postmortem dissection (2). A landmark in the history of clinical trichinellosis was Zenker's demonstration in 1860 that encapsulated larvae in the arm muscle caused the illness and death of a young woman. Even after 1860, many cases of trichinellosis were undoubtedly not diagnosed because of the diffi culty in recognizing the infection clinically. Since its discovery, *Trichinella* has been the subject of a very large body of research as well as global efforts to prevent human exposure through meat inspection and other mitigations.

Species of *Trichinella*

Nematodes of the genus *Trichinella* are ubiquitous in animals, both domestic and wild. *Trichinella* was long thought to be monospecific, with just a single species, *T. spiralis*, being recognized. As biochemical and, more recently, molecular tools have been used to characterize *Trichinella* isolates, it has become clear that the phylogenetics of this genus is much more complex. Species and genotypes of *Trichinella* are differentiated by geographic distribution, host distribution, and host infectivity, as well as subtle morphological, physiological, and biochemical differences. These differences in traits and characters which loosely group the species and genotypes are supported by extensive molecular studies (3, 4). The genus *Trichinella* is divided into two groups, or clades, based on

whether the larva is encapsulated in host musculature or nonencapsulated. The encapsulated group currently includes six species and three genotypes: *T. spiralis, T. nativa, T. britovi, T. murrelli, T. nelson, T. patagoniensis, Trichinella* T6, *Trichinella* T8, and *Trichinella* T9. The nonencapsulated group includes *T. pseudospiralis, T. zimbabwensis, and T. papuae.*

Among these nine species and three genotypes, only *T. spiralis* is commonly associated with the domestic pig and circulates widely in the synanthropic cycle in temperate regions of the world. Other species and genotypes found in temperate regions include *T. murrelli* and *T. britovi*, which circulate primarily in the sylvatic cycle in North America and Eurasia, respectively (5). Both *T. nativa* and *Trichinella* T6 show resistance to freezing temperatures and are distributed in the Holarctic (*T. nativa*) and the northern parts of the United States and Canada (*Trichinella* T6). *Trichinella* T6 has been shown to be closely related to *T. nativa* based on interbreeding studies (6). *T. nelsoni* has been reported from wild animals in countries of the southeastern region of Africa, and its range overlaps that of *Trichinella* T8 (which through interbreeding studies has been determined to be closely related to *T. britovi*) (3). *Trichinella* T9 has a range limited, so far, to sylvatic carnivores in Japan. This genotype interbreeds with *T. britovi* but has been shown by molecular studies to be more closely related to *T. murrelli*. The most recently named species, *T. patagoniensis*, was isolated from a mountain lion in the Patagonian region of Argentina. Extensive genetic analyses identified this isolate as a new species, phylogenetically very close to the ancestral root species *T. spiralis* (7, 8).

Among the three nonencapsulated species, *T. pseudospiralis* has been found in North America, Europe, Asia, and Australia in mammals and carnivorous birds (9, 10). Originally identifi ed in crocodiles, and found only in Africa, *T. zimbabwensis* has a broad host range, including lizards and lions, but has also been shown in experimental studies to be capable of infecting many other homeothermic and poikilothermic hosts (11, 12). The

nonencapsulated species *T. papuae* has been reported to occur in Southeast Asia and Australasia in wild pigs as well as saltwater crocodiles and has been reported as a source of human infection in those areas (13).

Life Cycles of *Trichinella* Species

All species of *Trichinella* complete the life cycle within one host; no intermediate host or extrinsic development is required. When larvae contained in raw or inadequately cooked meat are ingested, the muscle fibers and capsules that enclose the parasites are digested in the stomach. In the intestine, the liberated larvae burrow into the lamina propria of the villi in the jejunum and ileum. Four molts occur within 48 h, and by the third day, the worms are sexually mature. The small tapered head of the adult worm has a round, unarmed mouth that opens into a tubular esophagus. Approximately one-third of the anterior portion of the body is composed of the stichosome, consisting of stacks of discoid stichocytes. The secreted contents of these cells are important for serological detection of infection and also contain antigens that confer protective resistance to the host. Immediately below the esophagus and the stichosome lies a thin-walled intestine, the hind portion of which terminates in the rectum, a muscular tube lined with chitin. The adult female worm measures about 3.5 mm in length and possesses a vulval opening about onefifth the body length from the anterior end (Fig. 24.1). The male measures 1.3 to 1.6 mm in length and possesses a single testis that originates in the posterior portion of the body and extends anteriorly to near the posterior end of the esophagus, where it turns posteriorly to form the vas deferens, which becomes the enlarged vesicula seminalis. The vesicula seminalis becomes the ejaculatory duct to join the copulatory tube in the cloaca. The copulatory tube forms the copulatory bell that is extruded during copulation (Fig. 24.2). There are two ventrally located copulatory appendages on each side of the cloacal opening, which possibly serve to clasp the female during copulation. Between these appendages lie four tubercles, or papillae.

In the host, sexually mature adult worms reenter the lumen of the small intestine, where copulation takes place. The adult males die shortly after copulation. The female worms burrow back into the mucosa and begin to larviposit, usually into the central lacteals of the villi, about 7 days after infection and may continue to do so for a period up to a few weeks. Each female worm can shed approximately 1,500 newborn larvae, but this number varies based on species and host.



Figure 24.1 Scanning electron micrograph of a female adult worm of *T. spiralis* with its prominent vulval opening (magnification, ×2,450).

The tiny newborn larvae (100 by 6 mm) are carried from the intestinal lymphatic vessels to the regional lymph nodes and into the thoracic duct and the venous blood, passing through the right side of the heart, through the pulmonary capillaries back to the left side of the heart, and into peripheral circulation. During migration, the larvae may enter many tissues, including those of the myocardium, brain, and other sites, but here they either are destroyed or reenter the bloodstream. Generally, only larvae that reach striated muscles are able to continue development. There they penetrate the sarcolemma of the fibers, where they mature, reaching approximately 700 to 1,100 mm in length. They become coiled within the fibers and, in the case of most species, are encapsulated as a result of the host's cellular response. This host-

parasite complex, called the nurse cell, is capable of supporting the infective larvae for months or even years. An increased vascular supply to the nurse cell provides nutrients and oxygen vital to the parasite's survival. The encapsulated cyst eventually becomes calcifi ed, and as a result, the larva dies.

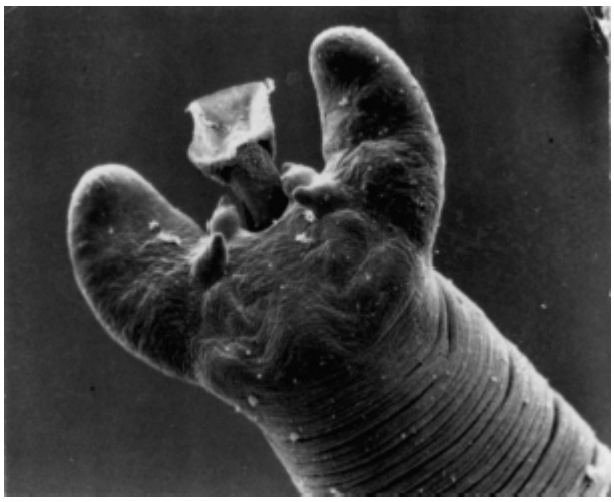


Figure 24.2 Scanning electron micrograph of a male adult of *T. spiralis* with its copulatory bell (magnification, ×1,400).

Infection in humans represents a dead end in the parasite's life cycle. However, in animal hosts, the carcass serves as a source of infection for carnivores and scavengers. Typically, infected animals are refractory to subsequent infection. However, molecular evidence has documented dual infection in animals (14), and it is possible that these dual infections result from multiple exposures.

Epidemiology

Trichinellosis, the human disease resulting from infection with species of the genus *Trichinella*, is considered a zoonosis because infection occurs as a result of ingestion of raw or poorly cooked meat from infected animals. *Trichinella* infection has been documented in domestic animals from 43 countries and in wildlife from 66 countries (15).

In the period of 1986 to 2009, the WHO reported 65,818 cases of human trichinellosis from 41 countries (16). Of these, 86.47% were

from the European region and another 10.90% were from the Americas. These numbers do not include reports from China. The mortality rate for these cases was 0.064%. Countries of Eastern Europe accounted for the majority of cases in the European region, while Argentina had the most cases in the Americas. Outbreaks in the period were linked to domestic pork and game meats in most countries, with the notable exception of horsemeat-related outbreaks in France, Italy, and Mexico and isolated outbreaks associated with dog meat.

The transmission patterns of *Trichinella* spp. can be divided into domestic-animal (synanthropic) and wildlife (sylvatic) cycles (14). Humans can become involved in both of these cycles. The domestic cycle, by definition, must involve domesticated (farm) animals. There are various patterns of transmission within the domestic cycle. Pigs may become infected through the deliberate feeding of uncooked or undercooked animal flesh containing infective larvae or by scavenging on infected animal carcasses. Infected pigs may then serve as a source of infection for other pigs through cannibalism. Within the domestic cycle, rats may serve as a reservoir or bystander host, acquiring infection from wildlife, carrion, or pigs. Rats may also transmit infection directly to pigs that ingest them.

The species of *Trichinella* most frequently associated with the domestic cycle in pigs is *T. spiralis*; however, there are an increasing number of reports of *T. britovi* as a cause of human infection caused by meat from domestic pigs (17). Low infectivity of other *Trichinella* species for the domestic pig diminishes their ability to circulate in the domestic cycle. Because cooking, freezing, and other processing methods kill *Trichinella* larvae in meat, most human infections have resulted from instances where meat preparation was not adequate. Pork products such as fresh sausage, summer sausage, and dried or smoked sausage have allbeen implicated as sources of human trichinellosis.

In several countries of the European Union, where slaughter inspection of pigs for *Trichinella* infection has been required for more than 100 years, the parasite has been essentially eradicated from domestic swine, and consequently, human trichinellosis resulting from domestic pork has not been reported for several decades. These countries do report sporadic outbreaks due to game meats or imported meats. Pork and pork products continue to serve as a major source of infection in many parts of the world (18–20). For example, in Romania, between 1980 and 1989, a total of 4,345 cases of human trichinellosis were reported. Between 1990 and 2004, a total of 23,948 cases were reported. In the year with the greatest number of human cases, 1993, more than 250 human cases were reported per 1 million inhabitants (21). This spike in human infection is attributed to changes in the pork production systems due to turmoil in the social and political systems. Reemergence of trichinellosis as a serious health risk has likewise been reported in Serbia (22), where infection rates in pigs between the years 1995 and 2006 were nearly 1.0%, and in Bulgaria, where 145 outbreaks were reported between 1990 and 2006 (23).

Trichinella continues to be a significant public health risk in parts of Asia and in Central and South America. Wang and Cui (24, 25) reported 23,004 cases of trichinellosis in China between 1964 and 1999. Almost allcases (95.8%) were linked to pork. From 2000 to 2003, there were 17 outbreaks involving 828 individuals and 11 deaths (1.33%) (26), and from 2004 to 2009, there were 11 outbreaks involving 1,387 individuals and 4 deaths (0.29%). All outbreaks occurred in southwestern China and involved ethnic groups which eat raw meat. In a serological survey in 10 provinces, autonomous regions, and municipalities, an average positive rate was 3.19% (range of 0.26 to 8.43%) (27).

In most cases, poor hygienic conditions for raising pigs and inadequate veterinary control at slaughter are the reasons for risk of human infection. Pigs raised outdoors, fed raw garbage, or raised in contact with large rodent populations are at risk of acquiring infection. Inadequate veterinary inspection may result from

insensitive or improperly performed testing methods or from pigs that are butchered outside of normal inspection channels (backyard pigs). In addition to poor animal management and gaps in inspection programs, culinary habits (improper cooking) impact the risk of human exposure, and this is exemplifi ed in some Asian countries where epidemics have been reported, including Thailand, Laos, Japan, China, and Hong Kong.

Another domesticated animal that emerged as a major source of human trichinellosis is the horse. Outbreaks in 1975 and 1985 in the southern suburbs of Paris were attributed to the consumption of raw horsemeat in the form of steak tartare (28). Fifteen outbreaks of human trichinellosis were associated with consumption of horsemeat in France and Italy between 1975 and 2005. These outbreaks involved a total of 3,334 individuals with five deaths (0.15%). As a result of these outbreaks, mandatory testing of horse meat was required in France beginning in 1985 and throughout the European Union as of 1991 (29).

Three species have contributed to human trichinellosis from horsemeat: *T. spiralis*, *T. britovi*, and *T. murrelli* (30). Following the first outbreaks, there was speculation that the horsemeat was contaminated with meat from other species (31). However, the recovery of larvae from suspected meat (32) and identification of naturally infected horses (33) have confi rmed this source of human exposure. The route by which horses become infected has been the subject of considerable speculation. The majority of horses linked to human outbreaks in Italy and France originated from countries or regions where a high incidence of *Trichinella* infection is known to occur in pigs, rats, and other animal species. One epidemiological study reported that the intentional feeding of animal products and kitchen waste is a common occurrence among horse owners in parts of Eastern Europe and that horses are more willing to consume meat than was previously realized (34). With better awareness of the risk of acquiring trichinellosis from horsemeat, inspection programs have been implemented, and horsemeat has not been implicated in any recent outbreaks.

The sylvatic cycle of *Trichinella* involves more than 100 species of animals, and many species pose a risk to humans who eat raw or undercooked game meats. In the sylvatic cycle, wild carnivorous or omnivorous animals scavenge the carrion of dead animals or eat meat from prey. Higher infection rates typically occur in animals near the top of the food chain. For example, in the arctic environment, *Trichinella* is quite prevalent in polar bears, grizzly bears, foxes, and wolves. Transmission in the arctic environment is further facilitated by the survival of larvae in frozen meat. In Alaska, allhuman cases of trichinellosis have been traced to the consumption of bear or walrus meat (35), and the etiological agent of these infections is the freezing-resistant species *T. nativa* (36–38). The repeated outbreaks of trichinellosis caused by the consumption of infected walrus meat have led to an inspection program for harvested walruses prior to distribution of the meat to Inuit communities (39).

The meat of wild boars is becoming an increasingly important source of *Trichinella* as these animals expand their range in many countries and has now become the second most common source of human infection (16, 40). Serological surveillance reported that up to 4% of wild boar tested had evidence of exposure to *Trichinella* spp. A review of human outbreaks between 2005 and 2015 reported 16 common source outbreaks due to wild boar meat from 13 countries involving 426 people and causing 12 fatalities (41).

Pathogenesis and Pathology

Disease caused by *Trichinella* infection varies with the intensity of the infection and the tissues invaded. The in-and-out movements of the adult worms, especially the females in intestinal tissue, cause an acute inflammatory response and petechial hemorrhages. The cellular response consists primarily of neutrophils with eosinophils. This is followed by an infi ltration of lymphocytes, plasma cells, and macrophages that peaks at about 12 days after infection, gradually declining thereafter. Thus, lesions in the intestine are due to the host's response to the adult worms or their protein products.

The newborn larvae cause an acute inflammatory response as they pass through or become lodged in various tissues and organs. The infiltration consists of lymphocytes, neutrophils, and especially eosinophils. Although there is myocarditis, viable larvae are more numerous in the pericardial fluid than in the myocardium. Pulmonary hemorrhage and bronchopneumonia may be observed during this stage of larval migration through the capillaries. Rarely, encephalitis may result if the larvae migrate through cerebral capillaries.

When larvae encyst in striated muscle fibers, there is an immediate tissue response consisting of inflammation of the sarcolemma of the involved muscle fibers. The disturbance of ultrastructure and metabolic processes in muscle fibers results in basophilic transformation. This is followed by destruction of the muscle fibers and the eventual formation of a capsule. The larvae gradually die, provoking an intense granulomatous reaction or foreign-body cellular response that culminates in calcification. The most heavily parasitized striated muscles include the diaphragm, intercostal muscles, and ocular and masseter muscles. Larvae that enter tissues other than striated muscles disintegrate and are eventually absorbed.

Clinical Manifestations

The vast majority of individuals infected with *Trichinella* spp. are asymptomatic, probably because low numbers of larvae are ingested. Classical trichinellosis is usually described as a febrile disease with gastrointestinal symptoms, periorbital edema, myalgia, petechial hemorrhage, and eosinophilia (42). During the intestinal stage of infection, gastrointestinal symptoms such as nausea, vomiting, and “toxic” diarrhea or dysentery, as well as fever (over 38°C) and sweating, may be observed. Malaise can be severe and lasts longer than fever. The onset of intestinal symptoms occurs usually within 72 h after infection and may last for 2 weeks or longer.

Generally, from the second week, when the newborn larvae are migrating and can reach almost any tissue within the body, a characteristic edema is noted around the eyes and in some cases around the sides of the nose, at the temples, and even on the hands. Periorbital edema is present in about 85% of patients and is often complicated by subconjunctival and subungual splinter hemorrhages that disappear within 2 weeks. Eosinophilia greater than 50% is the most consistent manifestation. In severe cases, however, the number of eosinophils may be low. As the larvae enter striated muscle fibers, a striking myositis and muscular pain are evident. In addition to inflammation and pain, eosinophilia is observed during this phase. Serum creatine phosphokinase and transaminase levels can be elevated as a result of leakage of these enzymes from muscle fibers into the serum. Higher levels of enzymes in serum may not correlate well with the severity of the clinical condition. Hypoalbuminemia, which is believed to be due to a demand for protein by the parasite, is usually accompanied by hypopotassemia. Muscle atrophy and contractures may also occur.

Respiratory symptoms, including dyspnea, cough, and hoarseness, may either be due to myositis of respiratory muscles or be secondary to pulmonary congestion. Neurologic manifestations are rare but may result from invasion of the brain by migrating larvae. In severe cases, death may result following cardiac decompensation or respiratory failure, cyanosis, and coma.

Intimate contact between the parasite and host tissues stimulates the production of antibodies that can be demonstrated in the serum. The exact role of antibodies in acquired immunity is not entirely clear, but antibodies appear to be involved in destruction of newborn larvae. On the basis of experimental animal data, it would be safe to assume that in most cases in humans, the severity of infection is reduced considerably by the development of immunity from previous subclinical infections. Local gut immunity in mice has been shown to be T-cell dependent (43).

It has been reported that different species of *Trichinella* present somewhat different clinical pictures in humans. For example, Serhir et al. (44) described a diarrheic syndrome in outbreaks caused by *T. nativa* in arctic regions of North America. This syndrome, characterized by persistent diarrhea and little edema or fatigue, differs from the traditional myopathic syndrome characterized by edema, fatigue, fever, and rash. These distinct syndromes occur concurrently in outbreaks; following the accumulation of serological and epidemiological evidence, it was concluded that the diarrheic syndrome is seen in patients who have preexisting immunity to *T. nativa* from prior exposure (44). Similarly, infection with *T. murrelli* results in a greater number of patients developing a rash (44%) and fewer patients developing facial edema (58%) (45). Only a few outbreaks of human trichinellosis have been attributed to nonencapsulated species of *Trichinella* (46, 47). The only clinical difference observed in these cases was a prolonged period of symptoms (fever and myalgias), which lasted twice as long as those reported for encapsulated species.

Diagnosis

It is difficult to clinically diagnose trichinellosis because it mimics so many other infections as a result of its dissemination throughout the body. Thus, individual or small groups of cases may go undiagnosed, whereas common-source outbreaks make diagnosis easier (46).

Diagnosis is based on a history of eating infected meat, symptoms, laboratory findings (including serology), and recovery of larvae from muscles (48). Classical symptoms of trichinellosis include fever, myalgia, and periorbital edema, although, as discussed previously, these symptoms may vary depending on the species of *Trichinella*. The most characteristic feature of laboratory tests is marked eosinophilia. Although eosinophilia is not restricted to trichinellosis, its presence in 30 to 85% of infected individuals is a constant and important diagnostic aid. Eosinophilia is initiated in the second week of infection, reaching its peak by about day 20. It

may be absent not only in patients with very severe and fatal cases but also in individuals with secondary infection. Levels of muscle enzymes, including creatine phosphokinase and lactate dehydrogenase, are frequently elevated in trichinellosis patients, as are overall leukocyte counts (46, 49).

The most definitive diagnostic method is muscle biopsy to detect encapsulated or nonencapsulated larvae. The diagnostic success of biopsy depends on chance distribution of larvae in the particular striated muscle that is sampled. Gastrocnemius, pectoralis major, deltoid, and biceps muscles are commonly used because of easy accessibility. The muscle strip is compressed tightly between two microscope slides and examined for the presence of larvae (Fig. 24.3). Part of the biopsy sample can be digested or fixed and then sectioned, stained, and examined. The presence of active larvae following digestion with artificial gastric juice indicates a recent infection.

There are numerous immunological tests available for the diagnosis of trichinellosis (50–52). Recommendations for the use of these tests have been summarized by the International Commission on Trichinellosis (53). The enzyme-linked immunosorbent assay (ELISA), using an excretory-secretory (ES) antigen collected from muscle larvae or a synthetic tyvelose antigen containing a dominant epitope, is the test of choice for human trichinellosis based on a high degree of sensitivity and specifi city. Both ES and tyvelose antigens are preferable to crude extracts of *T. spiralis* muscle larvae due to a risk of crossreactions with other helminth infections. A sensitivity of nearly 100% has been obtained for humans infected with *T. spiralis* when immunoglobulin G (IgG) is measured by ELISA (54). Serological tests measuring other classes of antibodies result in lower sensitivity (52); however, detection of specific IgM or IgA is indicative of a recent infection. The use of antigens from other stages (adults and newborn larvae) has not resulted in comparable serodiagnosis. Western blotting to determine the presence of antibodies to ES antigens in the range of 40 to 70 kDa

has been used as a confi rmatory test. The sandwich ELISA has also been used to detect circulating antigens in sera (55).

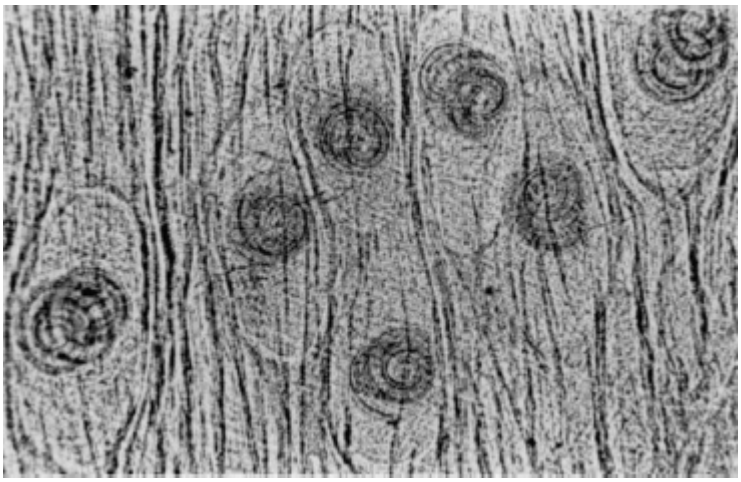


Figure 24.3 Example of *T. spiralis* muscle larvae in compressed muscle tissue viewed through a trichinoscope.

Trichinellosis patients typically become seropositive between the second and fifth weeks of infection, although this time varies with the species of *Trichinella*. The time required for seroconversion is inversely correlated with the infective dose. Thus, it is advisable to take multiple serum samples at intervals of several weeks in order to demonstrate seroconversion in patients whose sera were initially negative or to detect rising titers. Once patients become seropositive, antibody levels do not correlate with the severity of the disease (52). Patients typically remain seropositive for several years, but seropositivity has been reported to last for up to 35 years following infection (45, 56).

Treatment

The efficacy of treatment of trichinellosis depends on the intensity of infection, the species of *Trichinella*, the stage of infection, and the character and intensity of the host response. The purpose of treatment during the intestinal phase is to destroy adult worms and to interfere with the production of newborn larvae. The drug of choice is mebendazole (Vermox) at dosages of 200 to 400 mg three times a day for 3 days and then 400 to 500 mg three times a day for

10 days (42, 48). Mebendazole is also believed to be active against developing and encysted larvae but at dosages higher than those used against adult worms. Mebendazole is administered during the first week of infection before newborn larvae migrate, in moderate or severe infections in combination with corticosteroids, and in infections with *T. nativa*, which responds poorly to treatment with nonbenzimidazole compounds. Mebendazole is not recommended for women in the first trimester of pregnancy. Thiabendazole (Mintezol) is no longer used because of adverse reactions. Albendazole (Valbazen and Zentel) is better absorbed and is probably as active as or even more active than mebendazole (42). Pyrantel (Antiminth) at 10 mg/kg of body weight/day for 4 days and levamisole at 2.5 mg/kg/day (maximum of 1 to 50 mg) are active only against adult worms in the intestine.

To minimize hypersensitivity, it is recommended that corticosteroids be given in combination with anthelmintic drugs. Corticosteroids are recommended for acute severe trichinellosis not only for antiallergic action but also for anti-inflammatory and antishock actions. Supportive therapy, such as bed rest, is very important.

Prevention and Control

Prevention of human exposure to *Trichinella* from domestic pigs can be accomplished in a variety of ways: (i) by prevention of infection on the farm (preharvest), (ii) by testing animals at slaughter, (iii) by postslaughter processing using methods that have been proven effective for killing *Trichinella* larvae in meat, and (iv) by proper cooking by the consumer. The recommended application of these methods for the control of trichinellosis in pork has been summarized by the International Commission on Trichinellosis (57) and in guidelines published by the World Organisation for Animal Health (OIE) (58).

Prevention of Infection on the Farm

Knowledge of the routes of transmission of *Trichinella* to domestic pigs has allowed the design of swine management systems that essentially eliminate the risk of exposure of pigs to this parasite. By following a series of good management practices, including biosecure housing, effective rodent control, and good manufacturing and storage practices for feed, it is possible to certify the safety of pork without subsequent slaughter inspection or further processing. The requirements for producing pigs under conditions that greatly reduce or preclude exposure to *Trichinella* infection are outlined by several published sources. The International Commission on Trichinellosis provides guidance for *Trichinella*-free pig farming (57). The essential elements of these recommendations relative to pig farming are reiterated in EU Commission Regulation no. 1375/2015 of the European Union (59) and the OIE Terrestrial Animal Health Code (60). New guidelines from the Codex Alimentarius (61) provide that pork from pigs raised under the conditions outlined by OIE, and from territories where *Trichinella* is not endemic in domestic pigs, can be considered to have a negligible risk to humans, and therefore, testing and postslaughter mitigations are not necessary to protect consumer health.

Slaughter Testing

In virtually alldeveloped countries, animals slaughtered for human consumption are examined by a federal or state inspector to determine if the animal is healthy and suitable for use as human food. In many countries, the inspection process for slaughtered pigs includes testing each pig carcass for *Trichinella* infection by using one of several approved direct methods of inspection. Inspection methods for *Trichinella* infection have historically been performed by a trichinoscope examination, in which a piece of muscle is compressed between two glass slides and scanned under a microscope. This method is both tedious and ineffective, since it has a sensitivity of only about 5 larvae per g of tissue and nonencapsulated species of *Trichinella* are very difficult to visualize

(62); consequently, it is no longer recommended for routine use in testing pigs.

The development of a high-volume slaughter test, the pooled sample digestion method, takes advantage of the ability of *Trichinella* larvae to survive treatment with acidifi ed pepsin (as they do in the stomach of a host). Samples of tissue collected from sites of parasite predilection (diaphragm, cheek muscle, and tongue) are subjected to digestion in acidifi ed pepsin. Larvae, freed from their muscle cell capsules, are recovered by a series of sedimentation steps and then visualized and enumerated under a microscope. Requirements for performing the digestion test are found in the directives of the European Union (59), in the U.S. Code of Federal Regulations (63), and in the OIE *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (64).

Using methods of inspection testing as practiced on pig carcasses, the sensitivity of the digestion method is approximately 3 larvae per g of tissue (65, 66). This level of detection is considered effective for identifying pork that poses a significant public health risk. Although there is insufficient information to determine the exact number of larvae that are necessary to cause clinical human disease (and these figures will be affected by the type of *Trichinella*, the amount of meat eaten, and the health of the individual), it is generally considered that infections of >1 larva per g of tissue are a public health risk. Thus, most infections that could cause clinical human disease would be detected by currently employed direct testing methods. Through the application of rigorous slaughter testing using artificial digestion methods, *Trichinella* infection has been virtually eliminated from domestic pigs in some countries.

Postslaughter Meat Processing

Many countries have requirements for ready-to-eat pork products that have not otherwise been demonstrated to be free from *Trichinella* infection; they must be processed by heating, freezing, or curing to kill any potential *Trichinella* larvae. Commercial

processing methods that have been proven experimentally to render pork free from infective *Trichinella* larvae are described in the U.S. Code of Federal Regulations (63). Pork meat must be heated to 58°C or frozen at one of several time-temperature combinations. With flash freezing, *Trichinella* larvae are killed instantaneously at −35°C. The effectiveness of curing depends on a combination of salt concentration, temperature, and time. Each method should be tested experimentally to determine effectiveness, as no model for curing conditions has been devised.

Consumer Education

The most effective measure to prevent trichinellosis in regions where the disease is endemic is education of the public. The responsibility lies with consumers to ensure that larvae are killed before the pork is eaten. The U.S. Department of Agriculture recommends that fresh pork be cooked to an internal temperature of 145°F (63°C) before consumption in the home (15). Rapid cooking methods, such as the use of microwave ovens, may not heat the pork uniformly or for sufficient time to destroy the larvae (67). Commercial cooking of pork products requires lower temperatures to ensure inactivation of *Trichinella*, presuming that temperature is more closely controlled by commercial meat processors. The U.S. Code of Federal Regulations (63) requires that pork be frozen for 20 days at 5°F (−15°C), 10 days at −10°F (−23°C), or 6 days at −20°F (−29°C) to kill the larvae, provided that the meat is less than about 6 in. (15 cm) thick. These freezing temperature and time requirements are not effective for killing the arctic isolate *T. nativa* or other freezing-resistant types of *Trichinella*.

TAENIASIS

Until the mid-1980s, it was believed that taeniasis was the result of ingesting tissue cysts of either *T. saginata* from cows or *T. solium* from pigs; however, Fan (68) identifi ed and characterized a third human taeniid with adult morphological characteristics like those of

T. saginata and larval characteristics more in line with those of *T. solium*. Pigs were putatively identifi ed as the intermediate host for this unique taeniid, which had a predilection for liver tissue in a multitude of experimental hosts. At that time, controversy arose over the classification and therefore the naming of this third human taeniid. However, sufficient morphological, biological, and genetic differences have been identifi ed to warrant use of the name *T. asiatica* (69–71). In addition, a DNA sequence-based study using formalin-fi xed samples dating back to 1929 clearly showed a partitioning of the taeniids into *T. saginata*, *T. solium*, and a distinct third species, *T. asiatica*, from the East (72, 73).

T. saginata Taeniasis

The history of taeniids has been described in the ancient literature dating back to 1500 BCE, with intriguing theories as to the parasites' origin and nature. However, it was not until 1782 that Goeze (74) identified a difference between the bovine and swine parasites, which was later confi rmed in 1857 by Küchenmeister based on the morphologies of the scolices (75). In 1861, Leuckart was the first to identify a relationship between the adult worm in humans and the larval bladder worm in cattle.

Life Cycle of *T. saginata*

The most prevalent intermediate host of *T. saginata* is cattle; however, buffaloes, camels, horses, and giraffes can harbor the intermediate stages. The life cycle of *T. saginata* begins with cattle ingesting *T. saginata* eggs during grazing. The eggs hatch in the duodenum, liberating a six-hooked embryo that penetrates mesenteric venules or the lymphatics and reaches skeletal muscles or the heart, where it develops into the cysticercus larva. The cysticercus is essentially a miniature scolex and neck invaginated into a fluid-fi lled bladder that measures about 10 by 6 mm. The bladder larva becomes infective within 8 to 10 weeks following ingestion and can remain infective for more than a year.

A person eating raw or poorly prepared beef harboring viable larval cysts (often referred to as cysticercus bovis) is subject to infection whereby the larvae are released from their surrounding muscle tissues by digestion in the small intestine. The scolex of the cysticercus evaginates from the vesicle or bladder and attaches to the mucosa of the jejunum, where the larva develops into a mature adult worm in 8 to 10 weeks. A mature adult worm is characterized by a scolex with four hemispherical suckers 0.7 to 0.8 mm in diameter situated at the four angles of the head. The entire strobila can grow to 17 m in length and possess 1,000 to 2,000 proglottids or segments immediately following the neck in a symmetric series of immature, mature, and gravid proglottids as they proceed posteriorly. The mature and gravid proglottids contain both male and female reproductive organs, resulting in self-fertilization when distal proglottids come together by folding of the worm's body. The presence of only a single worm per defi nitive host suggests that the mechanism of genetic variation results from other than crossbreeding among adult worms.

The scolex attaches to the mucosal surface of the upper jejunum by means of the four suckers, but because of its length, the entire worm might extend down to the terminal ileum. The developing proglottids extend down the small intestine, where the most distal gravid proglottids (20 mm long and 5 to 7 mm wide) detach singly from the rest of the strobila and independently migrate through the rectum to the outside. Each gravid proglottid contains about 80,000 eggs, which are expressed from the proglottids and deposited on the perianal skin. When gravid proglottids come to rest on the ground, eggs are extruded. The eggs may also be present because of indiscriminate, promiscuous defecation. Cattle ingest *T. saginata* eggs during grazing to complete the cycle.

Epidemiology

Taeniasis results when raw or inadequately cooked beef containing tissue cysts is consumed. Raw or rare beef is popular in many countries, particularly in the form of beef tartare (basterma) in the

Middle East; the equivalent of steak tartare (kitfo) in parts of Africa, especially Ethiopia; shish kebab in India; lahb in Thailand; and yuk hoe in Korea. Although wild cervids as well as other herbivorous animals have been reported to contain cysticerci of *T. saginata*, beef remains the primary source of *T. saginata* taeniasis in humans.

With respect to the incidence of taeniasis in humans, geographic areas can be classifi ed into three groups: countries or regions where infection is highly endemic (prevalence exceeding 10%); countries with moderate infection rates; and countries with a very low prevalence (0 to 0.1%) (76). The areas of high endemicity are Central and East African countries, such as Ethiopia, Kenya, and Zaire; Caucasian South Central Asian republics of the former Soviet Union; Middle Eastern countries, such as Syria and Lebanon; and parts of the six republics that constituted former Yugoslavia. However, recent reports suggest that the infection rates are on the decline in some regions (77). In Europe, the predominant taeniid is *T. saginata*. In a recent retrospective study encompassing the years 1990 to 2015 and five international databases, infections were found in 12 of 18 countries of Western Europe (78). In North America, the incidence is low. The last reported case in Canada was in 2013.

In 2008, a population-based survey was performed in the southwestern United States along the border with Mexico (79) among neighboring cities. Results indicated a combined prevalence of 3%; however, compared with the residents of Juárez, Mexico, residents of El Paso, TX, were 8.6-fold more likely to be tapeworm carriers. This difference between the United States and Mexico was attributed to widespread use of anthelmintics in Mexican border towns. In 2013, the U.S. Centers for Disease Control and Prevention (CDC) estimated that there were fewer than 1,000 new cases of taeniasis annually in the United States. It should be noted that the 2008 study and the estimates from the CDC do not distinguish between the pig and bovine tapeworms.

A recent issue, predicated upon genetic studies, is the within-species diversity in *T. saginata*. Findings by scientists in Southeast Asia

concluded that there are at least five different subgroups of *T. saginata*, most of which reside in that region of the world (80). Currently, no distinct information is available as to whether the genetic diversity can be linked to physiological parameters such as host range or virulence; however, studies of this nature provide groundwork for genetically identifying the source of new outbreaks, especially in countries where the prevalence is low.

Persistence and change in human *T. saginata* infections correlate with factors that govern infections in cattle. Infections in cattle are directly associated with environmental factors and animal access to pastures or cattle pens contaminated with human feces (81), sewage effluent used as fertilizer (82), or irrigation ditches and surface water (83). The U.S. Code of Federal Regulations (84) prohibits carcasses harboring as few as one dead larva from being approved for human consumption unless the carcass is first treated to remove the visible cyst(s). Eggs of *T. saginata* are capable of surviving for long periods in the environment and are resistant to moderate desiccation, disinfectants, and low temperatures (4 to 5°C). Longevities of 71 days in liquid manure, 33 days in river water, and 154 days on pasture have been reported (85). The eggs can also survive many sewage treatment processes and can remain viable in fluid effluent or dried sludge. Reviews (82, 86) summarize both biological and nonbiological treatments of sludge to reduce egg viability; however, a 2005 study in France (87) demonstrated that cows raised on pastures fertilized with liquid sludge did not acquire live cysticerci provided that a 6-week delay occurred between application and grazing.

The rate of reported cases of human taeniasis in many developed countries remains low. However, the true incidence is unknown because of underreporting. Among affluent Americans, infection has been attributed to international travel and to preferences for exotic diets while abroad. The penchant for raw beef and steak tartare is not limited to affluent Americans and Europeans but is also found, for example, among Ethiopians who eat beef. A combination of poor sanitation and culinary preferences for raw and

rare beef in many regions of high endemicity are key factors contributing to the risk of infection and transmission.

Pathogenesis and Pathology

The scolex of the adult worm generally is lodged in the upper part of the jejunum. Usually, only a single worm is present. Although multiple infections have been reported, these have come under scrutiny with the identification of *T. asiatica*. It has been suggested that these multiple infections with *T. saginata* were misdiagnosed infections of *T. asiatica*, which is capable of synchronically infecting human hosts (88). The adult tapeworm of *T. saginata* does not cause pathologic changes; however, mucosal biopsies have shown some inflammation, suggesting that the presence of the worm can trigger irritation that in turn results in bowel distention or spasm. Migration to unusual sites is rare, but complications such as appendicitis, invasion of pancreatic or bile ducts, intestinal obstruction, or perforation and vomiting of proglottids with aspiration have been reported. A recent retrospective examination of 34 case reports and 2 case series dating from 1957 through March 2016 showed that acute appendicitis and gastrointestinal perforations and migration to biliary tract can accompany infection. All these cases caused disease detected only by surgical intervention (89).

Clinical Manifestations

Although most cases of taeniasis are asymptomatic, up to one-third of patients complain of nausea or abdominal “hunger” pain that is often relieved by eating. Epigastric pain may be accompanied by weakness, weight loss, increased appetite, headache, constipation, dizziness, and diarrhea. The patient usually becomes aware of the infection when a proglottid is passed in the stool or is found on the perianal area or even on underclothing.

The adult worm is weakly immunogenic, as manifested by moderate eosinophilia and increased levels of IgE in serum. Allergic reactions,

such as urticaria and pruritus, may be due to the worm and its metabolites. The adult worms induce the production of antibodies. The persistence for years of a large, actively growing worm reflects lack of protective immunity to resident worms but is consistent with concomitant immunity observed in other helminth infections.

Diagnosis

Morphological diagnosis is based on the proglottid, since the eggs of *T. saginata* cannot be distinguished from those of other species of *Taenia* or those of *Multiceps* and *Echinococcus* species. The gravid proglottid of *T. saginata* has 15 to 20 lateral branches of the uterus on each side of the main uterine stem, a characteristic feature (Fig. 24.4); however, a similar range of uterine branches has been identifi ed in *T. asiatica* as well, suggesting that the geographic source of the infection may play a role in diagnosis if uterine branches are used in identification. If the scolex is present, the four characteristic hookless suckers can be used as a distinguishing character.

The egg is nearly spherical, measures 30 to 40 mm in diameter, has characteristic radial striations on its thick shell, and contains a hexacanth embryo with delicate lancet-shaped hooklets (Fig. 24.5). Rather than looking for the eggs in the stool, it is better to use the commercial Scotch tape method to obtain the eggs or proglottids from the perianal region. Since morphological identification of eggs is nonspecific, molecular techniques have been developed. Earlier methods were based upon DNA hybridization probes; however, PCR (90) and DNA sequencing (91) have advanced the ability to discriminate among various *Taenia* species. A recent review describes the advantages and disadvantages of conventional end-point PCR, real-time PCR, multiplex PCR, and loop-mediated isothermal amplification (92) in differentiating taeniid species.

Although an antibody response is induced by the adult worm, specific serological tests are not routinely used for diagnosing taeniasis; the disease is not lifethreatening, and detection of proglottids shed in human feces is the easiest and most defi nitive

test. In contrast, serology tests for bovine cysticercosis have been scrutinized through the years (93, 94), though problems have arisen from the disparate immune responses among the hosts (95) and the lack of correlation between the level of parasitism and the level of the immune response. Consequently, neither the efficacy nor the sensitivity of these methods has proven superior to that of physical inspection, including tests based upon recombinant proteins, peptides, or secreted antigens (ES), though recent results have been promising (96). One additional political hurdle impedes the commercialization of such a test: a viable antibody test for cysticercus bovis might result in the condemnation of a greater number of animal carcasses.

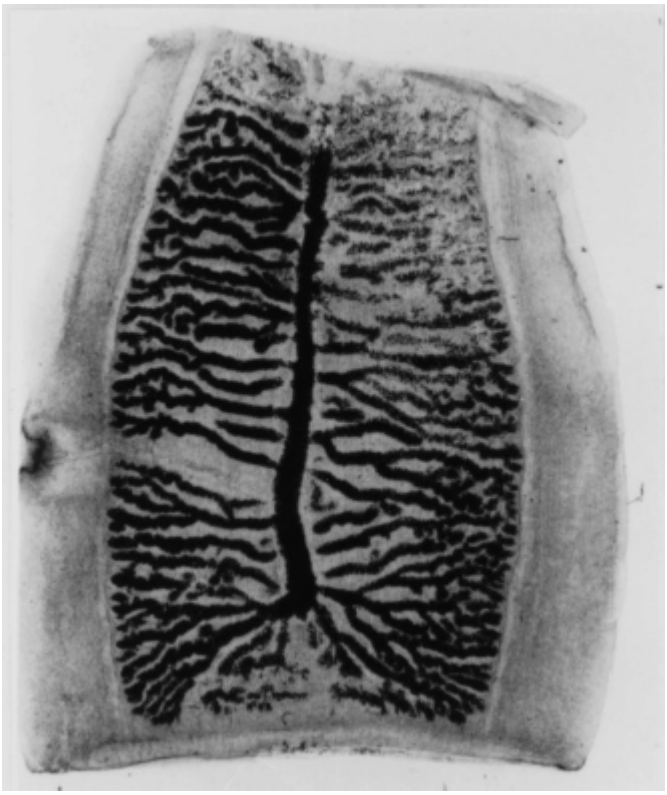


Figure 24.4 Taeniids are segmented flaworms consisting of proglottids. This micrograph depicts a gravid proglottid of *T. saginata* which possesses both male and female reproductive systems and at least 16 lateral uterine branches flled with eggs.

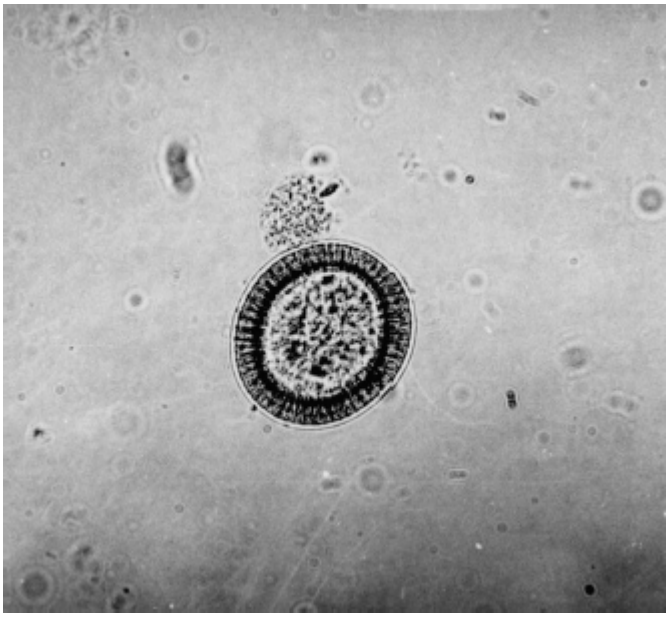


Figure 24.5 A gravid taeniid proglottid contains uterine branches filled with eggs (see Fig. 24.4). This micrograph shows a single *T. saginata* egg (magnifcation, ×590) extracted from the uterine branches.

Currently, meat inspection is used to detect cysticercosis in slaughtered cattle. Although practiced extensively, this method has limitations. Even routine examination of the heart and masseter muscles can miss a significant percentage of infected cattle. It is estimated that as few as 25% of infected cattle are detected by visual inspection (97). This is due to assumptions regarding predilection sites in host tissues, the inability to use the heart, diaphragm, cheek, and/or tongue as representative of wholebody infections, and the small number of tissue cysts generally found in areas of low prevalence.

In the United States, the percentage of carcasses inspected by state and municipal authorities varies. Cattle from small farms where sanitary conditions can be poor are frequently slaughtered in local abattoirs that tend not adhere to governmental inspection requirements. Clearly, allinfected carcasses should be condemned; however, approximately 70% of infected carcasses are trimmed of visible cysts and then passed unrestricted for human consumption.

This is prohibited by the U.S. Code of Federal Regulations (84), which also requires heavily infected carcasses to be condemned. Nonetheless, the combination of trimming the cysts with the less than 100% animal inspection requirement is consistent with the perceived low infection rates at the abattoir.

Treatment

The drug of choice for treating taeniasis is a single oral dose of 5 to 10 mg of praziquantel (Biltricide) per kg of body weight for adults or children. This treatment provides very high success rates (98). A good alternative is niclosamide (Niclocide and Yomesan) at 2 g orally once for adults and 50 mg/kg orally once for children. This taeniacide damages the worm such that a purge following therapy will often produce the scolex. Other drugs, such as albendazole and mebendazole, can also be used, but only under specialized circumstances. Reports of failure of treatment with allmajor drug classes (niclosamide, albendazole, and praziquantel) date back to 1970 and continue to surface today (99–101). It is unknown if these relate to unrecognized complications of the infection, rare naturally occurring isolates, misdiagnosis, or drug resistance. Given that the reports are sporadic and noncentralized and have not increased over the past 47 years, the failed treatments are unlikely the result of a resistant phenotype.

Prevention and Control

The best way to prevent taeniasis is to thoroughly cook meats before ingesting. In addition, one can limit cysticercosis in cattle by controlling sewage disposal so that tapeworm eggs are unavailable to the animals. Humans are the only defi nitive hosts of *T. saginata* and thus the only disseminators of eggs. Inasmuch as *T. saginata* adults can shed eggs at a rate of 480,000 to 720,000 daily, control depends on educating livestock producers and their employees about modes of transmission. Providing adequate toilet facilities in cattle feeding establishments can help to reduce the incidence of infection. Where toilet facilities are available, overloading of

systems should be avoided. In addition to education and monitoring, prevention should include sanitary protection of cattle feed as well.

The development of an effective cattle vaccine for use in areas of high endemicity would certainly reduce human infections. However, development of vaccines for bovine cysticercosis, natural or otherwise, has been slow. In addition to the difficulties in generating parasite vaccines, research in this area has not flourished because of the absence of human pathogenicity and the ease and economics of anthelmintic treatment. Going forward, it is anticipated that monetary and logistical issues will continue to deter development of vaccines as a prophylactic measure for bovine cysticercosis. If, however, developed countries see a benefit or if inspection criteria change to condemning allinfected carcasses, this mindset may change as well.

With regard to the consumer, public health education concerning the risks of eating raw or inadequately cooked beef is important. The cysticerci in meat are inactivated by freezing at –10°C for 10 days or –18°C for 5 days, heating to an internal temperature of 56°C, or salt curing under appropriate conditions. Major obstacles to this approach have been reluctance by consumers to modify preferred culinary habits and to break with long-established cultural traditions. Thus, attempts to eradicate *T. saginata* taeniasis have been unsuccessful.

T. solium Taeniasis

Taeniasis caused by *T. solium* was known at the time of Hippocrates. The Greeks described the larval stage in the tongue of swine as resembling a hailstone. In contrast to *T. saginata*, *T. solium* uses swine as an intermediate host, possesses an armed rostellum, has a smaller number of lateral uterine branches, and lacks a vaginal sphincter (102). Its phylogenetic classification as a unique species in 1758 is attributed to Linnaeus. It has been estimated that there are 50 million people worldwide with

neurocysticercosis, a plausible outcome of *T. solium*-derived taeniasis wherein an infected individual reacquires the infection from eggs rather than tissue cysts. In this case, human cysts form in the brain. One percent of these infected individuals die annually (103). For this reason, the World Health Organization lists neurocysticercosis as an important neglected tropical disease worldwide.

Life Cycle of *T. solium*

When pork containing a viable cyst of the larval stage of *T. solium* (often referred to as cysticercus cellulosae) is ingested, the head of the larva evaginates from the fluidfilled milky white bladder. The scolex bears four suckers and an apical crown of hooklets. The cysticerci are referred to as pork measles and are larger (5 to 20 mm in diameter) than cysticercus bovis. They attach to the wall of the small intestine and mature into adult worms in 5 to 12 weeks. The adult worm measures up to 8 m (usually 1.5 to 5 m) and has a scolex that is roughly quadrate, possessing a conspicuous rounded rostellum armed with a double row of large and small hooklets, numbering 22 to 32. A short cervical region is anterior to a series of proglottids or segments. Immature proglottids are broader than they are long, mature ones being nearly square, while gravid ones are longer than they are broad. The total number of distinct proglottids is less than 1,000. The terminal proglottids become separated from the rest of the strobila and migrate out of the anus or are passively expelled in the stool. A single gravid proglottid contains fewer than 50,000 eggs. Upon escape from the ruptured uterus of the gravid proglottid and after deposition on the soil, the eggs may remain viable for many weeks. The eggs of *T. solium* are more apt than those of *T. saginata* to appear in the stool.

In the normal cycle, the eggs are ingested by pigs, the usual intermediate host. The hexacanth embryo hatches in the duodenum, migrates through the intestinal wall to reach the blood and lymphatic channels, and is carried to the skeletal muscles and the myocardium. The embryo develops in 8 to 10 weeks into a

cysticercus. In the abnormal cycle, humans can serve as intermediate, but terminal, hosts and harbor cysticerci acquired by accidental ingestion of eggs or by the autochthonous cycle (an infection that results from the movement of eggs or gravid proglottids from the intestine back into the stomach by reverse peristalsis). The cysticercus develops most commonly in striated muscles and subcutaneous tissues but also in the brain, eye, heart, lung, and peritoneum.

Epidemiology

T. solium taeniasis has a worldwide distribution (104) and is important in allcountries where pork is consumed, especially those with suboptimal practices of sanitation and pig husbandry. Humans are the only known natural defi nitive host for this parasite. It is very difficult to evaluate the prevalence of human infection, because coproscopical methods cannot differentiate between infections caused by *T. saginata* and *T. solium*. Serological methods have been developed for this purpose but have not yet been tested in large field studies.

Infections resulting from *T. solium can* have much more serious consequences than those caused by *T. saginata*, because the eggs of *T. solium*, which normally infect pigs, can also infect humans. Thus, humans can acquire both taeniasis and cysticercosis from *T. solium* tissue cysts and eggs, respectively. Cysticercosis in humans is an important public health problem in developing countries where hygiene and sanitary conditions are deficient or nonexistent and in areas where swine-rearing practices are primitive (105). Infection of humans with larvae results from inadvertent ingestion of eggs in contaminated water or foods, e.g., vegetables fertilized with night soil (human excrement), from indirect contact with hands of individuals already infected with the adult worm (external autoinfection), or from direct contact with a tapeworm carrier. Also, internal autoinfection can occur in which the eggs from proglottids are carried by reverse peristalsis back to the duodenum or stomach,

are stimulated to hatch, and then invade extraintestinal tissues to become cysticerci. This is a key cause of neurocysticercosis.

The prevalence of human neurocysticercosis is increasing in some parts of the world, and a considerable body of literature is devoted to the diagnosis, treatment, and prevention of this disease (106–108). The relevance of neurocysticercosis as a public health problem in the United States has been highlighted by reports of autochthonous cases (109). Furthermore, with the increase in illegal immigration and the number of migrant workers from Central America, cysticercosis has garnered attention as a neglected infection in the United States that is linked to poverty (110). Infections are quite common in Mexico, Central and South America, central and southern Africa, Asia, non-Islamic Southeast Asia, Slavic countries, and Southern and Eastern Europe. A recent endemicity map with corresponding levels of intensity is available through the World Health Organization (111). Although the disease is present in Mexico, current evidence suggests that the incidence and severity of infection did not increase between 1994 and 2009 (112), offering encouragement for advancing efforts to control this disease. It should be noted that most of these studies, as well as other similar studies, used indirect serology-based assays, which are prone to false-positive and false-negative results. In general, specific habits of eating raw or undercooked pork are linked to foci of taeniasis; however, in some regions there is no link between porcine and human cysticercosis, where those who do not eat pork have as great a chance of contracting the disease as those who do (113). An interesting development is that reports worldwide are showing an association between the presence of neurocysticercosis and the incidence of epilepsy, suggesting that many infected patients are being misdiagnosed as epileptics (114, 115).

Pathogenesis and Pathology

As in *T. saginata* infection, there is usually only a single adult worm, and pathologic changes are similar. There is mild local inflammation of the intestinal mucosa resulting from attachment

by the suckers and especially the hooklets. However, because of its smaller size, *T. solium* is less likely to cause intestinal obstruction. Rare instances of intestinal perforation with secondary peritonitis and gallbladder infection have been reported.

Pathologic changes due to cysticerci can be serious, depending on the tissue invaded and the number of cysticerci that become established. Damage results from pressure caused by encapsulated larvae on surrounding tissue, since the cysticerci produce occupying lesions. There may be no prodromal symptoms or only slight muscular pain and a mild fever during invasion of the muscles and subcutaneous tissues. In ocular cysticercosis, which accounts for about 20% of neurocysticercosis cases, there may be loss of vision. Invasion of the meninges, cortex, cerebral substance, and ventricles evokes tissue reactions, leading to focal epileptic attacks or other motor or sensory involvement. The reasons for the predilection of cysticerci for the central nervous system remain unknown.

Clinical Manifestations

Since only a single adult worm is present in *T. solium* taeniasis, there are no symptoms of epigastric fullness. Patients are generally asymptomatic and become aware of the infection only when they find proglottids in their stools or on perianal skin. However, there may be vague abdominal discomfort, hunger pains, anorexia, and nervous disorders. Rare instances of intestinal perforation with secondary peritonitis and gallbladder infection have been reported. Retrospective studies conducted in 2017 linked surgical endoscopic abdominal intervention to taeniasis (71). Eosinophilia, as high as 28%, and leukopenia can occur. The persistence for years of actively growing tapeworms is not consistent with the development of protective immunity. Seizures that often accompany neurocysticercosis are to a high degree misdiagnosed as epilepsy, primarily because of the inability to adequately diagnose the infection.

Diagnosis

Diagnosis of taeniasis is based on examination of stool specimens and perianal scrapings; sensitivity is increased by increasing examinations to three times daily. Since *T. solium* eggs cannot be distinguished from those of *T. saginata*, specific diagnosis is based on the identification of the gravid proglottid, which has fewer lateral branches (7 to 12) on each side of the main uterine stem (Fig. 24.6). If the scolex is obtained, it can be identifi ed by its hooklets and four suckers. Other tests involving PCR, multiplex PCR (116), loop-mediated isothermal amplification (117), and serology testing have also been developed (118).

Diagnosis of neurocysticercosis is more problematic. Clinical presentation is unpredictable but often accompanied by neurologic symptoms, intracranial hypertension, mental changes, or sudden unexplained death among individuals at highest risk for disease (for a review, see reference 119). Histologic studies are always the most definitive; however, whole-body imaging (120), neuroimaging (121, 122), and other lab results can assist in final diagnosis and treatment (118). A set of diagnostic criteria have been developed (123) that remain applicable today. Clearly, image scanning is the most sensitive; however, a study using cerebrospinal fluid to compare antibody and PCR-based diagnostic tests showed that PCR was the most sensitive of the tests (95.9%), but with variable specifi city (80 or 100%), and that most antibody-based tests had similar sensitivities (124).

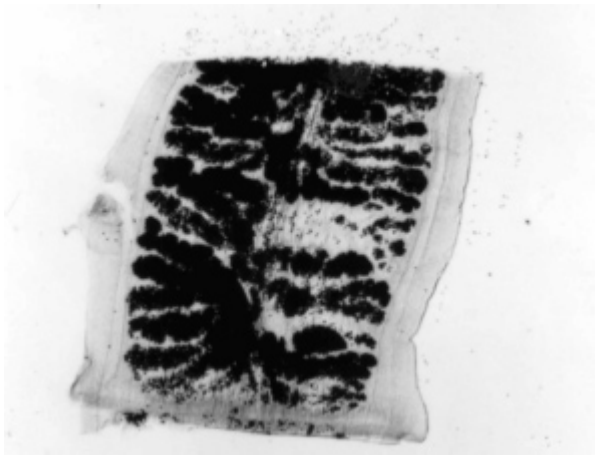


Figure 24.6 Gravid proglottid of *T. solium*. Note that there are fewer lateral uterine branches than in *T. saginata* (Fig. 24.4).

The sensitivity of serological tests for taeniasis and cysticercosis varies by the method and the clinical form of infection. Efforts have been made to improve the sensitivity of serology-based modern methods (118), although emphasis on the use of serology has shifted primarily to the diagnosis of cysticercosis rather than taeniasis. As with *T. saginata*, obtaining sufficient parasite antigen poses logistical problems in the absence of a validated recombinant product.

Treatment

Surprisingly, asymptomatic neurocysticercosis is left untreated. In contrast, treatment for symptomatic neurocysticercosis is contentious. Anthelmintic recommendations include praziquantel and albendazole. Unfortunately, the cysticidal efficacies of both drugs alone are not optimal. Further, studies showed that praziquantel can cause severe adverse conditions if given in large dosages or if given as the first course of treatment. This prompted a switch by clinicians to albendazole as the first-line treatment. In 2014, Garcia et al. (125) conducted a double-blind, placebo-controlled, phase 3 trial comparing combination therapy involving praziquantel and albendazole to albendazole alone. The study had three groups receiving treatment over a 10-day period: combination therapy (albendazole at 15 mg/kg per day plus praziquantel at 50

mg/kg per day), standard albendazole (15 mg/kg per day), or high-dose albendazole (22.5 mg/kg per day). The results clearly showed that combination therapy had increased parasiticidal effects, leading to better seizure control and long-term prognosis without increasing the side effects. However, drug treatment is not a panacea for allinfections. Distal lesions such as ocular, ventricular, and spinal cysts are best treated surgically, because anthelmintics can provoke tissue damage from irreversible, drug-induced inflammation. It is recommended, therefore, that alltreatments be accompanied by high-dose glucocorticosteroids and anticonvulsants to reduce the host inflammatory response as the cysts are destroyed, and thereby lower the risk of seizures.

With respect to taeniasis, even though the infections result in minimal side effects, it is imperative to treat patients harboring the adult worm, since cysticercosis can occur from internal autoinfection. The major concerns in treating patients with *T. solium* taeniasis are to prevent vomiting and to ensure rapid expulsion of disintegrated proglottids from the intestine. Niclosamide was once the drug of choice because of its effectiveness against the scolex, in which it causes necrosis of the head and adjoining segments by blocking sugar uptake. This in turn causes the tapeworm to lose its hold, after which it is eliminated in pieces during defecation. Praziquantel, however, is more effective, with fewer side effects, in purging the adult worm. After treatment with praziquantel, the tapeworm loses its ability to resist digestion in the gastrointestinal tract. Thus, only digested pieces are passed in the feces. Trials using a new drug, tribendimidine, that is effective against soil-transmitted parasites have shown promise in treating rodent cestode infections (126). One open-label study (127) also showed effective treatment against *Taenia* spp., but studies have not advanced beyond experimental treatments for this indication.

Prevention and Control

Prevention and eradication of *T. solium* adults in the intestine and of cysticerci in various tissues in humans and in pigs are a concern

in areas of endemicity where economic, social, and sanitary conditions are substandard. Without fundamental changes, the most scrupulous personal hygiene and eating habits will not prevent or eradicate infection in underdeveloped or developing countries. In developed countries, infection can be avoided simply by adherence to modern animal husbandry practices. The best preventive measure for interrupting transmission from humans to animals is to introduce and maintain proper sanitary facilities to dispose of contaminated human feces. Even with proper toilet facilities, measures must be taken to make sure that sewage treatment is adequate to kill the eggs.

Since one infected individual can infect thousands of pigs by contaminating feedlots, allpersonnel with access to the pastures must be educated regarding the parasite and the means of avoiding transmission. Prospective employees who will come into contact with animals should be examined for tapeworm infection before employment and periodically during employment; chemical toilets should be installed and properly maintained at convenient locations, and whenever possible, pigs should be kept in enclosures or indoors to prevent access to human fecal matter.

Meat inspection programs are only partially effective for identifying and condemning infected carcasses. Inspection for measly pork is performed in the United States for animals intended for interstate commerce, but as with *T. saginata*, organoleptic inspection can miss lightly infected carcasses. Thus, the consumer should make sure that meat is cooked to an internal temperature of 56°C or is frozen at −10°C for 14 days to kill cysticerci (128). Other means of inactivating cysticerci in pork, such as irradiation, have not been commercially applied. The best preventive measure is to avoid eating raw or uninspected pork.

Assuming that eliminating swine cysticercosis will greatly assist in eradicating human taeniasis and neurocysticercosis, efforts to immunize pigs with recombinant antigens have met with remarkable success in both laboratory studies and field trials (129,

130). Unfortunately, vaccination as a means of prevention and control is subject to problems other than a lack of efficacy. Economic and regulatory issues as well as dissemination and vaccine stability also come into play. Thus, a swine vaccine has yet to be made commercially available.

Other approaches are being entertained to control the disease. On a local scale, success has been achieved through a mass drug administration program within a community in Tanzania (131). Also, an 8-year, community-based public health intervention program in Honduras (132) reduced disease long term through deworming, education, the construction of health clinics and systems for water and sewage control, surveillance, animal husbandry training, and local advertising.

T. asiatica Taeniasis

T. asiatica was first detected in the aboriginal population in the mountainous regions of Taiwan (68). Initially, the etiologic agent was thought to be *T. saginata* because of morphological similarities among the adult worms; however, notable differences, including shorter length, fewer proglottids, wider diameter of the scolex, and fewer testes in the mature proglottid of the agent, were observed (68). Molecular studies also showed that that *T. asiatica* was genetically distinct from *T. saginata* (133) and provided the ability to differentiate *T. asiatica* from other human cestodes (72, 134). Even though swine are the intermediate host for *T. asiatica*, the close relationship between *T. asiatica* and *T. saginata* in the absence of neurocysticercosis among populations exhibiting the highest prevalence of infection is consistent with *T. asiatica* not being a cause of human cysticercosis (135). Research suggests that *T. asiatica* and *T. saginata* are capable of hybridization (136); however, given that these studies involved single-gene analyses, normal genetic variation among geographic isolates could have become obscured. The application of comparative genomics will hopefully better address this issue in the future.

Epidemiology

T. asiatica infection has been found in several Asian countries, notably the mountainous regions of Taiwan, Cheju Island of Korea, and Samosir Island of Indonesia. It is estimated that public health costs in these areas alone exceed $35 million annually (137). In Taiwan, a study of 1,661 cases of Asian taeniasis among nine aboriginal tribes in 10 mountainous counties found an overall clinical infection rate of 76% (138). Multiple studies since that time have suggested that the infection rate is quite variable among the aboriginal populations of Taiwan; nonetheless, the rate remains comparatively high. Pigs, cattle, goats, wild boars, and monkeys can serve as intermediate hosts, and this has been validated in experimentally infected animals (139). Of these, the wild boar appears to be the probable natural intermediate host in Taiwan. This may be true also in Indonesia, where people have become infected, presumably from consuming pork (140).

The cysticercus of *T. asiatica* is armed with tiny rostellar hooklets like that of *T. solium* and develops in a period shorter than that required for either *T. saginata* or *T. solium*. Interestingly, it is found mainly in the parenchyma of the liver (68), whereas the predilection sites for cysticerci of *T. saginata* and *T. solium* are the muscles of cattle and pigs, respectively. Thus, the custom of eating the viscera, especially the liver, of freshly killed animals appears to be a major contributing factor to transmission.

Most recently, reports (141–143) on taeniasis and cysticercosis in Middle East countries revealed some interesting and previously unreported information on *T. asiatica*. First and foremost, the reports tremendously expanded the western limits of areas of endemicity of this pathogen far beyond its previously known boundaries, i.e., Southeast Asia. Indeed, an epidemiological study conducted in northern India indicated that nearly allcases were caused by *T. asiatica* and not *T. saginata* (144). Similar findings were obtained in a communitybased survey in Nepal (142). Equally important, for the first time, *T. asiatica* was found outside its

normal predilection site (liver) and in swine muscle tissue (144). Prior to this study, it was believed that muscle cysts in swine cysticercosis were characteristic of *T. solium* only. Thus, in the absence of additional data and given the expanded range of dissemination of *T. asiatica*, the presence of tissue cysts in swine can no longer be considered a diagnostic character delineating these pathogens. This heightens the risk of humans becoming infected with *T. asiatica* from undercooked swine meats.

Clinical Manifestations

Infected individuals pass proglottids in their feces, even for 30 years or more, suggesting that the life span of this form of *Taenia* is very long (138). Common clinical manifestations include pruritus ani, nausea, abdominal pain, and dizziness. Abdominal pain is usually localized along the midline of the epigastrium or in the umbilical region and varies in intensity from a dull, aching, gnawing, or burning feeling to an intense, colic-like, sharp pain. Infection is often accompanied by a change in appetite (positive or negative), headache, diarrhea, and/or constipation. In contrast to *T. solium* and *T. saginata* taeniasis, humans can simultaneously harbor multitudes of adult *T. asiatica* worms (88).

Treatment

Clinical trials performed some time ago in Taiwan showed that a single dose (150 mg) of praziquantel was highly effective against *T. asiatica* infection (145, 146). This treatment regimen remains current today. As with *T. saginata*, niclosamide is also effective.

Prevention and Control

The best preventive measure is to avoid eating raw, uninspected pork or other meat that contains the cysticerci. Since most cases of *T. asiatica* taeniasis result from the consumption of infected pork, the meat should be cooked thoroughly or should be frozen for at least 4 days at −10°C to kill the cysticerci, the same procedures used

to prevent *T. solium* taeniasis. Although new findings have identifi ed meat tissue as a predilection site for *T. asiatica* (144), the primary source of human infection is uncooked viscera.

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25
Protozoan Parasites

Protozoan parasites have long been associated with foodborne and waterborne outbreaks of disease in humans. Inactivation of these organisms is difficult because of their resistance to environmental stresses. Groups of protozoan parasites transmitted via foods include apicomplexans, flagellates, ciliates, and amoebae (Table 25.1).

A major characteristic of apicomplexan parasites is that a vertebrate host is required to complete the complex life cycle and produce infectious cysts. Of this group, *Cryptosporidium* species, *Cyclospora cayetanensis*, *Sarcocystis hominis*, and *Cystoisospora belli* frequently inhabit the intestinal mucosa and produce diarrheal illnesses in humans. These coccidian parasites affect immunocompetent as well as immunocompromised individuals, causing more severe and prolonged symptoms in the latter. Many species of *Cryptosporidium* can infect humans and a variety of animals, and some genotypes have been described as having host preferences or specificities. *Cryptosporidium hominis* and *Cryptosporidium parvum* are the two most prevalent species infecting humans. *C. cayetanensis* has been isolated exclusively from humans and has caused large foodborne outbreaks in the United States in the past 4 years. Another apicomplexan, *Toxoplasma gondii*, infects human tissues other than the intestinal mucosa and can cause birth defects, blindness, and chorioretinitis. *Sarcocystis* species in humans can also infect muscles and other organs. The life cycle stages of apicomplexan parasites are produced intracellularly in the host. For *Cyclospora*, *Toxoplasma*, and *Cystoisospora*, sporogony typically occurs outside the host, requiring the passage of time before oocysts are infective to a new

host, while *Cryptosporidium* oocysts are excreted already sporulated and are infectious when shed.

Microsporidia, once considered protozoan parasites, have been reclassified as fungi based on recent phylogenetic analyses. Microsporidia have been implicated in human foodborne and waterborne diseases and propagate and complete their life cycle intracellularly. Of the microsporidia, members of the following four genera have been most frequently implicated in human diseases: *Encephalitozoon*, *Enterocytozoon*, *Pleistophora*, and *Vittaforma*. None of these species is host, tissue, or organ specific, with the exception of *Enterocytozoon bieneusi*, which is known to infect only the human intestinal tract.

Flagellates, ciliates, and amoebae can propagate in more than one host. All three types of parasites can cause disease in humans and animals. The infectious stage of this group is the cyst. Once ingested by the susceptible host, the trophozoites, or motile forms, are released from the cyst and colonize the host's intestinal cells. In contrast with members of the *Apicomplexa*, microsporidia, and trypanosomes, flagellates, ciliates, and amoebae do not present intracellular stages but instead attach with specialized structures to the luminal surface of the intestinal cells and feed off the nutrients and cellular debris of the intestinal epithelium. *Giardia lamblia* (also called *Giardia intestinalis* or *Giardia duodenalis*), a flagellate previously considered a commensal organism, is now well recognized as an etiological agent of acute or chronic diarrhea in humans. *Balantidium coli*, a ciliate, also causes diarrhea in humans and, if the infection is not treated, can cause ulcerative colitis. Various species of amoebae can infect humans. Most of them are commensal, not producing disease; however, others can cause diarrhea, dysentery, or amebomas if the infection is not treated.

Table 25.1 Protozoa of medical importance acquired from food and water

Phylum or group	Protozoan	Infective stage
Apicomplexa	*Cryptosporidium parvum*	Oocyst
	Cyclospora cayetanensis	Oocyst
	Cystoisospora belli	Oocyst
	Toxoplasma gondii	Oocyst/tissue cyst
	Sarcocystis hominis	Oocyst
	Sarcocystis suihominis	Oocyst
	Sarcocystis spp.	Sarcocyst
Ciliophora	*Balantidium coli*	Cyst
Microspora[a]	*Enterocytozoon bieneusi*	Spore
	Encephalitozoon intestinalis	Spore
Sarcomastigophora	*Acanthamoeba* spp.[b]	Trophozoite
	Dientamoeba fragilis	Trophozoite
	Entamoeba dispar[c]	Cyst
	Entamoeba histolytica	Cyst
	Giardia intestinalis	Cyst

	Naegleria fowleri[b]	Trophozoite
	Euglenozoa Trypanosoma cruzi	Metacyclictrypomastigote/

[a] Microsporidia were originally considered parasites but have been reclassified as fungi.

[b] Species of normally free-living amoebae that invade through the mucosa and can in some instances infect the central nervous system of humans. Amoebic meningitis is usually fatal.

[c] Commensal, morphologically similar to *E. histolytica*, and not normally capable of inducing disease.

Trypanosoma cruzi is a vector-borne flagellate parasite that has been responsible for various outbreaks of foodborne illness. If the infection is not treated effectively during the acute phase, infection is usually lifelong, as there is no treatment for the chronic stage of the infection. The outcome is heart disease and may be complicated with “mega-” syndromes, particularly megaesophagus and megacolon (1). In Venezuela, an outbreak of American trypanosomiasis affected 103 individuals in a college community and was associated with consumption of guava juice. Of those, 75% were symptomatic, 44% had documented parasitemia, and one child died (2). In Brazil, 178 cases of acute Chagas’ disease were associated with consumption of açai paste and drinking açai juice. Among exposures tested, drinking açai juice at the local health facility was significantly associated with illness (3). The emergence of Chagas’ disease in the United States is possible, as opossums (sylvatic reservoir) and insect vectors infected with *T. cruzi* have been identified in the southern part of the country (4). Foodborne transmission of this parasite has been reported as the result of ingestion of foods contaminated with reduviid bugs or their feces that were inadvertently present in the fruits. Foodborne transmission is a significant route of infection in the Brazilian Amazon (estimated to be responsible for 50% of all cases between 1968 and 2000). In 2005, 25 cases of acute Chagas’ infection were reported in 2005 and were associated with consumption of sugar cane. There have also been reports of cases associated with

consumption of improperly cooked meats or blood of mammalian sylvatic hosts that served as reservoirs of the parasite. Studies designed to develop a vaccine to control Chagas' infection have also revealed that in mice, the parasite can cause illness via the oral, esophageal, gastric, or intestinal mucosa (5).

CRYPTOSPORIDIUM

Cryptosporidium was first isolated in 1910 from the intestines of mice. Various species of *Cryptosporidium* capable of infecting animals have since been described. It was not until 1976 that *C. parvum* infection in humans was first described. Since 1982, *C. parvum* has been one of the most frequently identified opportunistic pathogens associated with diarrhea and wasting syndrome in patients with AIDS. Cryptosporidiosis in immunocompromised patients produces a life-threatening, prolonged, cholera-like illness (6). In immunocompetent patients, *C. parvum* causes self-resolving, acute diarrheal disease with variability in the severity of symptoms. Isolate variations and associations with the severity of disease have been subjects of intensive investigation for some time. All *Cryptosporidium* infections were initially thought to be caused by *C. parvum*, even though there was some evidence that more than one *Cryptosporidium* species might be implicated. As molecular tools were developed, species-level identification of *Cryptosporidium* isolates allowed the description of *C. hominis*, previously recognized as *C. parvum* genotype I, which is isolated exclusively from humans. Genotype II, which belongs to the *C. parvum* group, was also described. This genotype is isolated from cattle and humans and is readily infective to laboratory animals. Subsequently, other *Cryptosporidium* species, originally called *C. parvum*, were described.

PCR targeting the small-subunit rRNA gene is usually employed to genotype *Cryptosporidium* isolated from humans, animals, and water samples. Genotype differentiation based on the sequencing of

the thrombospondin-related adhesive protein (TRAP-C2) has also been used (7, 8). Currently, the gp60 gene is being used to subtype *Cryptosporidium*. Six subtypes have been described for *C. hominis* and 11 subtypes for *C. parvum*. The denomination of each subtype includes the group type (I or II) and the type and number of the TCA, TCG, or TCT repeats. In some cases, other repeats (of 6 bp or 13 to 15 bp) located after the trinucleotide repeats are also included in the *C. parvum* or *C. hominis* subtype name, respectively. Human infections with *Cryptosporidium* are most frequently caused by five *Cryptosporidium* species (*C. hominis*, *C. parvum*, *C. felis*, *C. canis*, and *C. meleagridis*) and occur most frequently in HIV-positive individuals (9–15). Other species occasionally identified in humans include *C. muris*, *C. andersoni*, *C. suis*, and *Cryptosporidium* cervine, horse, rabbit, skunk, and chipmunk I genotypes (16).

Cryptosporidiosis is acquired after ingestion of food or water contaminated with infective *Cryptosporidium* oocysts. Once ingested, the oocysts excyst (Fig. 25.1). The released sporozoites proceed to invade the enterocytes. A parasitophorous vacuole is formed as the parasite enters the cell. This structure contains the intracellular parasites and communicates with the host cell via a feeder organelle. The parasite goes through two asexual multiplication stages called merogony: type I meronts contain eight merozoites, which are released and proceed to invade other enterocytes to form type II meronts, each containing four merozoites. Once merozoites from type II meronts are released, they infect enterocytes but differentiate into sexual stages identified as macrogametocytes (female) and microgametocytes (male). The union of a microgametocyte and a macrogametocyte produces a zygote (immature oocyst), which matures within its host into a fully sporulated oocyst (Fig. 25.2). Two types of oocysts are produced and excreted. Thin-walled oocysts excyst endogenously, resulting in autoinfection. Thick-walled oocysts, which are environmentally resistant, are shed in the feces and are immediately infectious to other hosts (17, 18). An additional life stage that may represent an extracellular trophozoite/gamont stage has been described for *C.*

andersoni, a parasite that infects the gut of cattle. This stage has also been reported for *C. parvum*. This finding is still under debate, as other groups have not been able to reproduce it. Phylogenetic analysis indicates that *Cryptosporidium* has a closer phylogenetic affinity with the gregarines than with the coccidian (19–21).

To date, 20 species of *Cryptosporidium* have been identified in humans, including *C. hominis*, *C. parvum* (cattle, sheep, and goats), *C. meleagridis* (of turkeys), *C. felis* (of cats), *C. canis* (of dogs), *C. ubiquitum*, *C. cuniculus*, *C. viatorum*, *C. muris*, *C. suis*, *C. andersoni*, *C. tyzzeri*, *C. fayeri*, *C. bovis*, *C. scrofarum*, *C. erinacei*, and others (22, 23). *Cryptosporidium* infects the brush border of the intestinal epithelium and causes villous atrophy, though the exact mechanism for this pathological alteration is not yet fully understood. Immunocompetent patients develop a profuse watery diarrhea accompanied by epigastric cramping, nausea, and anorexia, which is usually self-limiting and lasts for about 15 days.

Extraintestinal dissemination has been observed in immunocompromised patients. *Cryptosporidium* may be found in epithelial cells from other organs, such as those of the respiratory tract and the biliary tree (24). Immunocompromised patients (e.g., AIDS patients or those receiving immunosuppressive drugs) develop severe diarrhea (3 to 6 liters/day), which persists for several weeks to months or even years. These cases are the most severe and life threatening, with continuous shedding of oocysts. In patients with HIV infections, the $CD4^+$ cell count is the best marker for the ability of the immune system to self-resolve the infection. In the developed world, patients with $CD4^+$ counts of 180 cells/mm^3 or higher usually develop self-limiting cryptosporidiosis, whereas those with counts below 180 cells/mm^3 usually develop chronic and profuse diarrhea, which is exacerbated by the lack of effective therapy (25). The pathogenesis of the diarrhea is not clear, and the presence of a toxin has been suggested but not yet confirmed.

The mean prevalence of *C. parvum* infection in Europe and the United States is 1 to 3%, and the prevalence is considerably higher

in developing countries. Outbreaks of disease associated with *Cryptosporidium* have been reported in the United States as well as in other countries (18). *Cryptosporidium* infection is closely associated with travel abroad, exposure to farm animals, and person-to-person transmission in settings such as day care centers and medical institutions. A large number of waterborne outbreaks of cryptosporidiosis have been documented, and some of the most notable were those in Milwaukee, Wisconsin (26), and in Georgia (27), with a total of more than 400,000 cases reported. In Minnesota in 1995, *Cryptosporidium* was associated with cases of acute gastroenteritis experienced by attendees of a social event. This outbreak was epidemiologically associated with the consumption of contaminated chicken salad (28). In 1993 in central Maine and in 1996 in New York, apple cider was associated with outbreaks of cryptosporidiosis (29, 30). In 1998 in Spokane, Washington, another foodborne outbreak affected about 50 people, but it could not be traced to a specific type of food. In 2000, an infected food handler was responsible for a foodborne outbreak at a cafeteria of a university in Washington (31). *Cryptosporidium* has been identified in produce intended for human consumption. In 2008, a foodborne outbreak of cryptosporidiosis affecting 72 individuals in Helsinki, Finland, was associated with a salad mixture (32).

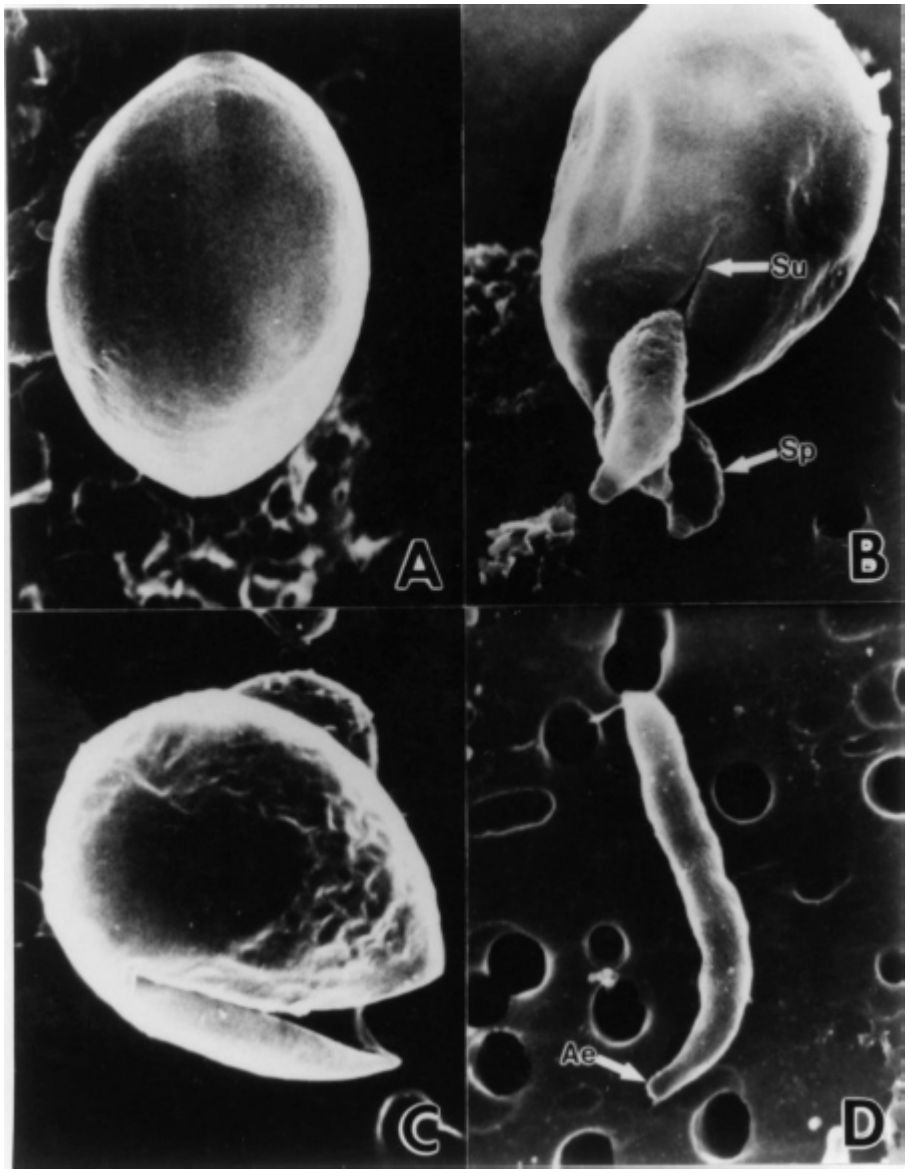


Figure 25.1 Scanning electron micrographs of oocysts and excysting sporozoites of *C. parvum*. (A) Intact oocyst prior to excystation (magnification, ×11,200). (B) Three sporozoites (Sp) excysting from oocyst simultaneously via the cleaved suture (Su) (magnification, ×11,200). (C) Empty oocyst (magnification, ×11,200). (D) Excysted sporozoite (magnification, ×9,800). Ae, apical end. Reprinted from reference 224.

In Denmark in 2005, an outbreak of diarrhea in 99 individuals occurred, of whom 13 were positive for *C. hominis* infection. The infection was associated with eating food from the canteen salad

bar. Peeled whole and grated carrots and red peppers were the likely source of the outbreak (33).

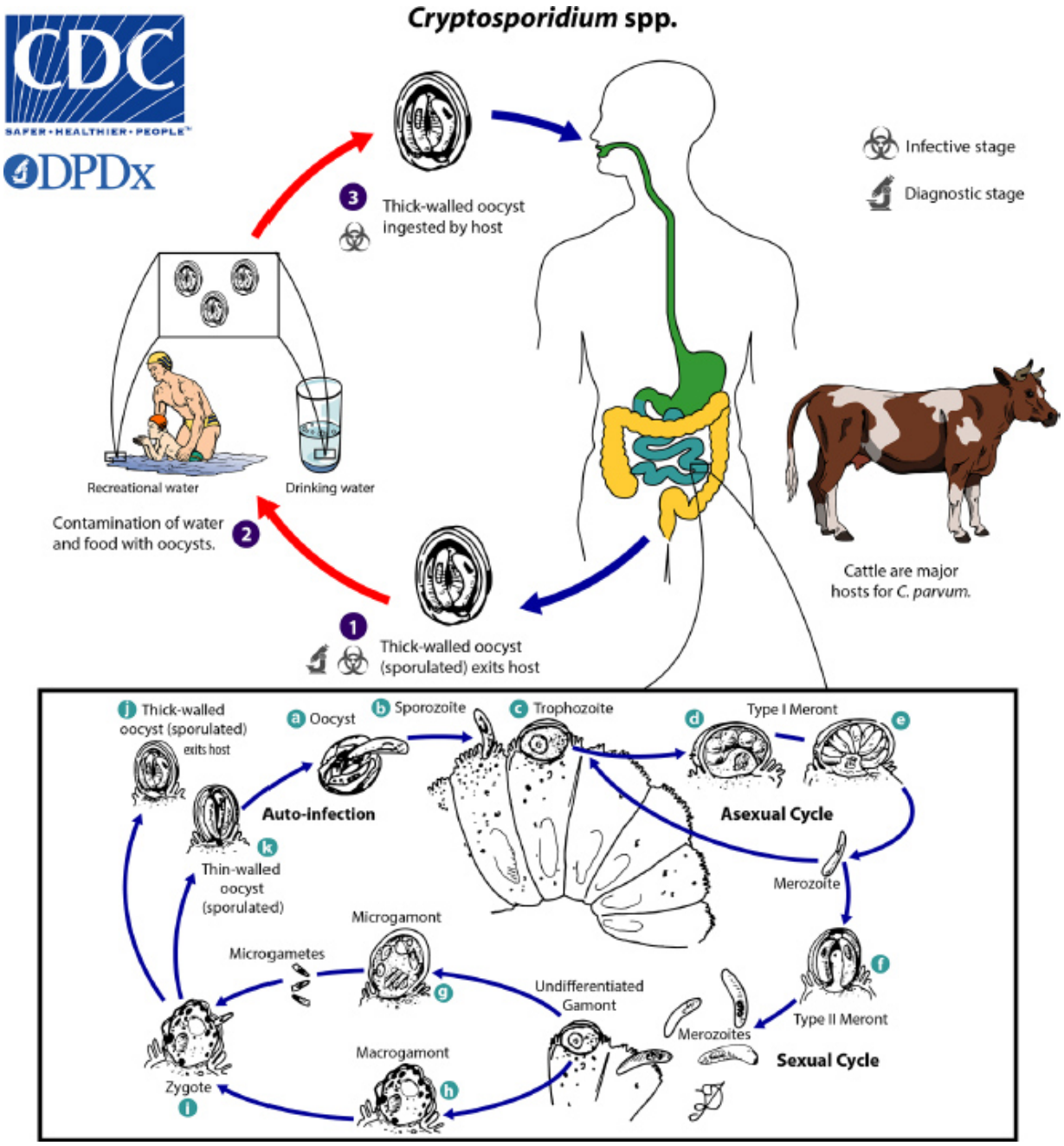


Figure 25.2 Life cycle of *Cryptosporidium*. From reference 225.

In Norway, 26% of lettuce samples and 74% of mung bean sprout samples assayed were *Cryptosporidium* positive. *Giardia* was identified in dill (20%), lettuce (20%), mung bean sprout (30%), radish (10%), and strawberry (20%) samples (15). The risk of

acquiring cryptosporidiosis and giardiasis based on frequency and level of mung bean sprout consumption was assessed to be 20 cases per 100,000 (34, 35). Analysis of produce obtained from a regional market in Peru revealed that 14.5% of samples contained *Cryptosporidium* oocysts (36). The immunofluorescence assay specific to *Cryptosporidium* was used for this study and is considered the gold standard assay. An alternative method for identifying *Cryptosporidium* from fresh produce uses polyclonal sera raised to a recombinant viral capsid protein of an RNA virus that is a symbiont of *Cryptosporidium* (37).

In a recent report, it was estimated that 8% of cases of cryptosporidiosis are foodborne, with 57,616 episodes of domestically acquired foodborne illness due to *Cryptosporidium* spp. occurring annually (38). This is much lower than the number estimated in 1999.

Although there is no effective and definite therapeutic agent for the treatment of cryptosporidiosis, spiramycin reportedly decreases diarrhea in the early stages of infection (39–41); however, it is not effective in treating advanced infections. Azithromycin, nitazoxanide, and paromomycin reportedly are effective in treating AIDS patients with cryptosporidiosis (42–45). Alternative experimental therapies that have been evaluated in humans include passive immunotherapies, such as hyperimmune bovine colostrum. Monoclonal antibodies and polyclonal hyperimmune hen yolk against *Cryptosporidium* have also been tested in animal models, with some success (46–48).

To date, nitazoxanide is the only drug approved by the Food and Drug Administration for the treatment of cryptosporidiosis. This treatment has been most effective in malnourished children. Nitazoxanide, paromomycin, and rifamycin derivatives are reportedly partially effective in severely immunocompromised patients with cryptosporidiosis (49).

Cryptosporidium oocysts are 4 to 6 μm in diameter. Because of their size, they can be overlooked in examinations of feces or

confused with yeast cells. *Cryptosporidium* can be identified by light microscopy using acid-fast staining. Immunoassays (fluorescent-antibody immunoassay or enzyme immunoassay) are available and are very sensitive and specific diagnostic tests (50–52). *Cryptosporidium* can be mistaken for *Cyclospora*, which is also positive in acid-fast staining but larger (Fig. 25.3). It is important that laboratories carefully measure the diameters of cells, particularly if the cells appear to be larger than those of *Cryptosporidium*. New molecular diagnostic tests such as PCR assays are being developed and could improve the sensitivity in detecting *Cryptosporidium* oocysts in produce (37, 53–58). It has been determined that shellfish can concentrate large volumes of *Cryptosporidium* oocysts along with other particulate matter (56, 59–63). *Cryptosporidium* has also been identified in shellfish organs by using conventional diagnostic assays, and molecular tools (multiplex nested PCR) have been evaluated to optimize parasite identification (56, 60, 64, 65). The use of natural grassland buffers may assist in controlling the parasite load that may potentially be deposited in water banks and oceans as a consequence of rangeland runoff, thus contaminating shellfish (66). To date, shellfish have not been associated with outbreaks of *Cryptosporidium* infections.

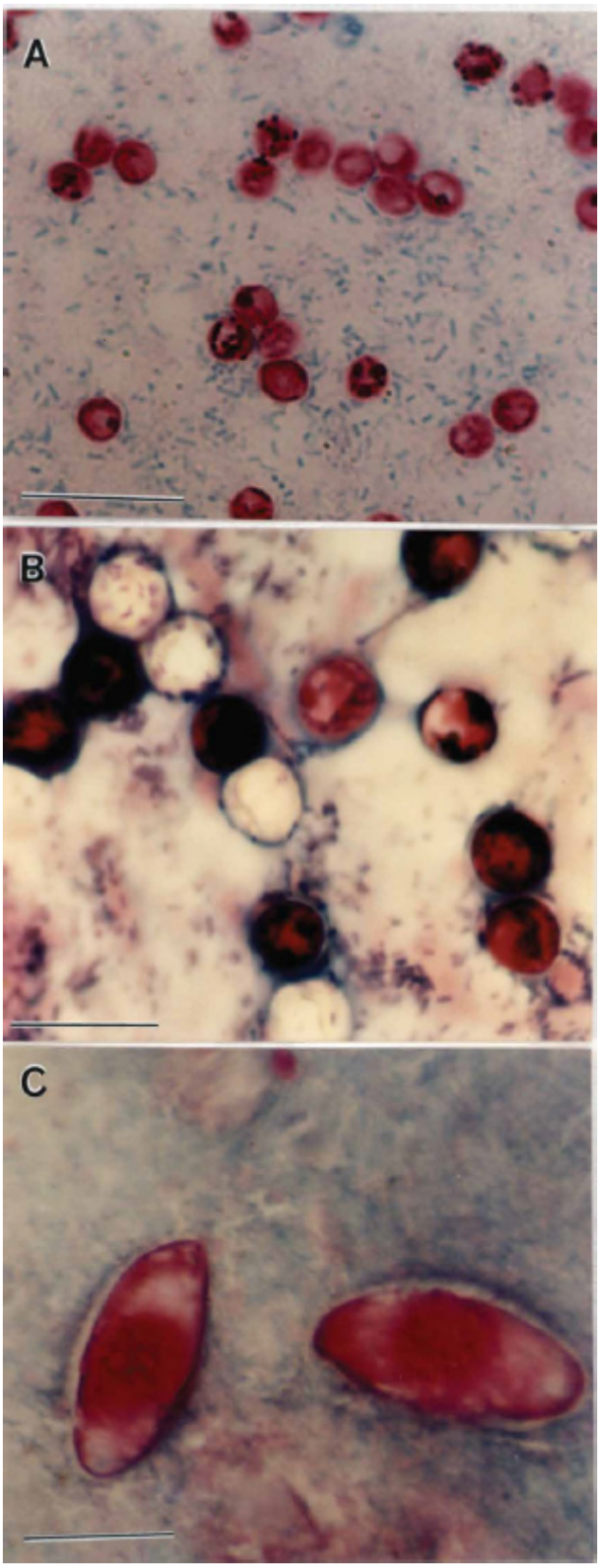

A
B
C

Figure 25.3 Acid-fast staining. (A) *C. parvum*. (B) *C. cayetanensis*. (C) *C. belli*. Bars = 20 μm.

Although *Cryptosporidium* oocysts are highly resistant to chlorine and chlorine dioxide, treatment with these chemicals at 1,000 μg/ml for 1 min resulted in 0.5- and 2-log reductions, respectively, according to the viability assay used (*in vitro* excystation or tissue culture infectivity) (67). More than 90% inactivation was achieved when *Cryptosporidium* oocysts were treated with ozone at 1 μg/ml for 5 min, chlorine dioxide at 1.3 μg/ml for 1 h, and chlorine and monochloramine at 80 μg/ml for 90 min (68). Various technologies effective at killing *Cryptosporidium* oocysts in drinking water and in treated wastewater have been developed, including pulsed UV light and pulsed-plasma gas (69) and chlorine dioxide at concentrations of 5 and 1.4 mg/liter in chlorinated recreational water venues (70).

Pasteurization of apple cider is effective for inactivating *Cryptosporidium* oocysts. Heating for 10 or 20 s at 70 or 71.7°C resulted in at least a 4.9-log oocyst viability reduction. When oocysts were exposed for 5 s, killing was 3.0 and 4.8 log, respectively. Hence, flash pasteurization used in the juice industry is sufficient for inactivating contaminating oocysts (71).

CYCLOSPORA

Cyclospora was probably first reported to be found in humans in 1979 by Ashford, who described it as an *Isospora*-like coccidian affecting humans in Papua New Guinea (72). Thereafter, other investigators found similar structures in fecal samples of patients with diarrhea, but because of the morphologies of the unsporulated oocysts and their autofluorescence, they were identified as coccidian-like bodies (or cyanobacterium-like bodies) (CLBs) (73–75). In 1993, conclusive identification was made, and the CLBs were fully characterized as coccidian parasites and placed in the genus

Cyclospora with the proposed species name *Cyclospora cayetanensis* (76, 77).

Cyclospora belongs to the family *Eimeriidae*, subphylum *Apicomplexa*. *Cyclospora* species infect moles, rodents, insectivores, snakes, and humans. In 1998, a *Cyclospora* species that morphologically resembles the *Cyclospora* isolated from humans was isolated from nonhuman primates (78–80). However, phylogenetic analysis has revealed that these isolates are a different species (78, 79).

Even before their true identity was established, epidemiologic information on the intriguing CLBs was being collected worldwide. In Nepal, the prevalence of *Cyclospora* infection is highest in adult expatriates (81), whereas in areas of Peru where infection is endemic, children under 10 years of age are the most susceptible to infection, although most are asymptomatic (82). Adults from areas of endemicity do not develop symptoms of infection, but adults of medium to high socioeconomic status living outside the areas of endemicity, as well as foreign travelers, typically present with clinical disease. These observations suggest that prior exposure to the parasite may result in protective immunity.

Newly shed oocysts (Fig. 25.4A) of *C. cayetanensis* require 2 weeks to sporulate and become infectious (Fig. 25.4B) under optimal laboratory conditions (77). The requirement of this amount of time for the oocyst to become infectious suggests that contamination of produce occurs with oocysts that are fully sporulated or almost fully sporulated. Otherwise, unsporulated oocysts would require the optimal time and environmental conditions to induce oocyst sporulation, while the produce would still be edible.

In studies performed in areas of endemicity, *Cyclospora* oocysts have been isolated from produce with removal by washing thoroughly with distilled water. The washes are concentrated by centrifugation, and pellets are fixed and preserved in 10% formalin. Samples are examined directly by using epifluorescence microscopy and phase-contrast microscopy (77). Nested PCR designed to

amplify the 18S rRNA gene is also used with these preparations; however, further testing using restriction fragment length polymorphism is required, because the described PCR assay cross-reacts with *Eimeria* species. *Eimeria* parasites are infectious to animals but not to humans and can be readily found in the environment (83, 84). An optimized TaqMan real-time PCR assay was developed and compared to a nested PCR assay using 0, 5, 10, and 100 oocysts in 25 g of cilantro or 50 g of raspberries (85). Detection by both methods was statistically similar. The authors indicated that the real-time PCR assay was simpler, less laborious, and less prone to amplicon contamination (85).

Cyclosporiasis is characterized by mild to severe nausea, anorexia, abdominal cramping, mild fever, and watery diarrhea. Diarrhea alternating with constipation has been commonly reported. Some patients present with flatulent dyspepsia and, less frequently, joint pain and night sweats. The onset of illness is usually sudden, and symptoms persist for an average of 7 weeks (86, 87).

C. cayetanensis infects epithelial cells of the duodenum and jejunum of humans. Merogony and gametogony occur intracytoplasmically within the parasitophorous vacuoles in intestinal cells (Fig. 25.4C) (87, 88). Duodenal and jejunal biopsy specimens from patients with cyclosporiasis show various degrees of jejunal villous blunting, atrophy, and crypt hyperplasia (86, 87). Extensive lymphocytic infiltration into the surface epithelium is present, especially at the tips of the shortened villi. The reactive inflammatory response of the host does not correlate with the number of intracellular parasites present in the tissues (87).

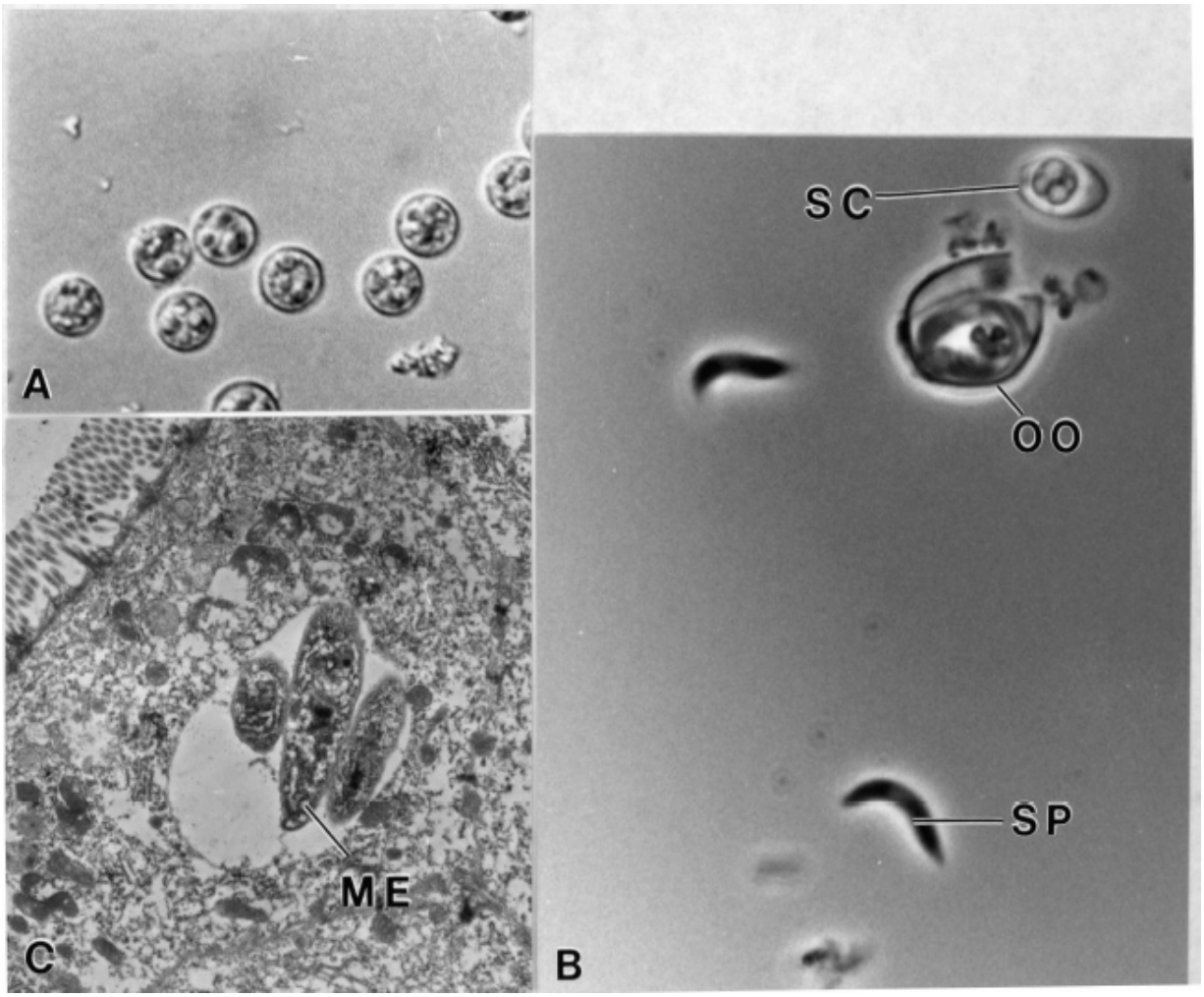


Figure 25.4 *C. cayetanensis* oocysts. (A) Phase-contrast microscopy of unsporulated oocysts. (B) Oocysts (OO) in the process of excystation. Note the two sporozoites (SP) free of the sporocyst (SC). (C) Transmission electron microscopy of human small intestine showing *Cyclospora* intracellular stages. ME, merozoite.

Routes of transmission for *Cyclospora* are undocumented, although the fecal-oral route, either directly or via water and food, is probably the major one. In the United States, epidemiologic evidence suggests that water has been responsible for sporadic cases of cyclosporiasis. In Utah, a man became infected after cleaning his basement that was flooded with runoff from a nearby farm following heavy rains. In 1990, an outbreak involving residents in a

physicians' dormitory in a Chicago hospital was epidemiologically associated with tap water from unprotected reservoir tanks that served the building and which had a broken water pump (89, 90). In Pokhara, Nepal, British Gurkha soldiers were confirmed to have cyclosporiasis; oocysts were isolated from their drinking water. The water, consisting of a mixture of river and municipal water, was routinely chlorinated and served the houses in the camp where the soldiers were stationed (91).

A few reports have described the isolation of *C. cayetanensis* oocysts from animals (chickens, ducks, and dogs) (92–95). At present, after several experimental studies attempting to infect these and additional animal species, it seems that *Cyclospora* species are host specific (96).

Since the early 1990s, *Cyclospora* has been linked to waterborne- and foodborne-disease outbreaks. Foodborne outbreaks have been epidemiologically associated with the consumption of contaminated fresh produce such as raspberries, lettuce, basil, and snow peas (97–100). In 1996, a large foodborne-illness outbreak occurred following a wedding reception at a restaurant in Boston, resulting in 57 guests having cyclosporiasis. Berries were implicated as the vehicle of transmission for *Cyclospora* oocysts (101). In 1999, another foodborne outbreak among attendees of two events was reported. Sixty-two cases of infection were documented, and the illness was associated with consumption of chicken pasta salad and tomato basil salad. The most likely vehicle of illness was fresh basil, grown either in Mexico or in the United States, which was included in both salads (102). However, *Cyclospora* oocysts were not recovered from or detected in the produce associated with these outbreaks. In the United States, most cases of cyclosporiasis have been reported in the months of April to August, suggesting a seasonal relationship.

In the United States in 1996 and 1997, *Cyclospora* infections were associated with imported Guatemalan raspberries (99, 100). It was speculated that the raspberries were contaminated when they were sprayed with insecticide that was possibly diluted with

contaminated surface water. Analysis of irrigation water revealed the presence of *Cyclospora* oocysts (103). In Peru, *C. cayetanensis* oocysts have been isolated from vegetables obtained from markets in areas of endemicity. In studies where vegetables were experimentally inoculated with *C. cayetanensis* oocysts, it was determined that washing with water does not remove all the oocysts (36). *Cyclospora* has also been isolated from produce from Nepal (104). In Germany in 2000, 34 individuals acquired *Cyclospora* infections that were associated with consumption of lettuce spiced with green leafy herbs which were all imported from southern Europe (105). In the same year, another outbreak occurred at a wedding reception in Philadelphia, Pennsylvania. The wedding cake tested *Cyclospora* positive, and cream filling containing raspberries was the food item strongly associated with illness (106). In 2001, in British Columbia, Canada, 17 cases of cyclosporiasis were reported. Eleven of these individuals had consumed Thai basil that was imported from the United States (107). In 2004, approximately 50 cases of cyclosporiasis were linked to consumption of raw Guatemalan snow peas (108). In 2009, a *Cyclospora* outbreak was reported in Sweden, involving 12 confirmed and 6 probable cases, and was associated with imported sugar snap peas from Guatemala (109).

In the most recent report from the Centers for Disease Control and Prevention, it is estimated that 99% of cases of *Cyclospora* infection in the United States are foodborne and that there are an estimated 11,407 cases annually (38). In the United States, in 2013, 643 cases of cyclosporiasis involving 25 states occurred during June to August. Three states had the largest number of cases: 279 cases were reported in Texas, 153 in Iowa, and 86 in Nebraska. The cases in Iowa and Nebraska in which the infected individuals ate at restaurants were linked to a salad mix produced by Taylor Farms de Mexico. The cases reported in Texas were associated with cilantro imported from Puebla, Mexico (110). In 2014, 304 cases of cyclosporiasis were reported; 207 of the infected individuals did not have a travel history within the 2 weeks prior to developing the

illness. One hundred thirty-three patients were from Texas, and their infections were associated with consumption of cilantro from Puebla, Mexico (111). In 2015, 546 cases of cyclosporiasis were reported; 319 of the individuals did not have a history of travel. Cases in Wisconsin and Texas were associated with cilantro as the suspect food vehicle (112).

As a result of these outbreaks, the FDA issued an import alert that indicated that shipments of cilantro (intact, cut, or chopped) from Puebla, Mexico, can be detained without physical examination from 1 April to 31 August of each year if the product is not from a firm listed on the Green List (firms that have been inspected and approved for import) (113). In 2016, 384 cases were reported from 25 states. No suspect food was identified (114). In 2017, there were 1,065 laboratory-confirmed cases in 40 states; at least 597 of the individuals did not report international travel prior to illness. No specific food vehicle was identified (115). Once again, in 2018, two multistate outbreaks of cyclosporiasis were identified in the United States. The first outbreak had 250 laboratory-confirmed cases from four states in which individuals reported consuming prepackaged Del Monte fresh produce vegetable trays that contained cauliflower, broccoli, and carrots. The second was associated with people from 15 states and New York City who reported consuming salads in McDonald's restaurants in the Midwest. The implicated romaine and carrot mix bags were distributed to McDonald's by the Fresh Express processor in Streamwood, IL. A total of 511 laboratory-confirmed cases occurred in this outbreak. So far, in 2018, 2,173 laboratory-confirmed cases of domestically acquired cyclosporiasis have been reported in the United States (116).

The cases reported from 2013 to 2017 occurred during the months of May to August, suggesting that the implicated food commodity was imported from a location where *Cyclospora* is endemic and where contamination occurred and that *Cyclospora* contamination is seasonal. Another possible explanation for this seasonal relationship observed in the United States could be that food commodities grown in the United States are contaminated in the

field by infected field workers. This could also explain the findings of Dixon et al., in which *Cyclospora* was identified in 1.9% of bagged salad mixes assayed. Most of the samples tested (97% of 544) were products from the United States sold in Canada (117). Similarly, in a study conducted in Italy, *Cryptosporidium* spp., *G. lamblia*, *T. gondii*, *C. cayetanensis*, *Blastocystis hominis*, and *Dientamoeba fragilis* were identified in ready-to-eat salads sold at retail (118).

Neither the minimum infectious dose of oocysts nor the sporulation and survival rates under different environmental conditions are known, although human volunteer studies have been attempted without success (119). Because of the potential low number of oocysts present in foods, molecular assays and methods to improve parasite recovery have been studied (83, 120, 121).

Analysis of the intervening internal transcribed spacer 1 (ITS-1) region sequence of *Cyclospora* isolates from the 1996 outbreak revealed that all were identical, suggesting a single source of contamination for the infection (122). Another study examined *C. cayetanensis* and *Cyclospora papionis* isolates. Although high sequence variability was present, conserved species-specific ITS-1 sequences were identified. This consistent and remarkable diversity among *Cyclospora* species ITS-1 sequences suggests polyparasitism and simultaneous transmission of multiple strains (123).

There is strong evidence to suggest seasonality of *Cyclospora* infections. In Peru, more than 6 years of prospective epidemiologic studies investigating endemic *Cyclospora* infections revealed that nearly all infections occurred between December and July (82). In Haiti, most *Cyclospora* cases occurred during the months of January to March (124). Rarely were infections documented at any other time of the year. In the United States, most cases occur between May and August. In Nepal and Honduras, most cases of cyclosporiasis occur between May and August (75, 125). In Mexico, a 10-year study revealed that most cases of *Cyclospora* infections occur during the months of June to August (126), similar to China, where most cases present during July to November (127). The

specific reasons for this marked seasonality have not been elucidated.

The only successful antimicrobial treatment for *Cyclospora* infections is trimethoprim-sulfamethoxazole (TMP-SMX) (82, 128, 129). AIDS patients appear to have higher parasite infestation levels than immunocompetent individuals infected with *Cyclospora*. However, the prevalence of *Cyclospora* infection among HIV patients is not higher than that among immunocompetent populations. This is probably due to the frequent use of TMP-SMX for *Pneumocystis jirovecii* (formerly *Pneumocystis carinii*) prophylaxis among HIV patients (128). Ciprofloxacin has also been evaluated as an alternative treatment for sulfa-sensitive patients (129).

Trace-back studies have been hampered by the lack of genotyping tools. Guo et al. (130) described a multilocus sequence typing (MLST) alternative. Sixty-four *Cyclospora*-positive specimens were analyzed using five genetic loci, including CYC3, CYC13, CYC15, CYC21, and CYC-22. Sequence data for 56 of them had at least four loci, and 34 specimens had all five loci. With this MLST method, the authors determined that there were at least 25 multilocus genotypes among these samples. Samples from China were genetically distant from those from the United States and Peru (130). In another study, Chinese investigators assayed 45 *Cyclospora*-contaminated samples from Zhenzhou and Kaifeng using MLST with five microsatellite loci and determined that there were 29 multilocus genotypes among the isolates in the samples. These isolates, including 34 reference isolates, produced three main clusters (131). The whole genome of *Cyclospora* has also been described by various research groups. Using comparative genomics, Liu et al. determined that *C. cayetanensis* and *Eimeria tenella* share similar genome organization, metabolic capabilities, and potential invasion mechanisms. However, *Eimeria* has unique surface antigens, and amino acid metabolism and post-translation modifications of proteins are some major differences between *C. cayetanensis* and other apicomplexans (132)

CYSTOISOSPORA

Cystoisospora is a coccidian parasite that infects humans. It is frequently identified in AIDS patients (133, 134) and can be acquired by ingestion of contaminated food or water. Oocysts excyst in the intestine, and sporocysts are released. The sporocysts in turn release sporozoites that infect intestinal epithelial cells. Asexual and sexual life cycle stages occur in the cytoplasm of enterocytes. Unsporulated *Cystoisospora* oocysts require 12 to 48 h to mature and become infectious outside the host.

Cystoisospora can be detected in stools from infected patients by observing unsporulated oocysts shed in feces. *Cystoisospora belli* oocysts (10 to 19 by 20 to 30 μm) can be detected in direct wet mount preparations in heavy infections during ova and parasite examination (Fig. 25.5). However, most infections are not heavy, and rates of shedding of oocysts may be variable; therefore, it is necessary to examine a series of samples and perform modified acid-fast staining (Fig. 25.3C). *C. belli* infects the entire intestine and produces severe intestinal disease (135). Deaths from overwhelming infections have been reported, especially in immunocompromised patients. Symptoms include diarrhea, nausea, steatorrhea, headache, and weight loss. The disease can persist for months to years (136).

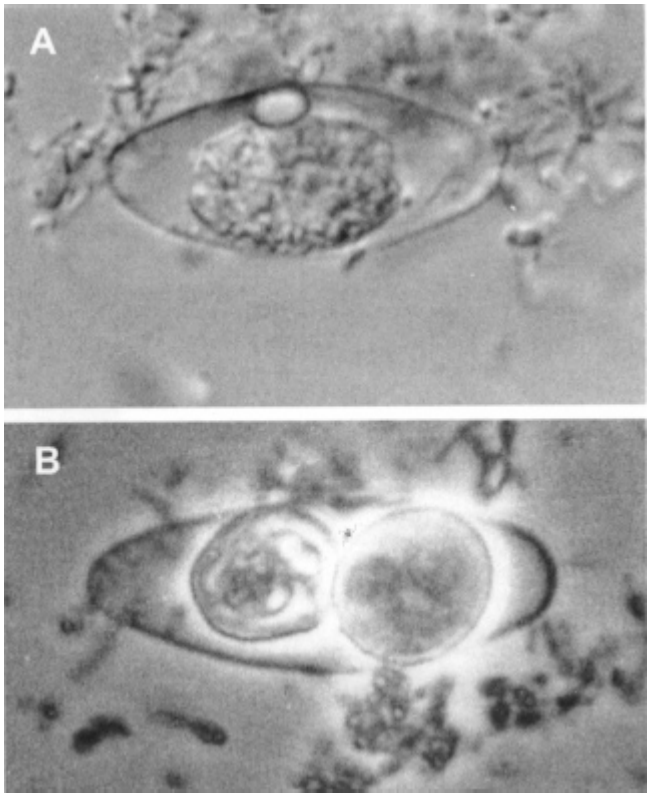


Figure 25.5 Bright-field photomicrograph of unsporulated (A) and sporulated (B) *C. belli* oocysts.

Cystoisospora infection is rare in immunocompetent people but occurs in 0.2 to 0.3% of immunocompromised AIDS patients in the United States and in 8 to 20% of AIDS patients in Africa and Haiti. *Cystoisospora* infection is endemic in many parts of Africa, Asia, and South America. The treatment of choice is TMP-SMX; however, recurrence in HIV patients is common after discontinuation of therapy (136).

TOXOPLASMA

T. gondii is a coccidian parasite that infects a variety of warm-blooded hosts. Cats are the definitive host, and other warm-blooded animals can serve as intermediate hosts. Cats excrete oocysts in their feces. Oocysts are environmentally resistant and can survive several years in moist, shaded conditions. Infections are acquired principally by ingestion of food and water containing oocysts,

ingestion of animal tissues containing cystic forms (bradyzoites), or transplacental transmission (137).

The unsporulated oocysts require 24 h outside the host to differentiate and become infectious. When oocysts are ingested by the intermediate host, the oocyst walls are ruptured and the sporozoites are released (Fig. 25.6). They invade epithelial cells and rapidly multiply asexually, producing tachyzoites. Tachyzoites multiply by endodyogeny, a process in which the mother tachyzoite is consumed by the formation of two daughter forms (Fig. 25.7). Eventually bradyzoites, which are slowly multiplying forms, develop, forming tissue cysts. Cysts persist for the duration of the life of the host. By encysting, the parasite evades the host's immune response and ensures its viability (138).

When animal tissues containing cysts are ingested by cats, proteolytic enzymes digest the cyst walls and the bradyzoites are released. They infect and multiply in the intestinal epithelial cells, transforming into tachyzoites. These in turn disperse via blood and lymph. When tissues of other animal species are ingested by felines, tachyzoites or bradyzoites begin the enteroepithelial cycle, and sexual multiplication also occurs (Fig. 25.6). Macro- and microgametocytes are produced. After fertilization, the zygote differentiates into oocysts, which are passed in the feces (138).

Although most *Toxoplasma* infections occur by ingestion of contaminated meat, other foods, or water, infections can also be acquired by organ transplantation or by blood transfusion (139–142). Disseminated toxoplasmosis may occur in patients who have received organ transplants and are receiving immunosuppressive therapy. Chorioretinitis is frequently observed in adults who acquire the infection. In immunosuppressed patients, toxoplasmosis can reactivate from latent infections (143, 144). It is estimated that 50% of cases of toxoplasmosis in the United States are foodborne, representing 76,840 cases per year (38).

Toxoplasmosis can also be acquired vertically by transplacental transmission when a pregnant woman becomes infected. After

multiplying in the placenta, tachyzoites spread into the fetal tissues. Infection can occur at any stage of pregnancy, but the fetus is most affected when infection occurs during the first months of pregnancy. Most infected children do not show any signs of the disease until later in life, when they may present with chorioretinitis and mental retardation (145–148).

The overall prevalence in humans and animals varies according to eating habits and lifestyle. The prevalence of *T. gondii* is highest in swine with outdoor access. Confined housing reduces swine exposure to cat feces and infected rodents (145, 149).

Toxoplasmosis can be acquired by ingestion of infected lamb, poultry, horse, and wild game meat. Cooking, freezing, or gamma irradiation will kill the *Toxoplasma* cysts and oocysts. Exposure to temperatures of 61°C or higher for 3.6 min inactivates the parasites, and freezing at −13°C kills the cysts (137, 150). *Toxoplasma* has also been identified in fresh produce and ready-to-eat salads (118, 151); however, the oocyst viability was not addressed in this study. Thus, the public health implications remain to be determined (118).

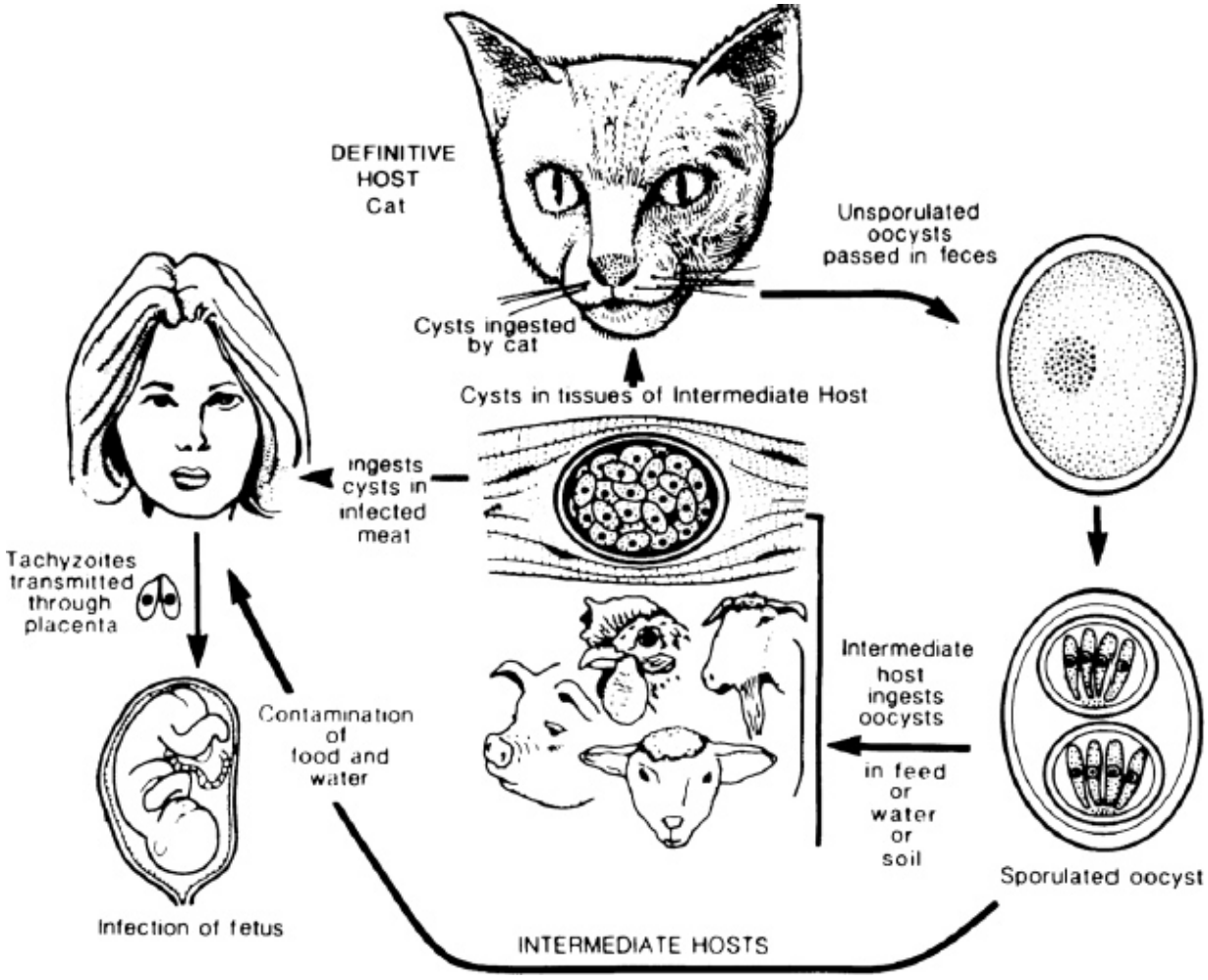


Figure 25.6 Life cycle of *T. gondii*. From Dubey and Beattie (137).

Pyrimethamine in combination with folinic acid or trisulfapyrimidine is the treatment of choice for acute infections. TMP-SMX is effective and frequently used to prevent recurrence of acute infections in AIDS patients (152).

There are several methods for detecting *Toxoplasma* in humans, such as enzyme-linked immunoassays, microarray assays, and molecular assays (153–162), the modified agglutination test, and indirect enzyme-linked immunosorbent assays using recombinant rMAG1 instead of soluble *Toxoplasma* lysate antigens in animals (163–170). For fresh produce, a loop-mediated isothermal amplification lateral flow dipstick was developed to detect *Toxoplasma* oocysts in ready-to-eat salads (171).

SARCOCYSTIS

Sarcocystis is another coccidian that causes a significant number of foodborne infections. Muscular sarcocystosis is caused by several species of *Sarcocystis*. The oocysts, which are excreted in the feces of an infected animal or individual, contain two sporocysts, each with four sporozoites. The oocyst cell wall is thin and usually breaks, and the sporocysts are more commonly identified in the feces. The intermediate hosts are pigs, cattle, sheep, etc., and humans acquire the infection when they ingest meats containing muscle cysts (sarcocysts) full of bradyzoites (172).

Humans can also be definitive hosts for *S. hominis* and *Sarcocystis suihominis*. These sarcocysts are released from the infected muscle and infect the intestinal lamina propria. The bradyzoites differentiate into sexual stages, and after fertilization, oocysts are produced. Oocysts measure 12.3 to 14.6 by 18.5 to 20 μm (172). Patients with muscular sarcocystosis present with musculoskeletal pain, fever, rash, cardiomyopathy, bronchospasm, and subcutaneous swelling. The intestinal infection is characterized by nausea, vomiting, diarrhea, dyspnea, tachycardia, and stomachache (63). The infection can be acquired by ingestion of raw or undercooked meats, other foods including vegetables, and water contaminated with feces of a variety of animals containing oocysts or sporocysts.

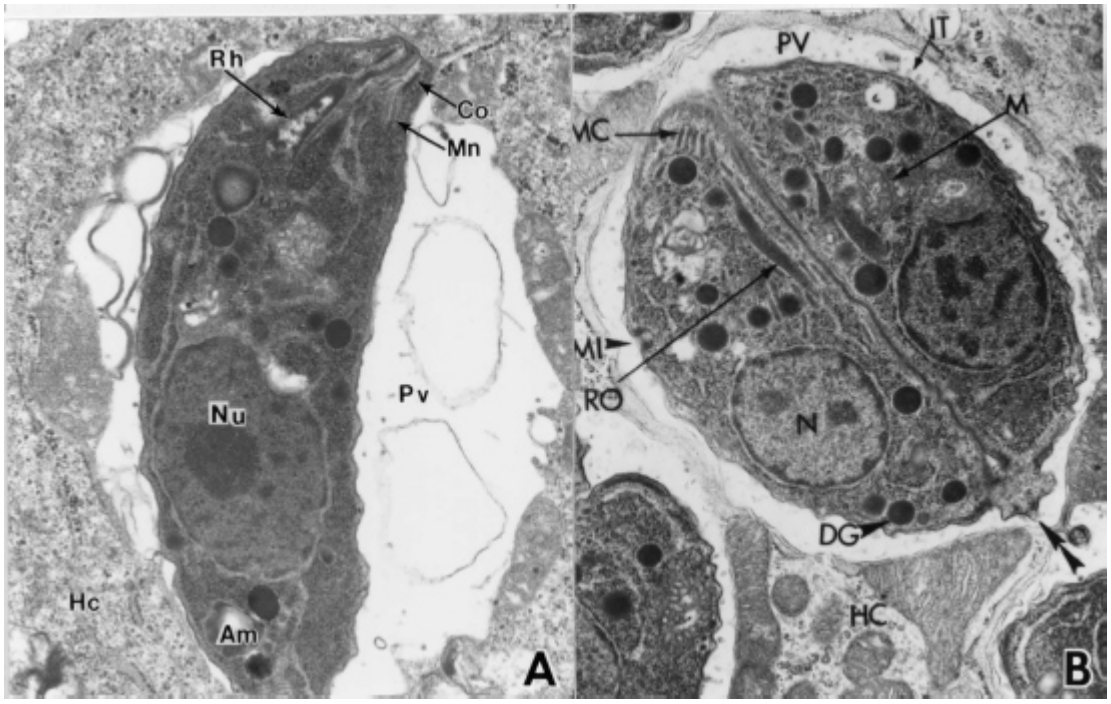


Figure 25.7 Transmission electron micrographs of *T. gondii*. (A) Sporozoite in parasitophorous vacuole (Pv) of host cell (Hc) at 24 h after inoculation. Am, amylopectin granule; Co, conoid; Mn, microneme; Nu, nucleus of sporozoite; Rh, rhoptry. (B) Final stage of endodyogeny to form two daughter tachyzoites that are still attached to the posterior ends (arrowheads). DG, dense granules; HC, host cell; IT, intravacuolar tubules; M, mitochondrion; MC, microneme; MI, micropore; N, nucleus; RO, rhoptry. From Dubey and Beattie (137).

During 2011 to 2012, a cluster of 68 cases of acute muscular sarcocystosis occurred in persons who had traveled to Tioman Island, Malaysia. Of those, six were laboratory-confirmed, and *Sarcocystis nesbitii* DNA was recovered from one muscle biopsy (173). The source of these infections was not determined but was suspected to be raw or undercooked meats (174).

Presumptive diagnosis of human intestinal sarcocystosis is based on symptoms and travel history. Definite diagnosis is determined by identifying sporocysts in the stools of the patients. *Sarcocystis* species cannot be differentiated by direct microscopic observation. Muscular sarcocystosis can be diagnosed by the clinical

presentation and by linking it to the patient's travel history. Muscle biopsies can be used to detect tissue cysts or sarcocysts present; however, this method is not very sensitive. Myositis, myonecrosis, perivascular and interstitial inflammation, vasculitis, and eosinophilic myositis have been observed in cases of muscular sarcocystosis (174). Molecular tools have been developed and used for testing animal tissues and meat for human consumption (175–183).

MICROSPORIDIA

Most microsporidium infections occur in AIDS patients (134). Several species of microsporidia have been associated with human infections, in particular *Enterocytozoon*, *Pleistophora*, *Encephalitozoon*, and *Vittaforma*. *E. bieneusi*, the only species that seems to be tissue specific, is associated only with human enteric infections. Microsporidium spores, which are highly resistant to environmental stresses, vary in size among the different species. They are ovoid or piriform and 1 to 2 μm in diameter. *Encephalitozoon intestinalis* spores are larger than those of *E. bieneusi*. These spores contain a polar filament that is used to eject the sporoplasm and penetrate the host cell cytoplasm. After entering the cell, the parasite multiplies asexually and eventually forms spores, lysing the host cell and invading neighboring cells. Although the mechanisms of transmission are not clear, it is believed that the infection can be acquired by ingesting spores in contaminated water and produce (184).

Infection with microsporidia is characterized by watery and large-volume stools. Microsporidia are occasionally associated with biliary tract disease and may potentially cause cholangiopathy, which occurs in AIDS patients (185).

Microsporidium spores can be histologically identified in tissue, but identification in fecal samples requires special staining methods. Microsporidia can be identified in tissues by using various

conventional stains such as hematoxylin-eosin, Gram, and Giemsa stains. These spores can be detected in fecal samples by using nonspecific staining techniques such as Calcofluor white and a modified trichrome with Chromotrope 2R; however, small structures and yeast spores are also stained by these procedures (149, 186, 187). Identification of microsporidia in the environment presents an additional challenge. Most species of the animal kingdom can be parasitized by species of microsporidia, and noninfectious species can be confused with those infectious to humans. Definitive identification is limited to transmission electron microscopy (188–191). New tests using specific monoclonal antibodies and molecular tools such as PCR assays are being developed to aid in the detection of microsporidia in tissues and in fecal and environmental samples (57, 192–194).

Infections caused by *E. intestinalis* can be treated with albendazole or with metronidazole and atovaquone. To date, there is no effective treatment for infections caused by *E. bieneusi* (195); however, nitazoxanide has been reported to resolve the infection in an AIDS patient (196).

GIARDIA

Giardia is a protozoan flagellate that belongs to the phylum *Sarcomastigophora*. Initially thought to be a commensal organism in humans, *Giardia* is now clearly recognized as a common cause of diarrhea and malabsorption. *Giardia* infects millions of people throughout the world in both epidemic and sporadic forms. Most human infections result from ingestion of contaminated water or food or direct fecal-oral transmission, such as would occur in person-to-person contact in child care centers and in male homosexual activity (197).

Three species of *Giardia* have been described based on differences discernible in cysts and trophozoites by light microscopy: *Giardia agilis* from amphibians; *G. muris* from rodents, birds, and reptiles;

and *G. lamblia* from various mammals, including humans. Two additional species that are indistinguishable from *G. lamblia* by light microscopy, *G. ardeae* from herons and *G. psittaci* from psittacine birds, have been identified based on ultrastructural morphologic differences (198).

G. lamblia is not host restricted, and wild animals such as beavers and muskrats have been implicated in waterborne outbreaks of giardiasis in humans. Molecular classification using small-subunit rRNA has placed *Giardia* as one of the most primitive eukaryotic organisms (199). Two major molecular groups or assemblages (A and B) of *G. lamblia* have been recognized as infecting humans but can also infect nonhuman primates, dogs, cats, and wildlife. Other assemblages infect animals exclusively: dogs and other canids (C and D); cattle, goats, sheep, and pigs (E); cats (F); rats (G); and marine mammals (H) (200).

Giardia can be observed in two forms: the trophozoite and the cyst. The cyst is the infectious form and is relatively inert and environmentally resistant. After cysts are ingested, excystation occurs in the duodenum after exposure to the acidic gastric pH and the pancreatic enzymes chymotrypsin and trypsin. Each cyst releases two vegetative trophozoites. The trophozoites replicate in the crypts of the duodenum and upper jejunum and reproduce asexually by binary fission. Some of the trophozoites then encyst in the ileum, possibly as a result of exposure to bile salts or cholesterol starvation (197). The trophozoites and cysts are excreted in the feces.

Cysts are round or oval. Each cyst measures 4 to 11 by 7 to 10 μm, has four nuclei, and contains axonemes and median bodies. Trophozoites have the shape of a teardrop (viewed dorsally or ventrally) and measure 10 to 20 μm in length by 5 to 15 μm in width. The trophozoite has a concave sucking disk with four pairs of flagella, two axonemes, two median bodies, and two nuclei. The ventral disks act as suction cups, allowing mechanical attachment to the surface of the intestine (Fig. 25.8) (201). Infections may result

from the ingestion of 10 or fewer *Giardia* cysts. Boiling is very effective to inactivate *Giardia* cysts, but cysts can survive after freezing for a few days.

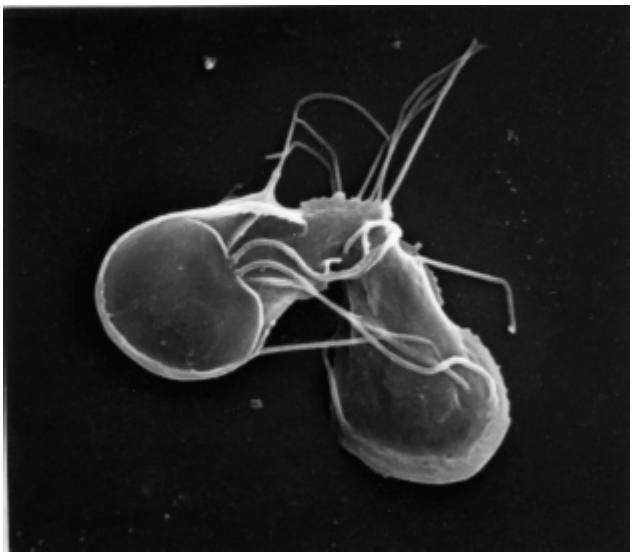


Figure 25.8 Scanning electron micrograph of *G. lamblia* trophozoites. One trophozoite shows the dorsal surface and the other shows the ventral surface with sucking disk and flagella.

Ozone is an excellent disinfectant and can even be used to inactivate microorganisms such as protozoa, which are very resistant to conventional disinfectants. The apparent activation energy for the inactivation of protozoa is 80 kJ/mol. By-products of ozonation include bromate, iodate, and chlorate, which may be of concern depending on the chemical composition of the water to be disinfected. A 2-log reduction of *G. lamblia* and *G. muris* cysts with ozone requires a contact time 2.4 times longer than that recommended by the surface water treatment rule.

A 2-log inactivation of *Cryptosporidium* in drinking water can be achieved using ozone; however, in other waters, including recreational waters, a 3-log reduction can be achieved using 5 mg/ml of free chlorine with or without the addition of 2.6 ml/liter of free chorine at contact times of 105 and 128 min. Ozone has also been evaluated, and 99% oocyst inactivation was achieved when the

integrated ozone residual was 0.5 mg/liter with a 5.2-min concentration time (202–205).

G. lamblia is prevalent worldwide and is especially common in areas where poor sanitary conditions and insufficient water treatment facilities prevail. Seasonality of giardiasis has been reported during late summer in the United Kingdom, the United States, and Mexico. Most *Giardia* infections are asymptomatic, but they can present as chronic diarrhea. Travelers to areas of endemicity are at high risk for developing symptomatic giardiasis. A study in Leningrad (now St. Petersburg), Russia, revealed that 95% of travelers developed symptomatic giardiasis. Hikers and campers are also at increased risk, since *Giardia* cysts, often of animal origin, can be found in freshwater lakes and streams. The prevalence of *Giardia* infection can be as high as 35% in children attending child care centers. Although these children are frequently asymptomatic, they may infect other family members, who may develop symptomatic giardiasis (206–208).

Giardia has been the most common agent in waterborne outbreaks of diarrhea in which the etiologic agent was identified. Waterborne transmission is usually a result of inadequate water treatment or sewage contamination of drinking, well, or surface water. Giardiasis has also been associated with exposure to contaminated recreational water, such as that in swimming pools. *Giardia* cysts can be inactivated by ozone and halogens; however, the concentration of chlorine used for drinking water is not sufficient for inactivation. Inactivation by chlorine requires prolonged contact time, and filtration is the recommended means for purifying water (209–211). It is estimated that 76,840 *Giardia* episodes associated with foodborne illness occur in the United States annually (38).

Symptomatic patients present with loose, foul-smelling stools and increased levels of fat and mucus in fecal samples. Flatulence, abdominal cramps, bloating, and nausea are common, as are anorexia, malaise, and weight loss. Blood is not present in stools. Fever is occasionally present at the beginning of the infection. In

contrast with most other forms of acute infectious diarrhea, *G. lamblia* infection results in prolonged symptoms. Although giardiasis may resolve spontaneously, the illness frequently lasts for several weeks and sometimes for months if left untreated. Those with chronic giardiasis have profound malaise and diffuse epigastric and abdominal discomfort. Although diarrhea may persist, it may be replaced by constipation or even by normal bowel habits (197).

Malabsorption associated with giardiasis may be responsible for substantial weight loss. Even in asymptomatic infections, malabsorption of fats, carbohydrates, and vitamins may occur. Reduced intestinal disaccharidase activity may persist even after *Giardia* is eradicated. Lactase deficiency is the most common residual deficiency, occurring in 20 to 40% of cases (212).

Villous blunting, lymphocytic infiltration, and malabsorption are observed in biopsy samples in symptomatic cases. No tissue invasion is observed, and large numbers of trophozoites are sometimes present in the crypts without obvious pathology. To date, the presence of a toxin has not been identified and no other potential mechanisms by which *Giardia* causes diarrhea have been elucidated.

Giardia infection can be diagnosed by finding cysts or, less commonly, trophozoites in fecal specimens (Fig. 25.9A). *Giardia* in feces can be detected by enzyme immunoassays, indirect and direct immunofluorescence assays using monoclonal antibodies, or PCR assays. All of these procedures are highly sensitive and specific for environmental and stool samples.

Genotyping of *Giardia* has been achieved using 18S ribosomal DNA and triosephosphate isomerase PCR assays, among others. In some patients with chronic diarrhea and malabsorption, results from stool examinations are repeatedly negative despite ongoing suspicion of giardiasis (213–215). *Giardia* cysts were found in 41.5% of nondepurated mussels from the Galician coast that were destined for human consumption (216).

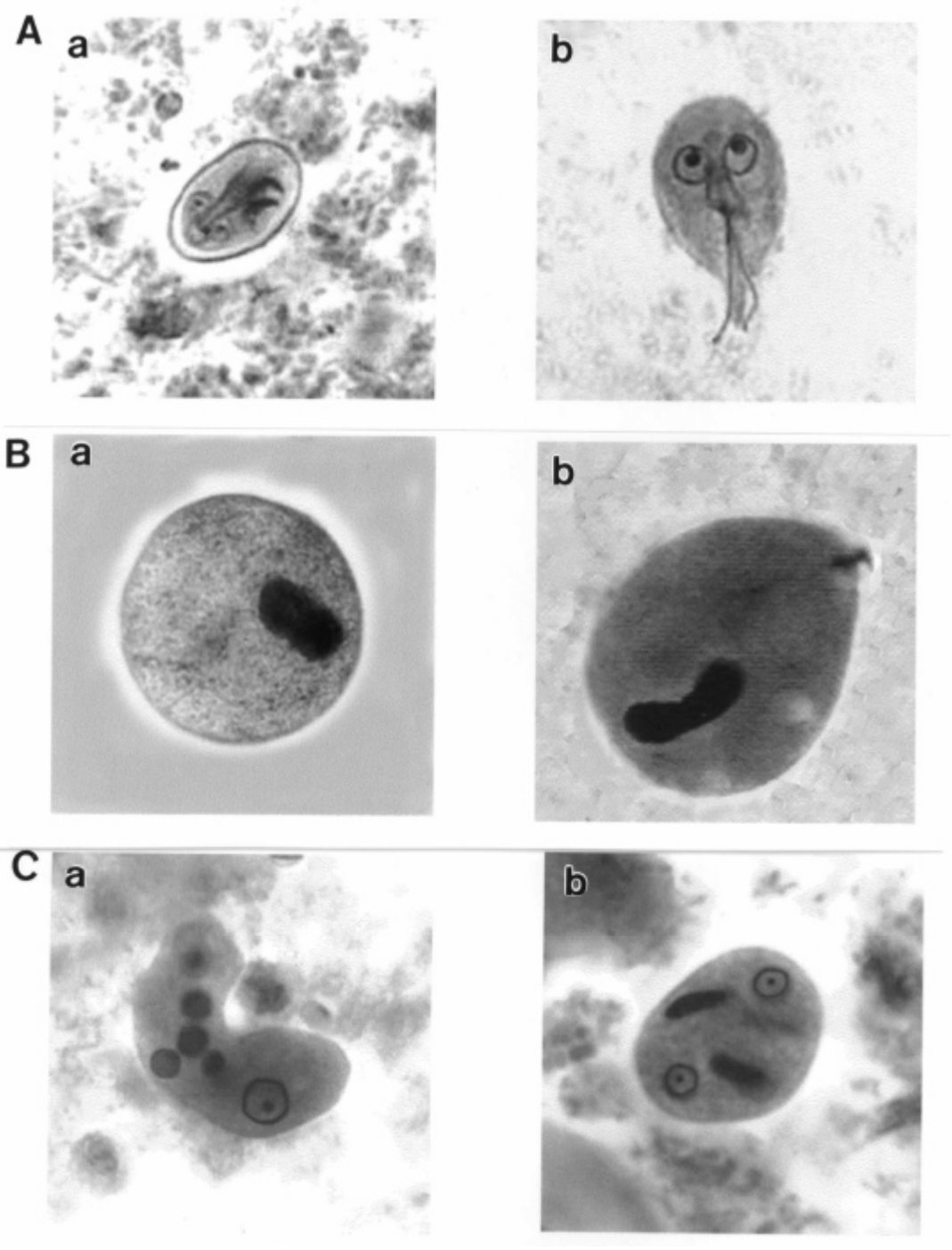

A
a
b
B
a
b
C
a
b

Figure 25.9 Hematoxylin and eosin staining. (A) *G. lamblia* cyst with two nuclei and a trophozoite (a) and showing two nuclei and the median body visible at one pole (b). (B) *B. coli* cyst showing a large macronucleus and cilia beneath the cyst wall and trophozoite (a) and showing an oval macronucleus (b). (C) *E. histolytica* trophozoite showing a nucleus and a few red blood cells in the cytoplasm and cyst (a) and showing two of four nuclei and rodshaped inclusion bodies with rounded ends (b). Courtesy of Lynne S. Garcia.

Effective treatment for patients with symptomatic giardiasis is mainly a single treatment course with metronidazole. In refractory cases, multiple or combination treatments have occasionally been required. Tinidazole is widely used throughout the world, and a single dose is effective for the treatment of giardiasis (217, 218).

BALANTIDIUM

Balantidium coli is a ciliate parasite that, although found worldwide, is not highly prevalent. It is a commensal parasite of pigs. The trophozoites reside in the large intestine and multiply by binary fission. In humans, *B. coli* can cause ulcerative colitis and diarrhea. The ulcers differ from those caused by *Entamoeba histolytica* in that the epithelial surface is damaged but with lesions more superficial than those caused by amoebae. The parasite encysts and is excreted in the feces. The cyst, which is the environmentally resistant form, is large and oblong, 45 to 65 μm in diameter (Fig. 25.9B). Both cysts and trophozoites contain two nuclei. Trophozoites move via their cilia and rotate on their longitudinal axis (219). The preferred treatment of infection is tetracycline and, alternatively, iodoquinol and metronidazole.

AMOEBAE

Various amoebae can infect humans as commensals; however, *E. histolytica* is pathogenic to humans. *Entamoeba dispar*, which is

morphologically similar to *E. histolytica*, is not pathogenic. Cysts and trophozoites of *E. histolytica* are excreted in the feces of infected individuals (Fig. 25.9C), with cysts being environmentally resistant. Once cysts are ingested, the trophozoite excysts, colonizes the large intestine, and multiplies by binary fission followed by encystation.

Most infected individuals are asymptomatic even when shedding cysts in their feces. In other instances, parasites can invade the mucosa and cause an ulceration that goes from the luminal surface of the intestine through the lamina propria and to the muscularis mucosae. The parasite then spreads laterally, forming a flask-shaped ulcer. The trophozoites feed on cell debris and red blood cells (220). Infection can progress, producing ulcerative amoebic colitis and causing perforation of the intestinal wall. Patients complain of diarrhea with stools containing blood and mucus, back pain, tenesmus, dehydration, and abdominal tenderness. Fulminant colitis is characterized by severe bloody diarrhea, fever, and abdominal tenderness due to transmural necrosis of the bowel. Another presentation of amoebiasis is formation of amebomas, which resemble carcinomas and are not necessarily associated with pain. Amoebae can also disseminate to the liver. Complications with amoebiasis are observed when the liver parenchyma is gradually replaced with necrotic debris, inflammatory cells, and trophozoites. Patients present with hepatomegaly, weight loss, and anemia (220, 221).

Asymptomatic amoebiasis can be treated with iodoquinol, paromomycin, or diloxanide. If mild to severe infection and hepatic abscesses are present, amoebiasis can be treated with metronidazole or tinidazole followed with iodoquinol to treat asymptomatic amoebiasis.

CONCLUDING COMMENTS

Inactivation of the protozoan parasites is challenging. Enrichment media are not available for cultivating parasites; therefore, isolation and identification methods need to be extremely sensitive and specific to detect small numbers of various forms of parasites that may be present in foods. Molecular tools such as PCR, restriction fragment length polymorphism analysis, and variations of these techniques are being developed to improve the sensitivity and specificity of detection and identification processes.

Treatment of drinking water by chlorination at permissible concentrations is relatively ineffective at inactivating spores, cysts, and oocysts of parasites and cannot be recommended as a sole method for water treatment. Boiling can inactivate cysts, oocysts, and spores. Irradiation of produce to inactivate pathogens that may be present has also been evaluated. This method effectively inactivates *Toxoplasma* cyst forms and oocysts. There has been an increase in consumers' acceptance of purchasing irradiated produce (222), providing additional incentive to use irradiation as a treatment to reduce or eliminate at least some genera of parasites in food and water.

Finally, we face new challenges to ensure the safety of produce and other foods. Foodborne-disease outbreaks caused by parasites are being documented with increased frequency. The consumption of fresh fruits and vegetables in developed countries has increased, and local production cannot meet this demand. Thus, importation of produce and other foods from countries where sanitary standards are minimal or lacking makes it even more difficult to control foodborne diseases (223).

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V
Preservatives and Preservation Methods

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26
Novel Physical Methods for Food Preservation

There is a growing consumer demand for safe foods that are free of additives, minimally processed, and fresh-like in appearance and taste. While conventional thermal processing methods can address microbial safety considerations, the severity of processing invariably lowers the quality of food. To address these needs, numerous non-thermal processing methods are under investigation. In this chapter, we highlight some of these physical, non-thermal processing technologies and discuss their mechanisms of action, their benefits over conventional technologies, and their potential limitations.

DENSE-PHASE CARBON DIOXIDE

Dense-phase carbon dioxide (DPCD) processing is a non-thermal processing technology. In DPCD processes, carbon dioxide (CO_2) is held under high pressure in the liquid or gaseous phase, as well as in the supercritical state (1). A supercritical fluid can be defined as a substance held at a pressure and temperature at which there is no distinction between the liquid and the vapor phases. Supercritical conditions take place above a specific combination of pressure and temperature known as the critical point. For the case of CO_2, supercritical conditions exist above ~7.38 MPa and ~31°C (2). The use of CO_2 entails some safety benefits due to its thermodynamic stability, as well as its lack of flammability and toxicity (3).

DPCD processing has largely been studied for the processing of liquid foods. The liquid food is often heated to a mild temperature (30 to 50°C) and then is subjected to pressure above the critical point of CO_2. The CO_2 is injected into the liquid food, and these conditions are maintained for a specific holding time, which is followed by the release of CO_2 through depressurization (1, 4–6) (Fig. 26.1). The application of DPCD has also been studied in solid foods. However, this technology is less effective in solid foods than in liquid foods for inactivating microorganisms, possibly due to the lower diffusivity of CO_2 within solid matrices. Additionally, in contrast to what has been observed in liquid foods, in which there is a marginal effect on the sensory and nutritional attributes of the food, solid foods generally experience adverse changes in texture (loss of firmness) and discoloration when subjected to DPCD treatments (1). In general, there are still some limitations for the commercial application of DPCD, such as lower efficiency compared to heat treatments, costly equipment, and environmental concerns related to the release of

CO_2 (1). Table 26.1 shows some examples of studies in which DPCD has been applied.

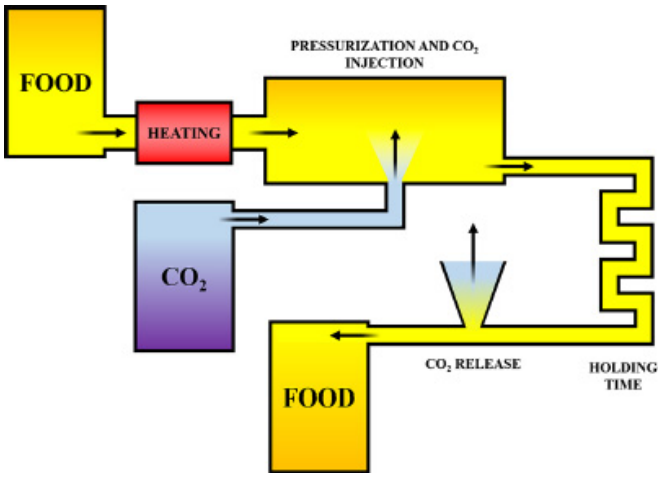


Figure 26.1 DPCD processing diagram.

Different phenomena have been proposed to explain the antimicrobial effect of DPCD. One probable contributing factor could be the decrease of pH caused by the incorporation of CO_2 into the liquid medium. Another mechanism could involve the denaturation of microbial enzymes, caused by the decrease in pH, the formation of bicarbonate complexes with enzymes, or precipitation of metal ions within the enzymes to form bicarbonate salts. Another accepted phenomenon is cell lysis, which may be caused by the expansion of CO_2 within the cells once it is released from the liquid food during the depressurization step. The expansion of CO_2 may also promote the release of vital biomolecules, affecting cell viability (1) (Fig. 26.2).

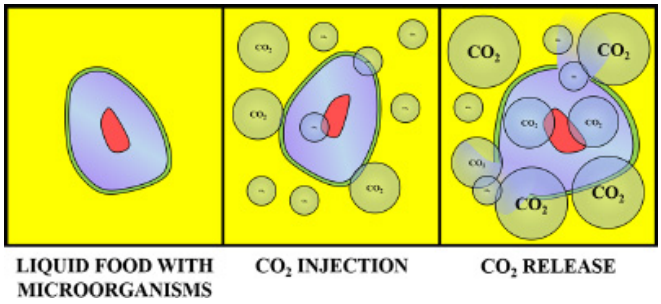


Figure 26.2 Possible mechanism for the antimicrobial effect of DPCD.

FILTRATION

In filtration, particles of different sizes are removed from a liquid medium by forcing the liquid through a porous matrix. The liquid that passes through the filtration medium is called filtrate, and the fraction that contains the removed particles is the retentate. Different mechanisms can serve as the driving force behind the removal of particles, such as a pressure gradient, a concentration

gradient, or an electric field (7). Filtration can be classified into microfiltration, ultrafiltration, nanofiltration, and reverse osmosis. Figure 26.3 shows the capabilities of each classification in terms of the range of particle sizes that can be removed. Microfiltration can be used to remove bacterial cells (8). For filtration to effectively remove microorganisms from the liquid medium, a pore diameter of 0.20 to 0.22 μm is needed, as well as a low initial microbial load in the liquid medium. Filtration offers the advantage of reducing the energy costs of the thermal treatments often used to inactivate microorganisms (7).

For sterilization, a filtration medium must efficiently remove particles that are the size of the different types of microorganisms, be able to have a high flow rate, prevent clogging, possess high thermal stability, possess adequate mechanical properties to ensure stability, be resistant to biofouling, and possess chemical stability to avoid release of chemicals into the filtrate (9). Innovations that have been developed for this technology have largely focused on approaches addressing the latter requirements.

Table 26.1 Recent studies on DPCD applications in foods

Food	Microorganism(s)	DPCD conditions	Logarithmic Reduction	Reference
Coconut water	Total aerobic bacteria	34.5 MPa, 30°C, 13% CO_2 for 6 min	6.18	4
Barley grains	*Penicillium oxalicum*	10 MPa, 44°C, 99.5% CO_2 (with water as cosolvent), 12 min	6.8	37
Bovine milk	Naturally present microflora	25 MPa, 50°C, 70 min	4.96	38
Grape juice	*Saccharomyces cerevisiae*, *Candida stellate*, *Kloeckera apiculata*	6.9–48.3 MPa, 25–35°C, 0–0.17% CO_2, 5 min	<6	
39	Orange juice	Aerobic mesophiles	7.6 MPa, 42°C, 8.5% CO_2, 20 min	2	6

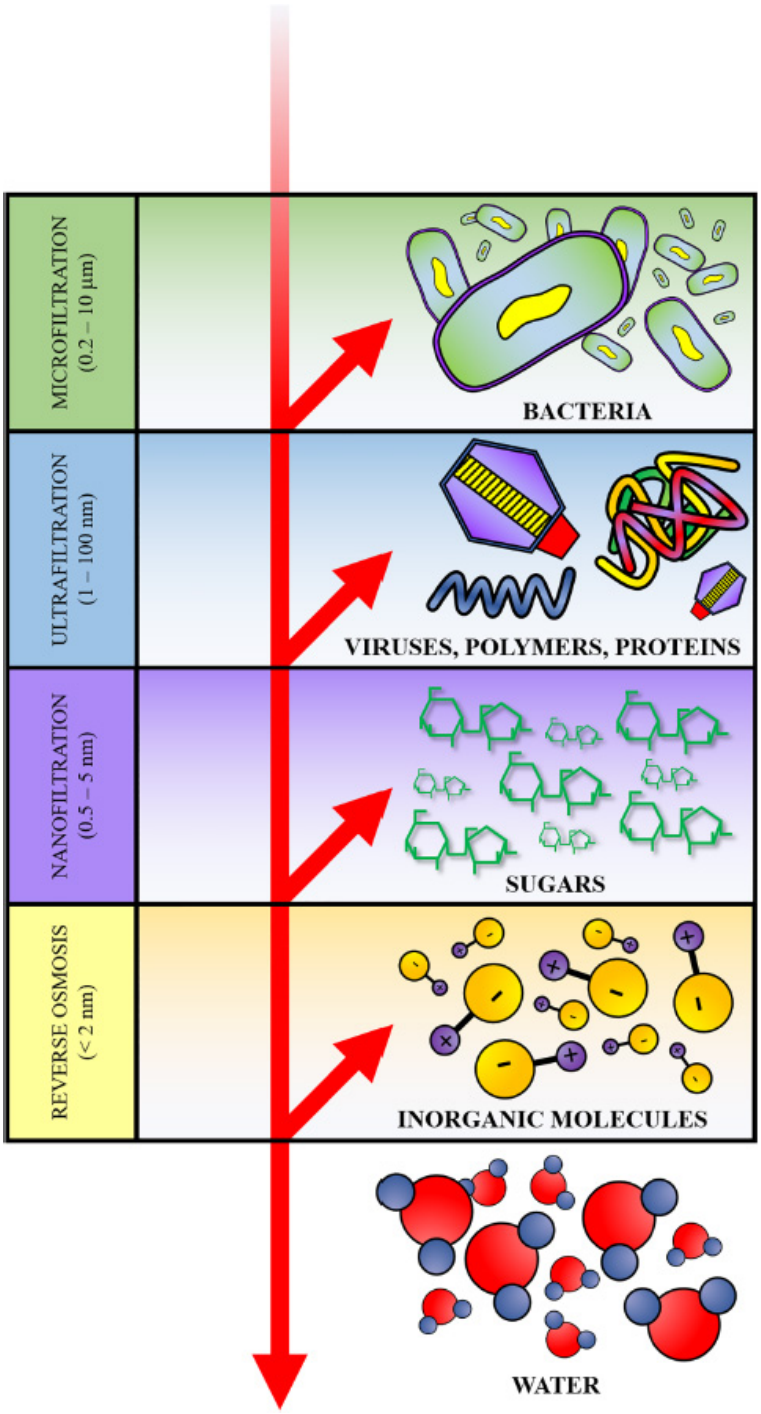


Figure 26.3 Capabilities for removal of different substances by different types of filtration (8, 12).

Most membranes used for filtration are made of polymeric materials which can provide greater flexibility and versatility to control pore size, surface chemistry, mechanical properties, and other functionalities that can help meet a specific objective for filtration. The polymers used for filtration membrane fabrication are normally hydrophobic. This feature has some drawbacks, such as resistance of

water to be filtered, as well as fouling by microorganisms and organic matter, which can subsequently result in microbial contamination of the filtrate or clogging of the membrane (7). Hence, this raises the need to modify the surface chemistry of the bulk materials used to fabricate filtration membranes. The different approaches that have been explored include blending of the bulk material with a variety of compounds that can provide an added functionality. Some of those compounds are amphiphilic substances that may reduce the affinity of organic molecules for the membrane (7), intrinsically antimicrobial polymers such as chitosan (7, 10), and antimicrobial metal nanoparticles (7, 11, 12).

Coating of the bulk material of the filtration membranes has also been used instead of blending (7, 13). Different techniques can be used in coating to incorporate an additional functionality, such as graft polymerization or surface activation to create reactive groups (on the bulk material) followed by covalent immobilization of different types of compounds (7). Another approach involves the fabrication of filtration membranes from electrospun nanofibers (7). During electrospinning, different polymers or compounds may be cross-linked to provide antimicrobial or antifouling properties to the resulting membrane.

Table 26.2 provides a summary of results of recent studies addressing antimicrobial applications of filtration technology.

COLD PLASMA

Plasma can be categorized as the fourth state of matter. Changes of state take place in substances when energy (in different forms, such as heat, radiation, light, or electricity) is applied to them. The transitions from the solid state to the liquid state and from the liquid state to the gas state demand increasing amounts of energy. If more energy is supplied to a substance that has reached the gaseous state, the inter- and intramolecular interactions (as well as the intra-atomic interactions) can be broken, which may result in a mixture of neutral molecules, electrons, and ions. These chemical species carry with them large amounts of energy. When they recombine or collide, that energy is released in the form of UV light (14–16). Plasma is a gas in which molecules and atoms have been ionized. The resulting high electrical conductivity allows a constant supply of electrical energy to be released through a wide range of methods, normally electric discharge (16, 17).

Plasma can be divided into hot and cold plasma (14, 16). Cold plasma refers to the type of plasma that can be generated at or near room temperature, which makes it an attractive nonthermal technology for food processing (14). Cold plasma can also be subdivided into thermal and nonthermal plasma. In thermal plasma, particles have high levels of energy and remain in equilibrium without transferring energy, whereas in nonthermal plasma there is an imbalanced level of energy between particles, which leads to energy transfer through collisions (14, 16). Nonthermal plasmas can be generated by electric discharge at low levels of pressure or at

atmospheric pressure (16). Cold nonthermal plasma at different levels of pressure is most frequently used in research addressing microbial inactivation (18–23). Plasma can also be generated from different types of gases, such as nitrogen, oxygen, mixtures of oxygen and nitrogen, and noble gases (14).

Table 26.2 Results of recent studies on antimicrobial filtration technology applications for foods

Antimicrobial material	Pore size	Microorganism tested	Logarithmic Reductions	Reference
N-Alkylated polyethyleneimine coating polypropylene microfiltration membrane	200 μm	*Staphylococcus aureus*	0.75–1.5	40
N-Chloramines on carboxylated thermoplastic of cardo poly(aryl ether ketone)		*Staphylococcus aureus* and *Escherichia coli* K-12	Inactivation below detection limit	41
Cellulose with chitosan	<1 nm	*E. coli*	Not reported (inhibition zone assay)	42
Quaternized cardo poly(arylene ether sulfone)	5–11 nm	*E. coli*	<1	43
Aminated poly(ether sulfone)		*E. coli*, *Bacillus subtilis*	Not reported (inhibition zone assay)	44

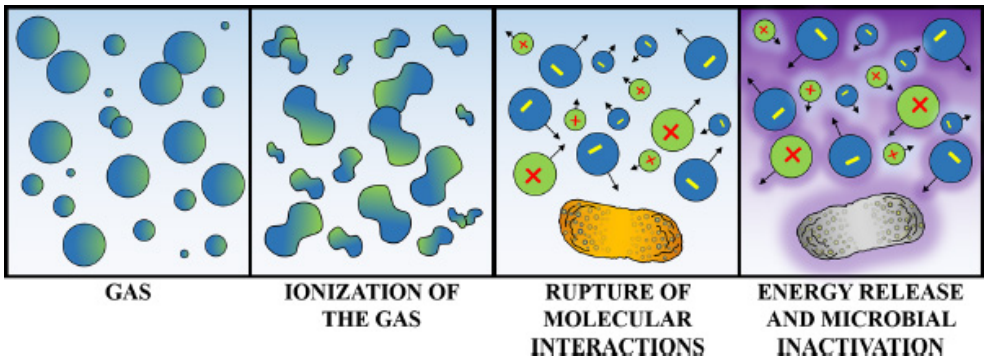


Figure 26.4 Antimicrobial effect of plasma.

The release of energy brought about by the collisions experienced between the different chemical species in plasma inactivates microorganisms (Fig. 26.4). Although the antimicrobial mechanisms of plasma have not been completely elucidated, they generally involve the degradation of cell membranes and essential biomolecules (such as genetic material, proteins, and enzymes) by exposure to the

UV radiation and the reactive species present in plasma (14). The antimicrobial effectiveness of cold plasma can vary widely depending on the plasma generation methods applied and the plasma composition. Some plasma-generating methods include radio frequency discharge, glow discharge, inductively coupled plasma, barrier discharge, plasma jets, and microwave regimes (14, 16). Although different plasma compositions are effective in inactivating microorganisms in different types of foods, the presence of oxygen enhances plasma's antimicrobial action (14).

Cold plasma has shown promise as a nonthermal processing alternative for microbial decontamination of foods. However, given the diversity of methods in which it has been studied (and the small scale in which they have been implemented), more research is needed to evaluate its scalability and applicability in food processing operations.

Table 26.3 summarizes results of studies involving plasma for reducing microbial loads in different types of foods.

COATINGS

The application of coatings to materials commonly used in food processing facilities (and throughout the transport chain) is a concept that has been widely studied and represents an attractive alternative to reduce microbial cross-contamination during the manufacture and distribution of foods (Fig. 26.5). According to the Centers for Disease Control and Prevention, the contamination of surfaces in the food preparation and processing environment is among the top five risk factors responsible for foodborne outbreaks (24).

Table 26.3 Results of studies on antimicrobial plasma applications in foods

Plasma type	Food	Microorganism	Logarithmic Reduction	Reference
Cold atmospheric plasma (air)	Cherry tomatoes and strawberries	*Salmonella enterica* serovar Typhimurium, *Listeria monocytogenes*, *Escherichia coli*	3.5–4.2	18
Cold atmospheric plasma (dry air or nitrogen)	Almonds	*Salmonella*, *E. coli* O157:H7	0–2	45
Cold atmospheric plasma (argon)	Whole black pepper	*Salmonella enterica*, *Bacillus subtilis*, *Bacillus atrophaeus*	2.8–4.1	46
Low pressure cold plasma (nitrogen, argon, helium)	Meat	Psychrotrophs, mesophiles, yeasts, and molds	1–6	21
Atmospheric pressure plasma (argon)	Lettuce, carrots, and tomatoes	*E. coli*	0–2	20

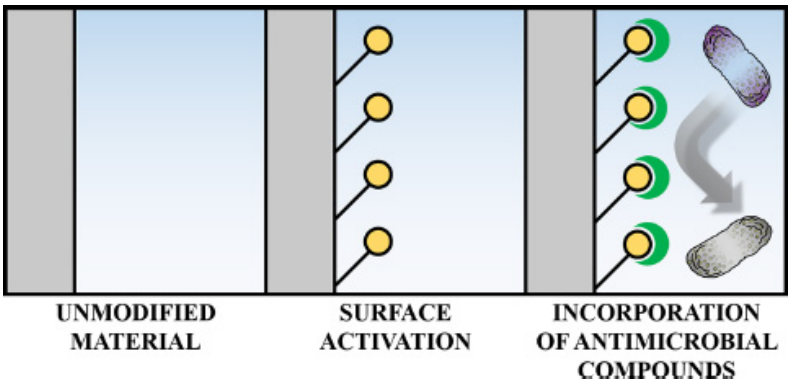


Figure 26.5 Application of a functional compound to an inert surface.

The surfaces of both organic (such as plastics) and inorganic (such as stainless steel) materials can be modified to provide a specific functionality. Most studies conducted in this field have focused on developing antimicrobial or antifouling materials (25). The rationale behind applying a thin coating on the surface of commonly used materials is that a thin coating would allow the bulk materials to retain their mechanical, chemical, and thermal properties, thereby enabling them to preserve their functionality and at the same time serve as an antimicrobial agent

(26). This could thereby decrease the use of chemicals for disinfecting food processing plants.

The materials used in food processing facilities are typically chemically inert. Before any type of coating is applied, the surfaces of these materials should be activated so that they contain functional groups, change their hydrophobicity, or change their topography and surface area. The functional groups (such as carboxylic, hydroxyl, amine, or thiol) can further react with different types of compounds. By changing the hydrophilicity or the topography, these materials can also retain different compounds or polymers on their surface through a variety of surface modification techniques (25, 27). Surface functionalization can be accomplished through the application of UV light irradiation, ozone, plasma, or corrosive chemicals such as chromic acid or piranha solution (25, 27–29).

The surface modification technique to be used depends on the type of material to be coated. For inorganic substrates, such as stainless steel, the most common techniques involve chemical vapor deposition and electroplating. In chemical vapor deposition, a compound is applied to a surface by condensation from a gaseous phase, which is created through high temperatures, with or without vacuum. This condition limits the range of compounds that can be applied, as they can normally be only low-molecular-weight compounds (25, 30). In electroplating, a compound is deposited on the surface of the material through diffusion. The driving force is normally a difference in voltage between the liquid medium that contains the active compound and the material to be coated (25).

Plastic materials are more flexible regarding the surface modification techniques that can be applied to them. After surface functionalization, antimicrobials or other active compounds can be applied through covalent immobilization, graft polymerization, layer-by-layer deposition, self-assembled monolayers, or reactive blending (25, 31). In each of these methods, bioconjugation techniques can be used to promote the formation of covalent bonds (32).

A wide range of antimicrobial compounds have been used to coat different materials (organic and inorganic), such as metal-based compounds, quaternary ammonium compounds, polycations, essential oils, peptides, and light-activated compounds. The mechanisms by which these compounds inactivate microorganisms include damage to the cell membranes and essential biomolecules. Because these antimicrobials are immobilized, they should not be released from the bulk material, thereby preventing contamination and colonization of surfaces by microorganisms (25).

Even though antimicrobial coatings can be effective against Gram-positive and Gram-negative bacteria, as well as against yeasts and molds, their application in food processing facilities has been limited. One of the main drawbacks of this technology is the limited stability and durability of coatings under adverse conditions (high temperatures or extreme pH). In addition, scaling up some surface modification techniques is a challenge (25). Table 26.4 provides examples of

immobilization of antimicrobial compounds on food contact surfaces and their biocidal efficacy.

Table 26.4 Examples of the use of antimicrobial coatings for food applications

Antimicrobial	Support	Microorganism	Logarithmic reductions	Reference
Lysozyme	Silicon rubber	*Micrococcus lysodeikticus*	Not reported (absorbance assay)	47
N-Halamines	Stainless steel	*Listeria monocytogenes*	2.5	29
Nisin	Clay (montmorillonite)	*Enterococcus faecium*	Not reported (inhibition zone assay)	48
Polyethyleneimine (polycation) with or without *N*-halamines	Polypropylene	*Escherichia coli* O157:H7	0.6 to >4	31
Antimicrobial peptide	Titanium	*Porphyromonas gingivalis*	<1	49

UV IRRADIATION

The UV range of the light spectrum includes wavelengths from 200 nm to 400 nm. UV radiation in the range of 200 to 280 nm (UV-C) inactivates microorganisms, which is attributed mainly to the dimerization of pyrimidine units, which alters or eliminates DNA replication, causing cell death (33). In spite of its proven efficacy against microorganisms (predominantly bacteria and viruses, although molds and yeasts may also be affected), the use of UV irradiation in food processing has been limited by its ability to penetrate foods, so the technology has been applied more frequently to the disinfection of surfaces (33, 34), which includes surfaces in the processing environment that have direct contact with foods or raw materials, such as conveyor belts, utensils, and processing equipment (35).

Two of the main factors that influence the antimicrobial efficacy of UV irradiation are the applied dose and its intensity. The intensity is normally expressed in units of energy per unit of time and area. Vegetative cells are more sensitive to UV irradiation than spores. Molds and yeasts require higher doses and intensities than bacteria to be affected. In general, doses high enough to cause irreversible damage in DNA are needed to provide a lethal effect (33). Another factor that determines the efficacy of UV irradiation is the medium that contains the microorganisms. The efficacy of UV irradiation tends to be higher when it is applied to liquid foods

(although the presence of organic matter and suspended particles [Fig. 26.6] may compromise its effect by absorbing part of the light irradiation). In contrast, a shielding effect can be observed in solid foods, where the roughness of the surfaces may leave microorganisms unexposed (33, 35), which may diminish or even eliminate any biocidal effect. However, UV irradiation has the potential to be applied as a complementary treatment to reduce microbial contamination in solid foods, in combination with other antimicrobial agents or technologies (35). This could also lead to a greater preservation of the chemical integrity of macro- and micronutrients, as they can be adversely affected by UV irradiation (33, 35, 36).

Several UV light sources are available. Their effectiveness depends on their intensities, as well as the amount of penetration that can occur through the sample. These light sources have largely been tested on liquid foods. Mercury lamps are among the most common sources and are used for water disinfection. They consist of bulbs that contain mercury and an inert gas (normally argon), which are ionized sequentially (argon excites mercury) through the application of an electric current. The ionization of mercury produces UV light at ~254 nm, which inactivates microorganisms. Mercury lamps are of low or medium pressure, which relates to the partial pressure of the mercury gas. Excimer lamps contain noble and halogen gas mixtures, in which ionization produces UV light in the 120- to 380-nm range. In microwave UV lamps, mercury gas is ionized by a magnetron instead of electrodes. The magnetron is used as the source of the electromagnetic waves that ionize argon and mercury (36).

Different designs of UV lamps have been developed to reduce microbial loads in liquid foods. Most consist of UV mercury lamps protected by a quartz cylinder. The liquid food is circulated around the UV lamps, allowing the liquid to form a very thin film, which allows sufficient penetration of the UV irradiation and greater antimicrobial activity (33, 35). However, some of these designs are still limited in their applicability, due to difficulties in scaling up.

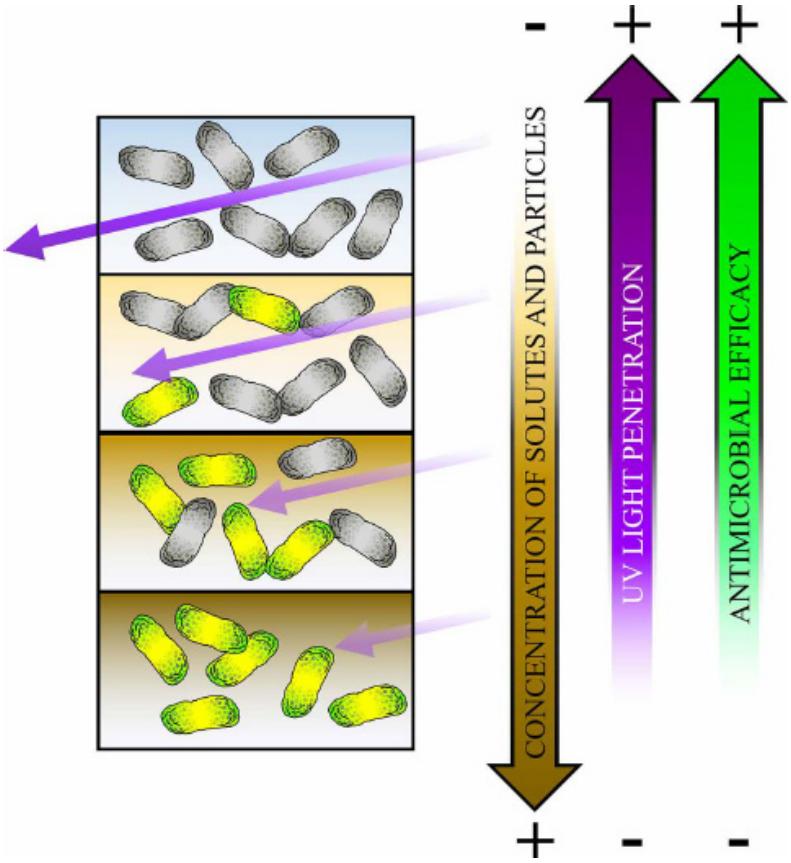


Figure 26.6 Effect of suspended particles and solutes on the antimicrobial activity of UV irradiation.

Table 26.5 provides examples of applications of UV irradiation to inactivate microorganisms in foods.

CONCLUDING COMMENTS

This chapter presents several emerging physical methods for food processing and preservation, such as DPCD processing, filtration, cold plasma treatment, antimicrobial coating, and UV irradiation. For each method, the principle of operation, mechanism of antimicrobial action, types of food products where these methods can be used, their unique benefits, and their potential limitations are discussed. Generally, these technologies have significant potential benefits over conventional processing technologies, such as improved energy efficiency, enhanced quality and safety of food, and longer shelf life. However, while these technologies are effective against pathogenic and spoilage vegetative microorganisms and viral particles, they are not as effective in inactivating spores. This gap can be addressed by a rational combination of two or more complementary processing methods. More research is needed to explore these combinations. Future work is also necessary to scale up these processes to a commercial level and achieve regulatory approval.

Table 26.5 Examples of UV irradiation for inactivating microbes in foods

UV light conditions	Food or medium	Microorganism	Logarithmic Reductions	Reference
UV-A light combined with gallic acid and lactic acid (320–400 nm)	Water	*Escherichia coli* O157:H7	≤4.7	50
UV-A light combined with gallic acid and lactic acid (320–400 nm)	Lettuce and spinach leaves	*E. coli* O157:H7	~2–3	50
UV-C light (254 nm) in combination with ultrasound and acidic electrolyzed water	Salmon	*Listeria monocytogenes*	0.5–0.8	51
UV-C light (200–280 nm)	Cheese	*Pseudomonas* spp.	0.8–2.1	52
UV-C light (specific wavelength not reported)	Cauliflower	*L. monocytogenes*, *E. coli* O157:H7, yeasts, and molds	0.17–1.8	53

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27
Chemical Preservatives and Natural Food Antimicrobials

The quality of food products declines from the point of harvest through animal slaughter and processing to consumption. Quality loss may result from microbiological, enzymatic, chemical, or physical changes to food structure and makeup. Consequences of microbiological changes include the introduction of hazards to consumer health due to the contamination and subsequent growth of microbial pathogens and the secretion of microbially produced toxins (e.g., mycotoxins and staphylococcal enterotoxin). Economic losses may occur through microbiological spoilage and resultant changes to the food, rendering it unpalatable to consumers. Food preservation technologies, including the application of antimicrobial preservatives, protect foods from the deleterious effects of microbial growth and inherent deterioration. Antimicrobial preservatives are often applied as one type of antimicrobial hurdle during food processing, in combination with other forms of food preservation: heating, refrigeration and/or freezing, water activity (a_w) reduction, nutrient restriction, acidification, use of modified-atmosphere packaging, fermentation, or various forms of nonthermal processes (e.g., application of high hydrostatic pressure and irradiation).

For the purposes of this chapter, food antimicrobials are defined as chemical preservatives added to or present in foods that retard growth of or kill microbes, but they do not include therapeutic antibiotic-type compounds used for growth promotion or disease treatment in food animals. Most food antimicrobials are bacteriostatic or fungistatic at usage levels, inhibiting microbial

growth rather than exerting microbicidal effects. Thus, food antimicrobials do not preserve food indefinitely. Food antimicrobials are often used in combination with other preservation procedures; targets for food antimicrobials are pathogenic and spoilage microorganisms. Food antimicrobials are legally defined as preservatives, though the term also describes additives functioning to preserve the food in question, including antibrowning agents (e.g., citric acid) and antioxidants (e.g., butylated hydroxyanisole) [Code of Federal Regulations Title 21 (21 CFR), §101.22(a)(5) (https://www.ecfr.gov/cgi-bin/text-idx?SID=0f2002a44dc3b3296966c07b0f4aadb6&mc=true&node=pt21.2.101&rgn=div5) and §170.3(o) (2) (https://www.ecfr.gov/cgi-bin/text-idx?SID=0f2002a44dc3b3296966c07b0f4aadb6&mc=true&node=pt21.3.170&rgn=div5)].

Herein, antimicrobial compounds are divided into the traditional and the naturally occurring. Antimicrobials are classified as traditional when they have been (i) used for many years, (ii) approved by many countries and/or regulatory bodies for inclusion in foods as antimicrobial agents, or (iii) produced by synthetic processes as opposed to natural extracts (e.g., industrial fermentation of nisin from *Lactococcus lactis* subsp. *lactis*). Ironically, many synthetic and traditional food antimicrobials are found in nature; examples include acetic acid from vinegar, lactoperoxidase in fluid milk, and nitrate species in some food crops. Finally, this chapter focuses on recent developments in food antimicrobial research and applications.

FACTORS AFFECTING ACTIVITY

The observed activity of antimicrobials depends on multiple factors related to the food product, its processing and postprocess handling, the antimicrobial itself, and the microorganism(s) targeted. Food preservation is best achieved when the microorganism(s) to be inhibited, antimicrobial type and concentration, food storage

conditions, food pH and buffering capacity, and the presence of other agents that affect shelf life are known and accounted for. Taylor et al. (1) reviewed these factors, differentiating intrinsic, extrinsic, process-related, and microbial factors. Davidson and Branen (2) further differentiated the microbial factors into those associated with the microorganism(s) targeted for inhibition and those related to the antimicrobial's structure and consequent functionality (i.e., physicochemistry).

Intrinsic factors are those inherent to the food product's physicochemistry, including food physical structure, nutrient availability and profile (including, by correlation, the product aw), pH and buffering capacity, the presence of physical barriers to microbial penetration into edible components of the food (e.g., hides, skin, husks, or rinds), the oxidation-reduction potential, and the presence of naturally occurring or fermented antimicrobials and compounds that may interact with these antimicrobials. Extrinsic factors include the temperature of food storage postprocessing, the duration of storage until the end of shelf life or consumption, the atmospheric composition of the storage environment or packaging, and the relative humidity of the storage environment and packaging. Process-related factors include changes in food composition, microbiota shifts, and changes in microbial populations and in food microstructure and/or moisture resulting from processing (e.g., thermal, dehydration, or high-pressure processes). These influence microbial growth in the food in an interactive manner, leading many food processors to apply a hurdle technology approach incorporating the use of antimicrobials in addition to control of other factors in order to prevent microbial growth and product safety or quality loss (3, 4).

Food pH is potentially the most significant intrinsic factor directing the effectiveness of many food antimicrobials, including the organic acids and different antimicrobial peptides (e.g., lysozyme and bacteriocins), though not always through similar mechanisms. The weak organic acids and the fatty acids are most effective in their undissociated state, as they are better able to passively diffuse

through the microbial membrane. This arises from a lack of charge and their organic nature, yielding greater hydrophobicity to the acid species that facilitates access and movement through the lipophilic core of the microbial membrane between its leaflets. Nevertheless, antimicrobial polarity has also been reported as highly important, as it affects the antimicrobial's capacity to be suspended or solubilized into the aqueous phase of a food product. Hence, many antimicrobials must possess both hydrophobicity and polarity for optimal access to, and inhibition of, microbial organisms in foods (2, 5). Finally, it is worth noting that many food antimicrobials discussed herein, particularly many of the spice-derived essential oil components, bearing GRAS (generally recognized as safe) status or affirmation do not do so for their food preservation or antimicrobial properties but for other functionalities in the food (e.g., as a flavorant or stabilizer or for pH control).

TRADITIONAL ANTIMICROBIALS

Organic Acids and Their Derivatives

A review of recent research suggests that the most effective organic acids used as food antimicrobials are monoprotic, including acetic, lactic, propionic, sorbic, and benzoic acids, as well as some of the fatty acids (e.g., caproic acid and butyric acid) (6, 7). Doores (8) reviewed the research available on food antimicrobial applications of organic acids and the tolerances to these preservatives of different foodborne bacteria and fungi, indicating that while some have antimicrobial activity, multiprotic acids (e.g., malic, tartaric, and citric acids) exhibited less antimicrobial activity and/or microbial inhibition than monoprotic acids. The antimicrobial utility of organic acids is thought to result largely from the undissociated acid's penetration into the microbial cytoplasm and the subsequent acidification of the cellular cytoplasm, resulting in initiation of the cell's acid tolerance and stress response systems. Thus, application of organic acids is generally limited to foods with

reduced pH (pH ≤ 5.5), as most organic acids possess at least one pK_a (acid dissociation constant) ranging from pH 3.0 to 5.0 (9).

Research suggests that the enhanced capacity of organic acids to penetrate the microbial membrane in the undissociated state versus the dissociated acid anion state (10). Nonetheless, over the last 2 decades, evidence has accumulated indicating that microbial inhibition is not solely the result of cellular acidification and energy regulation uncoupling (11–13). In fact, Koczoń (14) reported that benzoic acid salt species activity was less affected by pK_a than by the total undissociated-acid content. This would need to be balanced against the content of acid anion extracellularly and the organism's capacity to tolerate the acidulant applied for the microorganism(s) of interest (11). Taylor et al. (1) summarized these mechanisms, working to integrate these explanations for microbial inhibition via organic acid antimicrobials. In addition, perturbation of microbial membranes has been reported to occur, resulting in permeabilization of acid-treated cells, which increases the opportunity for leakage of their contents (15, 16).

It is known that microorganisms surviving sublethal environmental stressors may develop enhanced tolerance to subsequent exposure to antimicrobials, which may be termed tolerance, adaptation, or habituation based on the method of stress application and the cellular responses leading to the cell's enhanced survival (17, 18). In foods, adaptation of cells to sublethal stressors can be of concern also for the development of cross-protective effects against other stressors (19, 20). Although there is little evidence of antimicrobial adaptive responses being sustained over multiple generations, there is a recent report of foodborne *Escherichia coli* strains that possess and share mobile genetic elements carrying genes encoding resistance to quaternary ammonium sanitizers (21). Nonetheless, application of a more severe antimicrobial challenge to contaminating microbes will result in a lower likelihood of microbial survival during extended holding, even despite previous adaptation (22).

Acetic Acid and the Acetates

Acetic acid (pK_a, 4.75; 60.05 Da) (Fig. 27.1) is the primary component of vinegar and also may be microbially oxidized from ethyl alcohol, and its cationic salts, as well as diacetate salts and dehydroacetic acid, are some of the oldest known food antimicrobials and preservatives (23). In the United States, acetic acid and calcium and sodium acetates are identified by the FDA as direct food substances affirmed as GRAS (21 CFR §184) (https://www.ecfr.gov/cgi-bin/text-idx?SID=0f2002a44dc3b3296966c07b0f4aadb6&mc=true&node=pt21.3.184&rgn=div5), while dehydroacetic acid is approved for direct addition to certain foods (21 CFR §172) (https://www.ecfr.gov/cgi-bin/text-idx?SID=0f2002a44dc3b3296966c07b0f4aadb6&mc=true&node=pt21.3.172&rgn=div5). for pH control in foods, or as a pickling and brining agent. With respect to meat, poultry, and egg product manufacture, the U.S. Department of Agriculture (USDA) Food Safety and Inspection Service approves the use of multiple commercial products containing acetic acid or diacetate for pH control during meat animal harvest, in further product manufacture, or as antimicrobial agents for application onto or in formulations of various further processed meat products (e.g., fully cooked sausages) (24). Doores (8) summarized research that indicated that fungal microbes, including *Saccharomyces* and *Aspergillus*, were inhibited at higher concentrations than were the Gram-positive bacteria *Bacillus*, *Clostridium*, and *Staphylococcus*, as well as indicating that multiple Gram-negative bacteria were also susceptible to the acid. Geng et al. (25) recently reviewed the literature discussing the gene expression-mitigated tolerance of *Saccharomyces cerevisiae* to acetic acid, indicating over 300 genes encoding proteins participating in the organism's tolerance responses to the antimicrobial, including cell wall reconstruction, acetate efflux for detoxification, and metabolism of acetate to acetyl coenzyme A, as well as pH and proton efflux. However, Svoboda et al. (26) reported the inability to reduce yeast and mold counts on

melons following application of a surface sanitizer containing 0.78% acetic acid, peracetic acid, and hydrogen peroxide.

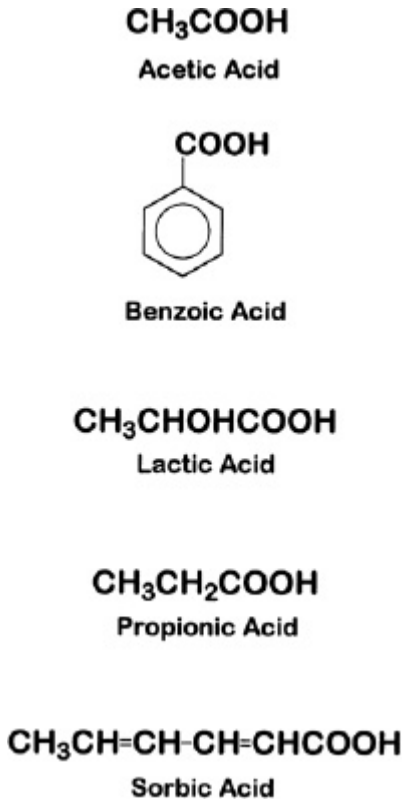


Figure 27.1 Organic acids used as antimicrobial food preservatives.

Bacteria sensitive to acetic acid application include both Gram-positive and -negative microbes (including pathogenic organisms), specifically *Listeria monocytogenes*, *Shigella flexneri*, *Salmonella enterica*, *Staphylococcus aureus*, *E. coli* O157:H7, and Shiga toxin-producing *E. coli* (STEC) (27). Sullivan et al. (28) likewise reported no differences in the inactivation of *L. monocytogenes*, *S. enterica*, and *E. coli* O157:H7 in egg pickling brine formulated to contain 2% acetic acid at pH 4.0 over 10 days' storage, though the Gram-positive *S. aureus* was still detectable after 5 days, whereas other pathogens were reduced to nondetectable levels. Similar results were obtained where *S. enterica*, *L. monocytogenes*, *E. coli* O157:H7, and *S. aureus* were reduced by 4.6 to 5.0 $\log_{10}$ CFU/g following 24 h of brining in a pickle solution containing 1.0 M acetic acid, 0.9 M NaCl, and 5 mM benzoate (29).

Bacterial pathogens have been reported to tolerate the application of nonlethal concentrations of acetic acid and the acetates by multiple mechanisms, including general stress response systems (e.g., *rpoS*) as well as various chaperone-type proteins and other acid tolerance response systems. Burin et al. (30) reported from a study of 79 isolates of *Salmonella enterica* belonging to multiple serotypes that *rpoS* was activated following 24 h of acid stress (4% [vol/vol] acetic acid, pH 6.0 and 5.0), but no stress response operon expression was detected in cells challenged with the acid adjusted to pH 4.0, presumably due to inactivation. These authors also reported that *Salmonella* cells were better able to withstand equivalent pH and concentration of lactic acid challenge, presumably due to a higher relative content of dissociated lactate versus acetate based on different pK_as of the acidulants and relative concentrations of dissociated and undissociated acids at the lowest experimental pH. Similar trends were also observed for *L. monocytogenes* isolates challenged with multiple organic acid and acid anion salts at pH 5.0 to 8.0, where acetic acid exhibited a lower MIC than did lactic, citric, malic, or propionic acid or salts of benzoate and sorbate at pH 5.5 (31).

Similar to *Salmonella* and other Gram-negative enteric pathogens, *L. monocytogenes* possesses the capacity for acid tolerance, possessing multiple systems for activating sublethal acid tolerance and cross-protection against other environmental stressors following acid shock (32, 33). Nonetheless, genetically encoded antimicrobial resistance systems and multidrug resistance systems identified for this pathogen, like others, do not appear to confer protective effects against food preservatives, including acetic acid and/or the acetates at concentrations sufficient to produce inhibition (34). *Campylobacter jejuni* possesses multiple stress-responsive systems, including acetic acid stress response and tolerance, though it has been reported to possess systems other than RpoS (35, 36).

Recently published research detailing the utilization of acetic acid or the acetates has focused on exploring novel methods of

antimicrobial delivery to the food system, as well as determining the capability of multipreservative and multihurdle processing technologies to produce microbial inhibition and control in various foods. Gaseous acetic acid application between 0.3 to 4.7 mM to surfaces of fenugreek seeds reduced *Salmonella enterica* serovar Enteritidis counts by 3.3 to 4.1 $\log_{10}$ CFU/g, and reductions of 3.6 to 4.0 $\log_{10}$ CFU/g in numbers of the pathogen were similarly achieved on black peppercorns following gaseous acid application (37). Greater reductions were observed on identically treated samples inoculated with *E. coli* O157:H7 following treatment, including a >5.0-$\log_{10}$-cycle reduction in *E. coli* O157:H7 on fenugreek seed and peppercorn surfaces treated with 0.6 or 4.7 mM gaseous acid for at least 3 h (37). Supplementation of 1% acetic acid with 8 mM zinc oxide nanoparticles produced 1.0- to 4.0-$\log_{10}$-cycle differences in numbers of *S. aureus*, *E. coli*, and *L. monocytogenes* organisms on fresh sheep meat surfaces following 12 days of refrigerated storage posttreatment, with no discoloration detected on treated meat for samples inoculated with Gram-positive pathogens (38). Bae and Lee (39) reported antagonistic effects between organic acids, including acetic acid, and NaCl when the acid was combined with 3% NaCl in a pickle brine against *E. coli* O157:H7 and *Salmonella*, though a synergistic inhibition effect between acid and NaCl was observed against *L. monocytogenes*. These authors concluded that acid structure in combination with osmotic pressure impacted subsequent interaction of antimicrobials, replicating similar effects achieved in previous research, though the impacts of dual membranes in the Gram-negative versus the Gram-positive pathogens were not fully elucidated (39). Conversely, 0.5 mg/ml acetic acid in combination with 5,000 ppm of the anionic surfactant sodium dodecyl sulfate (SDS) produced reductions in *S.* Typhimurium on blueberry surfaces equivalent to that produced by 200 ppm free chlorine, without significant changes to berry anthocyanin content or firmness (40).

Apart from acetic acid, the acetates and diacetates possess utility as food preservatives and continue to be incorporated into research

determining the efficacy of antimicrobial technologies for food safety and quality preservation. In comparing the inhibition of multiple Gram-positive and -negative bacterial pathogens, 3% acetic acid and potassium acetate subjected to electrolysis demonstrated equivalent reductions in *S. enterica* and *L. monocytogenes* populations, though the potassium acetate application produced significantly greater reductions in numbers of *S. aureus* during processing than protonated acid (41). Recent research has focused on the utilization of pH-buffered acetic acid-containing vinegar antimicrobial interventions for pathogen inhibition in fully cooked cured and uncured meat and poultry products. McDonnell et al. (42) reported that the addition of 2% buffered vinegar or 3% cultured sugar vinegar products to uncured sliced roast beef prevented *L. monocytogenes* growth over 12 weeks' storage at 4°C, though growth of the pathogen was observed for alternatively cured hams and turkey breast products similarly inoculated and formulated with antimicrobials. Similar inhibition of *L. monocytogenes* inoculated onto uncured ready-to-eat (RTE) turkey breast was reported with application of potassium and sodium-buffered vinegar antimicrobial solutions during 9 weeks of refrigerated storage, with a <1.0-$\log_{10}$ increase in pathogen loads detected (43). Nonetheless, outgrowth of *Clostridium perfringens* spores was inhibited in naturally cured (50 ppm ingoing NO_2) turkey breasts formulated with various clean-label antimicrobial products, including dry vinegar and lemon-vinegar blend products, although a fruit extract product appeared to be superior to acetate-containing products for spore germination prevention (44). Pork bologna products formulated with 0.0625, 0.1875, or 0.25% sodium diacetate and sodium lactate and surface treated with the bacteriocin pediocin demonstrated reduced *D* values at cooking temperatures for *L. monocytogenes*, indicating reduced survival of the pathogen (45).

Diacetates, similar to acetic acid and the acetates, have been explored in recent years for antimicrobial utility via multihurdle applications in animal-and plant-derived foods. Ahn et al. (46) summarized the research investigating the combination of

irradiation (ionizing) with antimicrobials for manufacture of RTE poultry products, identifying the combination of sodium diacetate with irradiation processing as effective for *L. monocytogenes* inhibition during postprocessing storage. More recently, incorporation of 5,000 ppm sodium diacetate into calcium alginate films along with plant-derived antimicrobial essential oil and the antifungal natamycin produced synergistic inhibition of *L. monocytogenes* on broccoli, enhanced by the application of 0.8 kGy gamma irradiation (47).

Benzoic Acid and the Benzoates

Chipley (48), citing Jay (49), identified sodium benzoate as the first federally approved food preservative in the United States. Currently in the United States, the acid species and the benzoates are identified as GRAS and approved for use at up to 0.1% in foods (21 CFR §184). Benzoic acid has a pK_a of 4.19 and is very poorly water soluble, while the sodium salt possesses significantly greater water solubility (66% [wt/vol]). Benzoic acid naturally occurs in cranberries, plums and prunes, cinnamon, and cloves, as well as many other berries (50). Koczoń (14) reported that while benzoate salt pK_a alone did not fully describe the antimicrobial activity against the yeast *Pichia anomala*, efflux mechanisms were activated upon benzoate application, resulting in large amounts of high-energy molecules being expended to combat cellular acidification, negatively impacting energy management within the cell. Critzer et al. and Creamer et al. (51, 52) also described multiple genetically encoded mechanisms for benzoate tolerance in bacterial pathogens including *E. coli* O157:H7 and *L. monocytogenes*. Nonetheless, recently reported data indicate that the pathogen surrogate *E. coli* K-12 can lose antibiotic resistance capacity through sacrifice of inducible multiple antimicrobial resistance (*mar*) operon elements following adaptation to benzoic acid (20 mM) stress over the course of multiple generations of the cell line (53).

Currently in the United States, the benzoates are primarily employed as antifungal agents during processing of foods, though research has also reported antibacterial effects. As cited by Davidson et al. (50), Chipley (48) identified benzoic acid as inhibitory towards yeasts at concentrations ranging from 20 to 700 μg/ml, with higher concentrations required for mold inhibition (20 to 2,000 μg/ml). In recent years the benzoates and benzoic acid have been repeatedly tested for foodborne microbe inhibition efficacy in combination with other processing interventions. Er et al. (54) reported that the minimum biofilm-inhibiting concentrations of sodium benzoate against poultry-recovered *Salmonella* capable of forming biofilms were 20- to >60-fold greater than the MICs of sodium benzoate and the *p*-hydroxybenzoate derivatives methyl- and propylparaben. Addition of 1,000 ppm sodium benzoate to orange and apple juices followed by application of ultrasound processing reduced the time to a 5.0-$\log_{10}$-cycle reduction in *E. coli* O157:H7 numbers by 22.4 and 35.6 min versus control juices, respectively (55). Sewlikar and D'Souza (56), however, reported no inhibition of the O157 and non-O157 STEC isolates by the application of 0.1% sodium benzoate (non-acidity controlled) or 0.1% pH 3.75 sodium benzoate versus controls at 25 or 37°C. These authors did detect reductions in STEC isolates of >6.0 $\log_{10}$ cycles following application of a commercial plant essential oil extract containing pH-adjusted (pH 3.75) benzoate antimicrobial product, indicating that the antimicrobial effects were primarily due to interaction of the acidulant and essential oil fractions of the antimicrobial product.

In addition to novel combinations of benzoate and other chemical and physical processing hurdles, innovations in delivery methods of benzoic acid and plant extracts containing benzoic acid were recently reported. Sagoo et al. (57) found that the coapplication of chitosan with benzoate enhanced the antifungal activity of the organic acid against spoilage yeasts. Recently, the encapsulation of benzoic acid and essential oils of spice plant extracts containing benzoic acid demonstrated similarly enhanced activity of the organic acid against foodborne bacterial and fungal microbes.

Khalili et al. (58) demonstrated inhibition of the mycotoxigenic mold *Aspergillus flavus* by the use of chitosan-benzoic acid nanogels, with and without encapsulated thyme essential oil, reporting that 1,500 mg/liter of nanogel is capable of fully inhibiting mold growth in medium. Encapsulation of cinnamon oil (0.05 or 0.1%) in chitosan nanoparticles produced significant bacteriostatic effects versus nonencapsulated essential oil or control-treated samples in beef patties during posttreatment refrigeration against *Enterobacteriaceae*, yeasts and molds, and *S. aureus* (59). Similar findings were obtained with beef cutlets treated with benzoic acid-chitosan nanogels entrapping rosemary essential oil against *S.* Typhimurium on fresh beef surfaces during 6 days of refrigerated storage posttreatment (60).

The benzoates, like other organic acids discussed herein, have been explored for their utility in limiting the shedding of human fecal pathogens in food animal production systems, allowing more comprehensive food safety management across the farm-to-fork continuum. Lynch et al. (61) reported that supplementation of a standard pig diet with benzoic acid did not result in significantly reduced *Salmonella* shedding versus controls but in a small subset did improve the recovery of *Salmonella* from animal lymph nodes (50% recovery from the treated group versus 77% recovery from the control group) following animal euthanasia and sample collection. Broom (62), reviewing the available literature on the use of organic acids in poultry production, noted similar findings indicating limited utility of benzoic acid in reducing pathogen shedding in turkeys and broiler chickens, though significantly less detection of the pathogen was mentioned. Benzoic acid, along with other antimicrobial organic acids, may exert multiple useful effects in modulating gut health of the livestock animal, resulting in reduced pathogen shedding and/or immuno-modulatory effects, leading to reductions in the risk of pathogen contamination during animal harvest and slaughter (62).

Lactic Acid and Lactates

Lactic acid possesses a pK_a of approximately 3.8 (90.08 g/mol) and occurs in both d- and l-isomers, though the l-isomer of the acid has been reported to be more effective than the d-form for inhibiting bacterial pathogens (63). Lactic acid is anaerobically fermented from glucose by the lactic acid bacteria through pyruvate via lactate dehydrogenase and the reducing equivalent NAD. Depending upon the application, lactates and lactic acid have been incorporated into food products as antimicrobials, antioxidants, acidulants, and/or flavorants, though they are described as having a clean flavor, not necessarily contributing significantly to product flavor at normal usage concentrations.

Similar to other organic acids, lactic acid and the lactates are reported to inhibit microbial growth by uncoupling of cellular metabolism regulation through cytoplasmic accumulation of acid molecules (64). Graves et al. (65) reported reduced ethanol production by fermentative yeast following 4% lactic acid exposure at multiple test pH values in a corn mash fermentation. Alakomi et al. (15) also described membrane permeabilization effects of lactic acid on Gram-negative bacterial membranes. Papadopoulos et al. (66) reported that lactate addition to processed meats discouraged lipid oxidation and product discoloration. More recent research has reported similar findings, where addition of 1.5% lactate to sausages resulted in reduced oxidation product formation compared to control sausages (67). The USDA Food Safety and Inspection Service currently approves the use of lactic acid to decontaminate meat animal carcasses and intact cuts up to 5% (application solution temperature may not exceed 55°C), a concentration previously reported to enhance the antimicrobial efficacy of lactic acid for pathogen reduction (68, 69). Additionally, lactic acid may be applied at up to 2.8% to beef heads and tongues, topically as a mixture with other organic acids to RTE meat products, to comminuted beef products, or as an antimicrobial fermentate-type product containing acetic acid and microbially fermented lactic acid for formulation of further processed meat and poultry products (24).

A great deal of research has explored the antimicrobial utility of lactic acid and the lactates for the manufacture of processed meat, poultry, and seafood items to prevent the growth of bacterial pathogens, including *L. monocytogenes*, *S. aureus*, *Salmonella*, and the clostridia (30, 70–73). As with other organic acid antimicrobial applications, recent research has sought to quantify the antimicrobial utility of the acidulant in combination with other antimicrobial hurdles. Mohan and Pohlman (74) applied organic acids with a nonionic surfactant to decontaminate beef trimmings and reported that the application of 30 g/liter lactic acid and surfactant did not outperform the application of lactic acid alone. Additionally, *E. coli* O157:H7 counts were not reduced by 30 g/liter lactic acid compared to controls (74). *E. coli* O157:H7 and non-O157 STEC were reduced by 1.2 and 1.6 $\log_{10}$ cycles on beefsteaks following application of 3 and 4% lactic acid to steak surfaces (75). Similar reductions were observed on pork surfaces following 4% lactic acid application (75). Verotoxin-producing *E. coli* was reduced by >3.0 $\log_{10}$ CFU/g on beefsteaks following application of 5% lactic acid and 0.8 to 0.9 kGy; similar reductions in *Salmonella* counts were also achieved (76). Aymerich et al. (77) likewise demonstrated synergistic inhibition of *L. monocytogenes* and *Salmonella* when applied in combination with high hydrostatic pressure. Finally, *L. monocytogenes* on carp fillets was prevented from growth by the application of thymol and 1 or 2% sodium lactate over 5 days at 4°C, while *S.* Typhimurium numbers declined on carp fillets following treatment (78).

García-Díez et al. (79) reported that the combined application of 3.3% sodium lactate and essential oils of oregano and garlic produced bacteriostatic effects on *Salmonella* and *L. monocytogenes* in frankfurters. Utilization of a cultured sugar-and-vinegar antimicrobial produced listeriostatic inhibition of *L. monocytogenes* in franks over 4 weeks of refrigerated storage, resulting in a 0.4-$\log_{10}$-cycle decline in pathogen numbers versus controls formulated with 1.68% lactate and nitrites (42). Combining 2.8% potassium lactate and 107 PFU/cm^2 anti-*L. monocytogenes*

phages produced a 4.0-$\log_{10}$-cycle reduction in pathogen numbers on sliced turkey held at 4°C after 28 days (80). Similarly, 0.75 to 3% sodium lactate and 10.5 or 21 g/kg lactic acid inhibited *L. monocytogenes* growth in queso fresco, resulting in a 3.0-$\log_{10}$-cycle reduction in pathogen growth versus treatments with caprylic acid, nitrite, and combinations of antimicrobials (81).

Sorbic Acid and the Sorbates

Sorbic acid and the sorbates are unsaturated small fatty acid and acid salts useful for the preservation of multiple types of human foods against fungal and bacterial microorganisms. Luck (82) and Stopforth et al. (83) provided reviews of the utilization of sorbic acid and its salts in the U.S. and European food industries. Stopforth et al. (83), citing previous works, indicated that sorbic acid was first recovered from the mountain ash tree, possesses a pK_a of 4.75, and, similar to benzoic acid, has much greater water solubility (32 to 50% depending upon sorbate salt species) as the dissociated acid anion than as the protonated weak acid (0.15 g/100 ml) (84, 85). A great deal of research has been published detailing the antimicrobial utility of the sorbates against foodborne microbes, bacterial and fungal alike, the concentrations and conditions of microbial inhibition in differing foods, and the potential mechanisms of microbial inhibition *in vitro* and in food product systems. Multiple fungal microbes, both yeasts and molds, are effectively inhibited by the sorbates, leading to their widespread utilization in various food products susceptible to fungal spoilage or mycotoxigenic mold growth and toxin secretion (83, 86–88). In the United States, sorbic acid and its calcium, potassium, and sodium salts bear GRAS status for use in foods (21 CFR §181 [https://www.ecfr.gov/cgi-bin/text-idx?SID=0f2002a44dc3b3296966c07b0f4aadb6&mc=true&node=pt21.3.181&rgn=div5] and §182 [https://www.ecfr.gov/cgi-bin/retrieveECFR?gp=&SID=0f2002a44dc3b3296966c07b0f4aadb6&mc=true&r=PART&n=pt21.3.182]). The World Health Organization's Codex

Alimentarius indicates that the sorbates are useful for inclusion in various products to maximal levels ranging from 200 to 3,000 mg/kg depending upon the food product and up to 10,000 mg/kg in edible casings used in various meat products (89).

In addition to the antifungal activity of the sorbates, antibacterial activity has been reported to occur against both Gram-positive and Gram-negative bacterial pathogens and food spoilers, including spore-forming bacteria. The sorbates have been repeatedly tested for their capacity to inhibit toxigenic clostridia, particularly *Clostridium botulinum*, and as preservatives in cured and uncured meat and poultry products, due to spore germination inhibition activity of the acids for both *Clostridium*- and *Bacillus*-produced spores (90–94). As short-chain fatty acids, sorbic acid and the sorbates may, in addition to their uncoupling-type mechanism of microbial inhibition, act on the membranes of targeted microbes in foods to enhance dissipation of transmembrane electrical potential, demonstrated previously in *Bacillus subtilis* cells (95–97). Lues and Theron (31) indicated that while at pH 5.5 the MICs of sorbate did not differ from those of other organic acids against *L. monocytogenes* strain CC77, as the pH of the test medium was increased to pH 7.0, the MIC of sorbate increased, ultimately requiring the greatest content of acidulant to inhibit *L. monocytogenes*.

Davidson et al. (50) described the sorbates as possessing useful inhibitory capacity against the clostridia and listeriae. The organic acid can prevent the germination of clostridial spores, a useful form of food safety and spoilage prevention for application in processed meats, cheeses, and other foods susceptible to these pathogens (92, 98–100). Ronning and Frank (90, 91) previously reported that sorbates dissipated or disrupted electrochemical potentials across cytoplasmic membranes in sporulating bacteria. As with other organic acid antimicrobials discussed herein, recent research has focused on the determination of sorbic acid and the sorbates' utility in food systems when combined with other preservatives and/or with different processing treatments. Combinations of salt, nisin,

and potassium sorbate at concentrations up to 4% at pH 5.5 and 7.0 yielded increasing inhibition and reduced predicted germination and growth of *Clostridium sporogenes* spores in nutrient broth as sorbate content was increased while other preservatives were held constant (101). Similar studies combining heat application (85°C, 10 min) and 3 mM sorbic acid significantly reduced the fraction of *B. subtilis* spores exhibiting germination and outgrowth versus no treatment and even 3 mM sorbic acid alone, declining from 83.3% of spores demonstrating germination and outgrowth down to 27.0% of spores demonstrating outgrowth (102). Non-spore-forming bacteria are also inhibited by sorbate application. Psychrotolerant *Lactobacillus sakei* organisms were inhibited by sorbic acid at 12.6 mM, a higher requirement for inhibition versus benzoic and citric acids (1.51 and 10.3 mM, respectively) (103). Use of sorbic acid in processed meat and fish products was described as not optimal for control of spoilage lactobacilli compared to benzoic acid or acetic acid, given the absence of maximal-use requirements for foods produced in European companies for sorbic acid versus acetic acid (103). Species of *Eurotium* and *Aspergillus* were inhibited by sorbic acid at pH 5.0 and an aw of 0.85 at levels of 0.05% (wt/wt), whereas MICs of sorbic acid at pH 6.0 and the same aw were between 0.1 and 0.3% (wt/wt), indicating the utility of food safety and spoilage prevention by hurdle processing (88).

Propionic Acid and the Propionates

Propionic acid, also identified as propanoic acid (CH_3CH_2 COOH; 74.08 g/mol), is a short-chain volatile fatty acid produced by multiple microbes residing in the human and animal guts (104–107). While the propionates are primarily applied as antifungal agents, particularly for mold inhibition, they also exert antibacterial effects against both Gram-positive and -negative spoilage and pathogenic genera (108–110). In the United States, the propionates are approved for use up to a concentration of 0.5% in RTE meat and poultry foods (24) and bear GRAS status (21 CFR §181 and §184). As with other organic acid antimicrobials, while its acid anionic salts

are more water soluble, propionic acid is the most potent antimicrobial form of the preservative (111).

Cronobacter spp. were inhibited by the application of sodium propionate in microbiological medium and in whole milk, where inoculated pathogen strains were inhibited in medium at 0.4% propionate. In milk samples, the addition of 0.1, 0.2, or 0.3% propionate reduced *Cronobacter* counts compared to controls, although the addition of the bacteriocin nisin or lacticin did not afford additional pathogen reductions compared to application of propionate alone (112). Application of mixed organic acids, including propionic acid, at 0.4 and 0.8% reduced *S.* Typhimurium to nondetectable levels 24 h after application on chicken skin; similar reductions in *E. coli* O157:H7 numbers were also achieved by exposure to acids (113). Likewise, reductions of *S.* Typhimurium and *E. coli* O157:H7 on celery surfaces achieved with application of propionate were enhanced by thermoultrasound treatment (114). The Gram-negative halophile *Vibrio harveyi* was inhibited by 0.05% propionic acid up to 100 h, in contrast to controls, at ambient temperature, while application of 0.06% acid completely inhibited organism growth up to 300 h of sample incubation (115).

L. monocytogenes isolates were reportedly inhibited by propionic acid in a pH- and strain-dependent manner, with MICs increasing 16- to 64-fold as the system pH was increased from pH 5.0 to 8.0 (31). Glass et al. (116) reported that propionic acid addition to the formulations of a cured deli turkey product at levels of 0.3 to 0.5% produced inhibition of *L. monocytogenes* growth to <1.0 $\log_{10}$ cycle on product stored at 4°C for 9 weeks, though the data did not indicate differences between treatments with respect to pathogen counts during storage. Other researchers indicated that the blending of levulinate and propionate into an uncured turkey deli product significantly outperformed propionate alone with respect to *L. monocytogenes* inhibition during refrigerated product storage (117).

Citric Acid and the Citrates

Citric acid (192.12 g/mol; $C_6H_8O_7$) and the citrates naturally occur in multiple sources, including food plants; calcium, iron (Fe^{3+}), and sodium citrates are considered GRAS alongside citric acid (21 CFR §184). Research has indicated that inhibition of Gram-positive and -negative pathogens occurs in microbiological media and in food products such as meat and fish at relatively low concentrations, in concentration- and pH-dependent manners (118–122). In a recent study of the impacts of feed additives on pathogen shedding in food animals, *Salmonella* shedding 2 days after treatment in pigs fed a citric acid-containing feed was significantly lower than that in control pigs, indicating that the acid could be safely fed and reduce pathogen fecal load, a risk factor for subsequent carcass and meat contamination (61). Kim et al. (123) demonstrated that citric acid application to sodium chloride solutions to produce acidified sodium chlorite functioned effectively to inactivate multiple bacterial pathogens through the acid-driven formulation of antimicrobial chloride species. As has been reported for other organic acid antimicrobials, citric acid exhibits greater activity against bacterial pathogens at reduced system pH, likely due to increased protonation of the acid molecule (31).

Byelashov et al. (124) reported that citric acid in combination with tripolyphosphate and NaCl performed as well as acetic acid or lactic acid in combination with tripolyphosphate with respect to inhibiting *E. coli* O157:H7 in beef knuckle following inoculation and refrigeration. Nevertheless, others reported minimal reductions (~1.0 $\log_{10}$ cycle) in STEC (O157 and non-O157) counts following exposure to citric acid at pH 3.75 (below the pK_a of citrate), indicating that acidity alone was insufficient to significantly reduce pathogen counts (56). Most recently, the application of 30 mM citrate to infant formula (pH 6.8), alone or in combination with the antimicrobials carvacrol and nisin A, produced reductions in *Cronobacter sakazakii* between 1.0 and 1.5 $\log_{10}$ CFU/ml (125). Similar experiments were conducted in apple juice (pH 3.2) and

showed that *C. sakazakii* was reduced to nondetectable levels after treatment with citric acid and nisin (125).

Given its occurrence in citrus fruits, citric acid has gained attention in recent years for its potential utility in decontaminating fresh produce. Sagong et al. (126) reported moderate sensitivity of *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* on lettuce leaves to the application of citric acid. In studies evaluating the dose-dependent impacts of the acid on the pathogens, reductions for allthree pathogens at the maximum applied concentration of acid (2.0%) were less than 2.0 $\log_{10}$ CFU/g. Coapplication of citric acid with power ultrasound treatment (40 kHz, 30 W) for 5 min led to reductions for all pathogens, with populations of *Salmonella* being reduced at 2% citric acid by 3.2 $\log_{10}$ CFU/g following acid and ultrasound application. Adler et al. (127) reported that the application of a lactic acid–citric acid blend (1%) or sodium hypochlorite acidified to pH 6.7 with citric acid to surfaces of jalapeno peppers reduced counts of aerobic bacteria by 1.1 to 1.3 $\log_{10}$ CFU/g. *Salmonella* counts, however, were not reduced to the same degree, with pathogen counts on inoculated peppers approximating 0.8 to 1.1 $\log_{10}$ CFU/g. Reductions in numbers of *Salmonella*, *E. coli* O157:H7, and *L. monocytogenes* ranged between 1.6 and 2.2 $\log_{10}$ cycles on lettuce leaves following application of a commercial citric acid preparation (128).

With respect to citric acid inhibition of foodborne molds, including mycotoxigenic molds, previous research indicated that the acid prevents toxin synthesis in the aspergilli (129). Recent research reported the ability of citric acid to reduce mycotoxin content in various foodstuffs. Five percent citric or lactic acid applied to feed experimentally inoculated with *Fusarium*-produced mycotoxins reduced deoxynivalenol by approximately half (130). The addition of 0.5 to 2.0% citric acid produced dose-dependent reduction in aflatoxin B1 in wet distiller's grains, producing a maximum reduction of 31%. Researchers compared citric acid to other decontamination strategies, reporting that while hypochlorite

outperformed citric acid in toxin reduction, it also produced undesirable bleaching of the feedstock (131). Likewise, application of lemon juice to whole pistachio kernels contaminated with 268 ng/g aflatoxin B1 followed by 4 days' storage and roasting at 120°C resulted in a 50% toxin reduction; application of lemon juice alone also effectively reduced toxin content by 23% (132).

Miller et al. (133, 134) reported that citric acid possesses enhanced buffering capacity and chelation activity versus other organic acids. Nielsen and Arneborg (135) reported that growth of *S. cerevisiae* and *Zygosaccharomyces bailii* in pH 3.0 citric acid reduced the maximum growth rate from 0.14 to 0.05 h^{-1}, though both organisms were inhibited to a greater extent in citric acid solutions adjusted to pH 4.0 and 4.5. Biomass output and ethanol fermentation by the yeast decreased in citric acid solutions, with lower output of biomass at pH 4.0 and 4.5 versus pH 3.0. Application of citric acid to medium-suspended cells of *S. aureus* and *L. monocyte-genes* resulted in physical damage to cells, but the coapplication of nisin and citric acid increased the extent of damage to cells, as indicated by scanning electron microscopy and cytoplasmic constituent release analysis (136).

Miscellaneous Organic Acid and Fatty Acid Antimicrobials

In addition to the organic acids described above, a number of other weak organic acids have been explored for microbial inhibition in media and in differing foodstuffs. Malic acid in particular has enjoyed much attention in recent years, particularly for decontamination of fresh food systems from bacterial pathogens. Lues and Theron (31) reported pH-dependent MICs for *L. monocytogenes* isolates in medium following application of malic acid. Dipping of pathogen-inoculated lettuce in malic acid (2.0%) produced reductions ranging between 1.6 and 1.7 $\log_{10}$ CFU/g for *E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes* (126). Over et al. (137) described bacteriostatic effects of malic acid (37.5 mM, pH

3.3) application *in vitro* against *L. monocytogenes* and *S.* Typhimurium, with bactericidal impacts against pathogens when the acid concentration was increased to 75 mM. Kang and Kang (138) reported reductions in *Salmonella*, *E. coli* O157:H7, and *L. monocytogenes* on paprika following submersion in 2% malic acid solution to nondetectable levels within 5 min of exposure. Multi-$\log_{10}$-cycle reductions of enteric pathogens on leafy green produce items have been reported in experiments in which malic acid was applied at different concentrations (139, 140). Mohan and Pohlman (74) reported that application of 30 g/liter malic acid to surfaces of beef trimmings reduced coliforms and inoculated *E. coli* O157:H7 numbers by 2.2 and 2.3 $\log_{10}$ CFU/g.

Levulinic acid (4-oxopentanoic acid; 116.2 g/mol) has been repeatedly tested in combination with surfactants for decontamination of foods and food process surfaces from viral and bacterial microbes. Cannon et al. (141) reported that the combined application of 0.5% levulinic acid and SDS reduced numbers of human norovirus surrogates to nondetectable levels within 5 min of application. Coapplication of 0.5% levulinic acid and 0.05% SDS reduced *E. coli* O157:H7 by 7.4 $\log_{10}$ CFU/ml following 30 min of application time, whereas no reductions in numbers of *Cryptosporidium parvum* oocysts were reported following application of higher concentrations of levulinate (2 or 3%) in combination with SDS (142). A combination of acetic, lactic, and levulinic acids (2% each) reduced *S.* Typhimurium counts by >5.0 $\log_{10}$ cycles following treatment of cherry tomato skin (143). Similar reductions in *S.* Enteritidis on unsprouted seeds treated with carvacrol-containing, 50 mM levulinate-acidified nanoemulsions were also reported, though following seed germination, the pathogen count returned to numbers that were not different from those following seed inoculation pretreatment (144). Researchers have also reported the utility of levulinic acid plus SDS to reduce the numbers of bacterial pathogens contaminating food contact surfaces, in some instances reducing pathogen counts by >5.0 $\log_{10}$

cycles versus controls or treatments using only sterile water (145, 146).

Multiple medium-chain fatty acids have been determined to possess antimicrobial activity against Gramnegative and -positive bacterial microbes, as well as fungal microbes, when applied alone or in combination with other intervention technologies. Previous reports have indicated the inhibition of pathogens such as *L. monocytogenes* and *Listeria ivanovii*, as well as other members of *Listeria*, following application of the fatty acids caprylate and caproate (6, 147). Chang et al. (148) demonstrated the susceptibility of Gram-negative enteric bacterial pathogens to caprylic acid. Patel et al. (149) reported similar activity against *E. coli* O157:H7 and *Salmonella* on lettuce treated with 25 or 50 mM caprylic acid, where pathogen loads were reduced to nondetectable levels following treatment. As with other antimicrobials, recently published research has focused on the coapplication of fatty acids in combination with other antimicrobials for purposes of enhancing foodborne microbe inhibition. Kim and Rhee (150) reported that the combined application of 0.5 or 1.0 mM caprylic acid with multiple plant-derived essential oil components, including thymol, carvacrol, and *trans*-cinnamaldehyde, produced 6.0- to 7.0-$\log_{10}$-cycle reductions in counts of *E. coli* O157:H7, whereas a single antimicrobial application resulted in only a 0.5- to 1.0-$\log_{10}$-cycle reduction. These researchers suggested that the membrane degradation activity of the fatty acid antimicrobial permitted easier access to the cytoplasm of plant essential oil components for subsequent pathogen inactivation. Combinations of caprylic acid and sodium caprylate with other antimicrobials inhibited the growth of *L. monocytogenes* in microbiological medium adjusted to pH 5.5 or 7.4, requiring significantly less fatty acid in acidified medium versus neutral pH medium (151). Nonetheless, combinations of sodium caprylate with ε-poly-l-lysine and acidic calcium sulfate were reportedly capable of synergistic *L. monocytogenes* inhibition in acidified medium (151).

Fatty Acid Esters

Monolaurin and Monocaprylin

In addition to the fatty acid antimicrobials, monoesters of multiple fatty acids are known to exert antimicrobial activity against microbial pathogens and spoilage microbes. Of those studied, monolaurin and monocaprylin have each been repeatedly tested and characterized for antimicrobial activity. Monocaprylin and monolaurin were evaluated for inhibition of *C. sakazakii* and *Cronobacter malonaticus*; monocaprylin was more effective as an inhibitor, requiring 1.6 to 1.7 mg/ml for bacterial inhibition, in contrast to monolaurin, which required >5.0 mg/ml (152). Lower MICs of the monoester of laurate were reported for various molds, including *Aspergillus niger*, *Alternaria alternata*, and *Penicillium roqueforti*, where MICs ranged from 0.16 to 1.5 mg/ml and were reported to not be greatly influenced by test medium pH (153, 154). *S. aureus* growth was reportedly inhibited at a 10-fold-lower concentration *in vitro* by monolaurin, and monolaurin interacted additively with other antimicrobials for pathogen inhibition on meat surfaces (155).

Synergistic interactions were observed for combined carbonation (1 to 5 MPa, 30 min), heat (80°C), and monolaurin for the inhibition of *Bacillus* and *Geobacillus* spores. Synergistic inhibition of *L. monocytogenes* by a combination of monolaurin and essential oils of tarragon and spearmint in broth medium was reported, while the combination of pennyroyal essential oil (0.6%) and 400 ppm monolaurin produced a 1.45-$\log_{10}$-cycle reduction in pathogen counts in inoculated white cheese (156). Currently, monolaurin is applied via engineered delivery systems for pathogen inhibition on foods. Nanoparticle-constructed monolaurin inhibited *S. aureus* cells at 0.1 mg/ml, not very different from previously reported free-monolaurin-specific MICs for *S. aureus* (155, 157). Entrapment of the bacteriocin nisin (10 μg) in monolaurin nanoparticles suppressed *S. aureus* growth over 175 h at 37°C (157). Zein-derived films impregnated with essential oil of *Zataria multiflora* (2%) and monolaurin (1%) produced inhibition zones of 74 and 57 mm against *L. monocytogenes* and *E. coli* O157:H7 cells in agar diffusion

testing (158). On minced-beef surfaces, antimicrobial encapsulates produced a 1.0-$\log_{10}$-CFU/g reduction in *L. monocytogenes* versus untreated controls (158).

LAE

Lauric arginate ester (LAE; *N*α-lauroyl ethyl ester) is a cationic surfactant antimicrobial compound, demonstrating inhibition of both Gram-positive and Gram-negative bacteria, as well as some fungal organisms, particularly yeasts belonging to the genera *Saccharomyces* and *Zygosaccharomyces* (159–162). Recently completed research indicated a synergistic inhibition of bacterial pathogens by the combined application of LAE with plant-derived antimicrobial essential oil components or extracts (162, 163). An additive interaction was recently reported by researchers applying 400 mg/kg LAE with an antimicrobial intervention containing anti-*L. monocytogenes* bacteriophages, where reductions in pathogen numbers on inoculated chicken breasts remained at >1.0 $\log_{10}$ cycle throughout 72 h of refrigerated holding posttreatment (164). Recent reports of combination application of eugenol and LAE against bacterial spoilage organisms (Gram-positive and -negative) indicated that the simultaneous application of sublethal concentrations of antimicrobials functioned synergistically to produce 6.0- to 6.5-$\log_{10}$-cycle reductions, whereas the application of antimicrobials in sequence produced smaller reductions in bacterial numbers (165). Ruengvisesh et al. (166) reported that eugenol-loaded micelles produced from a commercial LAE-containing antimicrobial product exhibited minimum bactericidal concentrations of 0.063% LAE-containing surfactant for both *S.* Typhimurium and *E. coli* O157:H7, indicating that the essential oil component and surfactant antimicrobial work effectively together for pathogen destruction. Ma et al. (163) and Nubling et al. (167) both reported enhanced sensitivity of Gram-positive bacteria to LAE versus Gram-negative bacteria, as indicated by differences in MICs, though differences in MICs vary only 2- to 4-fold.

It is suggested that LAE functions as a cationic surfactant, destabilizing membrane components of targeted microbes (lipopolysaccharide and membrane phospholipids) and allowing respiration and metabolism disruption and cytoplasmic content leakage; LAE was also recently reported to complex with DNA to produce large aggregates, thereby preventing proper transcription (168, 169).

Similar to the results of the studies described above, LAE has shown great utility when paired with one or multiple antimicrobials, as the compound functions to sensitize microbial membranes to subsequent or simultaneous attack by other preservatives. Fisher et al. (170) reported that an antimicrobial beef-marinating solution containing 5% chitosan, 2% (each) acetic, lactic, and levulinic acids, and 4% LAE reduced *L. monocytogenes*, *Salmonella*, *E. coli* O157:H7, and non-O157 STEC isolates to nondetectable counts within 6 h at 4°C. Pathogens were nondetectable throughout 48 h of refrigerated marination. In addition to applications utilizing multiple antimicrobials applied together or sequentially to a food product (i.e., hurdle processing), recent work has demonstrated the usefulness of LAE for food surface disinfection as a component of antimicrobial films or coatings. Application of chitosan-attached LAE to *L. monocytogenes* on frozen and thawed shrimp reduced pathogen counts by over 4.0 $\log_{10}$ CFU/g versus untreated controls (171). Similar reductions against *Listeria innocua* on deli turkey meat surfaces were achieved by application of chitosan-based films loaded with both LAE (0.388 mg/cm^2) and nisin (486 IU/cm^2) (172). Combining LAE at the same content with chitosan film and then applying a steam-type flash pasteurization produced a 5.1-$\log_{10}$-CFU/cm^2 reduction in numbers of the microbe on meat surfaces, thus demonstrating the opportunity for postlethality treatment of exposed meat and food surfaces for surface decontamination (173).

DMDC and DEDC

Dimethyl dicarbonate (DMDC) (Fig. 27.2) and diethyl dicarbonate (DEDC) are clear, colorless compounds with low aqueous solubility (DMDC: 3.6% water solubility). Whereas DEDC is not currently approved for use in foods in the United States, DMDC is currently approved for use in various beverages (wines and carbonated beverages) as an antimicrobial agent at concentrations not exceeding 250 ppm (21 CFR §172). The antimicrobial is added primarily as an antimycotic for the inhibition of wild yeasts in wines and spoilage yeasts in other beverages, including members of *Saccharomyces*, *Brettanomyces*, *Zygosaccharomyces*, *Candida*, and other genera. Research has also shown significant antimicrobial activity of both compounds against bacterial pathogenic and spoilage microbes (172). Basaran-Akgul et al. (174) reported that the application of 125 or 250 ppm DMDC in apple juice prepared from different cultivars of apples reduced *E. coli* O157:H7 to nondetectable levels within 24 h of application. Similarly, DEDC application at 1% in solution reduced numbers of nonpathogenic *Yersinia pestis* in liquid whole eggs over 40 days' storage at 4°C (175).

Research into the antifungal activity of DMDC for the preservation of wine and beverage quality has continued in recent years, using multihurdle strategies for inhibition of wine spoilage and beverage spoilage yeasts. A level of 200 ppm was fungicidal for the wine spoilage yeasts *S. cerevisiae* and *Rhodotorula mucilaginosa*, producing a 4.0-$\log_{10}$-CFU/ml reduction in yeast counts (176). Zuehlke et al. (177) reviewed the utility of DMDC in wines for spoilage yeast control, indicating its current use as a processing aid due to its short active period in the wine. The exact nature of the antimicrobial mechanisms of DMDC and DEDC is not known, given their rapid disintegration in food systems, but both are thought to inhibit cells by protein attack (172, 178).

NATURAL ANTIMICROBIALS

Phytoantimicrobial Agents

Research on plant-based antimicrobials is gaining attention in recent years due to the preferences of health-conscious consumers who want natural ingredients and processes in food production. These compounds are well known for their antimicrobial activity, including antiviral, antimycotic, antiparasitic, insecticidal, antitoxigenic, and antibacterial properties (179–181). Phytocompounds can be used in various forms, including essential oils and their active components, plant extracts and powders, and spices and herbs. These are designated GRAS by the U.S. Food and Drug Administration (182) and are applied by the food industry as part of the multiple-hurdle approach to better maintain the freshness, sensory attributes, and nutritional quality of the processed food products. These compounds can add unique flavors to foods and are also known for their health benefits and medicinal values (180, 183).

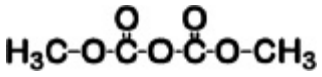


Figure 27.2 DMDC.

Since phytocompounds have multiple components responsible for antimicrobial activity, each compound could have multiple targets in a microbial cell. The exact mechanism of action for several phytocompounds is not yet clear. The mechanisms of action of some active components of essential oils are known. Cinnamaldehyde, the active component present in cinnamon oil and eugenol and the active component of clove bud oil, has been shown to interfere with the activity of enzymes. In *Enterobacter aerogenes*, cinnamaldehyde was found to interfere with the action of amino acid decarboxylases (184), and it also inhibited the separation of *Bacillus cereus* cells after cell division (185). Studies have shown that eugenol can inhibit ATPase, amylase, protease, and histidine decarboxylase (184, 186, 187). Carvacrol, the active component of oregano oil, is a lipophilic compound that can dissolve in the phospholipid bilayer of bacterial cell membrane and can cause

expansion and destabilization of the membrane, resulting in an increase in membrane fluidity and passive permeability (188, 189). Carvacrol was observed to cause leakage of phosphate ions from the treated cells of *S. aureus* and *Pseudomonas aeruginosa* (190). Carvacrol was also observed to cause disruption of the cell membrane and leakage of adenosine triphosphate in *E. coli* cells (191).

Plant essential oils and their active components have shown antimicrobial activity *in vitro* against microorganisms in numerous investigations. Carvacrol and cinnamaldehyde, at 0.1% or higher concentrations, demonstrated strong antimicrobial activity against both antibiotic-resistant and nonresistant *C. jejuni* strains (192) and *Salmonella* serotypes (193) in phosphate-buffered saline. Elgayyar et al. (9) evaluated the antimicrobial activity of numerous essential oils, including those from anise, angelica, basil, carrot, celery, cardamom, coriander, dill weed, fennel, oregano, parsley, and rosemary, against *L. monocytogenes*, *S. aureus*, *E. coli* O157:H7, *Yersinia enterocolitica*, *P. aeruginosa*, *Lactobacillus plantarum*, *A. niger*, *Geotrichum*, and *Rhodotorula*. Oregano, basil, and coriander essential oils showed strong inhibitory activity against both bacteria and fungi, while anise essential oil was effective only against the fungi (9). Lemongrass oil was effective against *Candida albicans*, *E. coli*, *S.* Typhimurium, *Serratia marcescens*, *S. aureus*, and other potential pathogens *in vitro* (194–197). Lemon, orange, and bergamot essential oils and their active components were found to be effective against *C. jejuni*, *E. coli* O157:H7, *L. monocytogenes*, *B. cereus*, and *S. aureus in vitro*, and among the compounds tested, bergamot, citral, and linalool showed the greatest activity (198).

The antimicrobial activity of plant extracts and plant powders *in vitro* against microorganisms has been the subject of research by many investigators. The survival of *E. coli* O157:H7, *S. enterica*, and *L. monocytogenes* in roselle (*Hibiscus sabdariffa*) calyx aqueous extracts or roselle leaf aqueous extracts over 72 h at 4, 8, and 25°C was investigated (199). No surviving *E. coli* O157:H7 or *Salmonella* organisms were detected in either of these extracts at 24 h at all

temperatures. *L. monocytogenes* populations were reduced by 5 and 3 log in the calyx and the leaf extract, respectively, at 24 h and at all temperatures, by 4 to 6 log at 4°C and 8°C, and to undetectable levels at 25°C, at 48 h (199). Roselle extracts were also effective against *S. aureus*, *Bacillus stearothermophilus*, *Micrococcus luteus*, *S. marcescens*, *C. sporogenes*, *E. coli*, *Klebsiella pneumoniae*, *B. cereus*, and *Pseudomonas fluorescens in vitro* (200). Olive leaf extracts exhibited antimicrobial activity against *P. aeruginosa* and *K. pneumoniae* as well as fungi including *C. albicans* and *Cryptococcus neoformans* (201). Olive pomace and olive juice freeze-dried extracts were effective against *E. coli* O157:H7, *S. enterica*, *L. monocytogenes*, and *S. aureus* (202). Commercial green tea leaf and rosemary powders were effective against *B. cereus*, *S. aureus*, and *L. monocytogenes in vitro* (203). Grapeseed extract was effective against *B. cereus*, *B. coagulans*, *B. subtilis*, *S. aureus*, *E. coli*, and *P. aeruginosa*; however, the concentrations required to inhibit Gram-negative bacteria (1,250 to 1,500 ppm) were higher than those required to inhibit Gram-positive bacteria (850 to 1,000 ppm) (204).

Plant antimicrobials can be applied in meat products as ingredients to improve the microbiological safety of these products. Roselle aqueous extract exhibited antimicrobial activity against *L. monocytogenes*, *E. coli* O157:H7, *S. aureus*, and *B. cereus* in ground beef (205). Grape-seed, pine bark, and rosemary extracts were added to raw ground beef at a concentration of 1%, and they brought about a 1-log reduction in populations of *S.* Typhimurium, *L. monocytogenes*, and *E. coli* O157:H7 after 9 days of storage (206). Oregano and cinnamon bark extracts were added to raw pork at a concentration of 4% as part of the curing juice and caused 2- and 3-log reductions in *S. enterica* populations, respectively, during storage at 20°C for up to 9 days (207). Garlic, ginger, and turmeric pastes inhibited the growth of multidrug-resistant *S.* Typhimurium DT104 in heat-treated ground beef stored at 4°C for 10 days (208). The antimicrobial effects of garlic, ginger, carrot, and turmeric pastes against *E. coli* O157:H7 were evaluated in ground beef;

commercial ginger paste demonstrated a 1- to 2-log-CFU/g reduction after 3 days of storage at 4°C (45). Cinnamon oil and olive extract were effective against multidrug-resistant *S.* Typhimurium in heated ground pork, resulting in 1.3- and 3-log-CFU/g reductions, respectively, after 7 days of storage at 4°C and these compounds were stable during cooking at 70°C for 5 min (209). Lemongrass oil inhibited the growth of foodborne pathogens in minced meat products (210). Carvacrol and cinnamaldehyde were effective against antibiotic-resistant *S.* Newport on oysters stored at 4°C for up to 3 days, exhibiting reductions of about 2 and 5 log CFU/g, respectively (193).

Plant antimicrobials have demonstrated dual benefits—antimicrobial and antioxidative activities—in grilled meats. Grilled ground beef patties are heated to reach an internal temperature of 71.1°C at the geometric center to inactivate *E. coli* O157:H7. The periphery of the patty can reach temperatures as high as 200°C, which can induce the formation of potentially carcinogenic compounds called heterocyclic amines. Epidemiological studies have shown a close correlation between consumption of well-done meats containing heterocyclic amines and incidence of cancer in humans (211, 212). Carvacrol simultaneously inactivated *E. coli* O157:H7 (2.5- to 5-log-CFU/g reduction) and reduced the formation of heterocyclic amines (58 to 78% reduction in the three major amines) in heated ground beef patties (213). Olive extract at 5% and lemongrass oil at 1% reduced *E. coli* O157:H7 population to below detection limits, and olive extract reduced the heterocyclic amines MeIQx and PhIP by 79.5% and 84.3%, respectively, in heated ground beef patties, while onion powder reduced PhIP by 94.3% (214). Olive and apple extracts, clove bud oil, and onion powder exhibited concentration-dependent effects against *E. coli* O157:H7 and the carcinogenic heterocyclic amines MeIQx and PhIP during grilling of ground beef patties (215).

Plant antimicrobials can be added to the wash water for washing produce postharvest, and this application may be particularly useful for organic produce, since growers of organic produce have limited

options. Oregano, lemongrass, and cinnamon essential oils were added to the wash water for organic leafy greens, including romaine and iceberg lettuces and mature and baby spinaches; concentration-, storage time-, and exposure time-dependent activities against antibiotic-resistant *S.* Newport were observed during storage at 4°C for 3 days (216–218). The antimicrobial activities of olive and apple powder extracts as well as hibiscus aqueous extract against *S.* Newport on organic iceberg lettuce, romaine lettuce, baby spinach, and mature spinach stored at 4°C for 3 days were concentration and storage time dependent, with olive, apple, and hibiscus extracts showing 2- to 3-log, 1- to 2-log, and 1-log reductions, respectively, while hydrogen peroxide (control) showed a 1-log reduction (219). On organic leafy greens (romaine lettuce, iceberg lettuce, and baby and mature spinaches), the concentration-dependent reductions in *S.* Newport populations by hibiscus tea, grape-seed, and green tea extracts ranged between 0.7 and 2.3 log CFU/g, 0.4 and 2.0 log CFU/g, and 0.2 and 1.6 log CFU/g, respectively (220). Carvacrol and cinnamaldehyde at 1% were effective against antibiotic-resistant *S.* Newport on celery (193). Cinnamaldehyde was effective against *E. coli* O157:H7 on baby spinach leaves (221). Carvacrol, cinnamaldehyde, and thymol exhibited antimicrobial activity against *B. cereus* strains in carrot broth (222).

Plant compounds can enhance product shelf life by inhibiting the growth of spoilage organisms. Basil essential oil was found to be effective against spoilage microorganisms such as *Aeromonas hydrophila* and *P. fluorescens* on lettuce (223). When applied to romaine lettuce, clove and tea tree essential oils demonstrated antimicrobial effects against various types of background microflora, including bacteria, yeasts, and molds (224). Cinnamon oil inhibited spore production and the hyphal growth of molds, including *Colletotrichum coccodes*, *Cladosporium herbarum*, *A. niger*, *Botrytis cinerea*, and *Rhizopus stolonifer* (225). Olive, apple, and hibiscus extracts were effective against background microflora of organic iceberg and romaine lettuce and baby and mature spinach, with reductions ranging from 0 to 2.8 log CFU/g (219).

A unique way to apply plant-based antimicrobials to food is by means of edible films (Fig. 27.3) that can be made from the pulp of a plant part, such as fruits, vegetables, leaves, or flowers. Edible films could help alleviate some of the adverse effects that plant antimicrobials may have on the sensory properties of the treated products. Antimicrobial edible films can be used as wrappings on meat and poultry products to improve the microbial safety and quality of these products. Edible films made of apple pulp containing three concentrations of carvacrol and cinnamaldehyde and used to wrap chicken breast with storage for 72 hours at 4 and 23°C and ham and bologna with storage at 4°C for up to 7 days were effective against *S.* Enteritidis, *E. coli* O157:H7, *C. jejuni*, and *L. monocytogenes* (226–228). Antimicrobial edible films can be added as ingredients in salad bags to inactivate foodborne pathogens. Apple-, carrot-, and hibiscus-based edible films containing carvacrol or cinnamaldehyde (Fig. 27.3) added to salad bags with organic leafy greens stored at 4°C for up to 7 days were effective against antibiotic-resistant *S.* Newport (229). When an alginate-based edible coating containing essential oils and their active components along with malic acid was applied on fresh-cut melons, the populations of *S.* Enteritidis were significantly reduced (230). Alginate-based films containing essential oils and their active components reduced the populations of *E. coli* O157:H7 on fresh-cut Fuji apples (231). Methylcellulose coatings containing chitosan applied to fresh-cut cantaloupe reduced the populations of mesophilic aerobes, psychrotrophs, lactic acid bacteria, and total coliforms by 3 to 4 log CFU/g (232). Plant antimicrobials have the potential to improve food safety, and commercial-scale trials will help the industry to determine the most effective applications of these healthful commodities in foods.

Natural Antimicrobials from Animal Sources

Lactoferrin and Lactoferricin

Lactoferrin is an iron-binding glycol-protein (molecular mass, 76.5 kDa) in milk and colostrum; it is also present in other physiological fluids and polymorphonuclear leukocytes (233). Lactoferrin is approved for application to beef in the United States (234). Lactoferrin exists in milk primarily as a tetramer with Ca^{2+} and possesses two iron-binding sites per molecule. For each molecule of Fe^{3+} bound by the enzyme, one bicarbonate (HCO^{-}) is required. Lactoferrin must be low in iron saturation and bicarbonate must be present for the protein to exert antimicrobial activity (132, 235). The exact biological role of lactoferrin is unknown; however, it may act as a barrier to infection of the nonlactating mammary gland and aid in protecting the newborn against gastrointestinal infection (236, 237). The lactoferrins exhibit antimicrobial activity through iron sequestration, modification of microorganism motility, disruption of biofilms, modification of virulence factors, and due destabilization of microorganism cell membrane (238). Lactoferrin can inhibit numerous foodborne microorganisms, including *B. subtilis*, *B. stearothermophilus*, *Carnobacterium viridans*, *L. monocytogenes*, micrococci, *E. coli*, and *Klebsiella* species (235, 239–241).

Payne et al. (242) found that bovine lactoferrin had to be reduced to 18% iron saturation to have bacteriostatic activity against four strains of *L. monocytogenes* and an *E. coli* strain at concentrations of 15 to 30 μg/ml in ultrahigh-temperature-treated (UHT) milk. At 2.5 mg/ml, it exhibited no activity against *S.* Typhimurium or *P. fluorescens* and little activity against *E. coli* O157: H7 or *L. monocytogenes* (243). Branen and Davidson (244) studied the interactive antimicrobial effects of combinations of lactoferrin with monolaurin, nisin, or lysozyme against *L. monocytogenes*, *E. coli*, *S.* Enteritidis, and *P. fluorescens* in culture medium and fluid milk. In culture medium, lactoferrin combined with nisin inhibited *L. monocytogenes* and lactoferrin combined with monolaurin inhibited *E. coli* O157:H7. None of the antimicrobial combinations with lactoferrin were effective in UHT milk incubated at 25°C; this

was attributed to interactions of the antimicrobials with food components and to the storage temperature (244).



Figure 27.3 Apple-, carrot-, and hibiscus-based antimicrobial edible films containing carvacrol and cinnamaldehyde that can be used as wrappings for meat products or added as ingredients in salad bags.

Lactoferricin B (i.e., hydrolyzed lactoferrin [HLF]) is a small polypeptide (25 residues) obtained from pepsin digestion of lactoferrin (245). Unlike bovine lactoferrin, lactoferricin is inhibitory to multiple genera of the lactic acid bacteria, including *Lactobacillus* and *Pediococcus* (246). Incubation of *S. aureus*, *E. coli* O157:H7, and enteropathogenic *E. coli* in 20 to 40 μM HLF resulted in only 10 to 15% growth of targeted microbes compared with untreated controls (247). Jones et al. (248) determined that HLF at concentrations of 1.9 to 125 μg/ml has bacteriostatic and/or bactericidal activity against *Shigella*, *Salmonella*, *Y. enterocolitica*, *E. coli* O157:H7, *S. aureus*, *L. monocytogenes*, and *Candida*. These

findings were recently confirmed and reviewed (249, 250). The addition of the chelator EDTA enhances the activity of HLF in liquid medium, indicating that decreases in the antimicrobial activity of HLF may be in part due to excess cations (251). Venkitanarayanan et al. (241) found that adding HLF to ground beef at 100 μg/g reduced *E. coli* O157:H7 populations by ~1.0 $\log_{10}$ CFU/g after 5 days at 4°C, whereas the pathogen populations in non-HLF-treated beef decreased by only 0.5 $\log_{10}$ CFU/g. In minimal culture medium, however, a decrease in *E. coli* O157:H7 populations of nearly 2.0 $\log_{10}$ CFU/ml was observed compared with medium containing no HLF (241).

Lactoperoxidase

Lactoperoxidase is an oxidoreductase in milk which plays an important role in protecting the lactating mammary gland and the intestinal tracts of newborn infants against pathogenic microbes. Lactoperoxidase is inhibitory towards both Gram-negative and Gram-positive bacteria (252). Lactoperoxidase contributes to the keeping quality of raw and pasteurized milk (253, 254). The combination of pasteurization at 60°C for 15 s with naturally occurring lactoperoxidase in goat milk inhibited the growth of *E. coli* O157:H7 for 6 hours postprocessing, resulting in a reduction in pathogen populations of 0.7 to 1.2 $\log_{10}$ CFU/ml compared with goat milk samples receiving the heat treatment but no antimicrobial (255). Adding the lactoperoxidase system (lactoperoxidase, potassium thiocyanate [KSCN], glucose oxidase, and glucose) to skim milk inoculated with *L. monocytogenes* and held at 25°C resulted in a 50-h lag phase, compared with an ~3-h lag phase in untreated control milk (256). The lactoperoxidase system also can inhibit growth of *S. aureus*, *L. monocytogenes*, *E. coli* O157:H7, *Salmonella*, and *Y. enterocolitica* in products such as beef, vegetable juices, milk, and liquid whole eggs (257, 258). For this reason, the lactoperoxidase system has been suggested as a preservative in foods. It has been applied in combination with alginate coating on chicken breast fillets during cold storage (259).

Lysozyme

Lysozyme is a powerful antimicrobial enzyme found in egg white, milk, and blood. It is classified as GRAS (234). It has been reported to act efficiently against food spoilage microorganisms. Its main target is Gram-positive bacteria, as it attacks the peptidoglycan in the cell wall (260, 261). Lysozyme is the predominant protein in egg albumen, being present at approximately 3.5% (262). Hen egg white lysozyme is the primary antimicrobial substance in albumen, although its activity is enhanced by ovotransferrin, ovomucoid, and alkaline pH (~9.3). Lysozyme possesses greater stability to heat at acidic pH (30 min at 80°C), but its thermal stability is reduced as pH is increased, an interesting phenomenon given the natural alkalinization of eggs during their storage after laying. The enzyme is most active from 55 to 60°C but maintains up to 50% of its activity at lower temperatures (10 to 25°C) (262). Lysozyme catalyzes hydrolysis of the α-1,4-glycosidic bond between C-1 of *N*-acetylmuramic acid and C-4 of *N*-acetylglucosamine, resulting in cell wall degradation and lysis in hypotonic systems.

The efficacy of lysozyme in inhibiting spoilage and pathogenic microbes on fresh and processed meat has been studied by several researchers. A combination of 250 or 500 ppm lysozyme, 250 or 500 ppm nisin, and 5 mM EDTA suppressed aerobic bacterial mesophile growth on ground ostrich patty surfaces during 7 days of aerobic or vacuum storage at 4°C (263). Applying 0.5 U of lysozyme/cm^2 on surfaces of turkey bologna followed by in-package pasteurization (65°C, 32 s) reduced *L. monocytogenes* to below detectable limits within 10 weeks of refrigerated vacuum-packaged storage; a combination of lysozyme and nisin inactivated the pathogen more rapidly, reducing *Listeria* populations to below detection limits after only 2 weeks of storage at 4°C (264). Conversely, adding lysozyme (100 or 200 μg/ml), EDTA (1.0 to 2.5 mg/ml), or a combination of these antimicrobials to UHT milk did not significantly inhibit *S.* Typhimurium or *P. fluorescens* but did exert a significant inhibitory effect on *L. monocytogenes* (243).

Likewise, growth of heat-shocked *L. monocytogenes* cells was significantly inhibited by 100 mg of lysozyme/ml of demineralized fluid milk, although fortification with divalent cations removed the observed inhibition (265). Lysozyme is approved for use in cheeses, frankfurter casings, and cooked meat and poultry products. It has been applied against *L. monocytogenes* Scott A in both plant and animal foods (266).

Ovotransferrin

Ovotransferrin is major iron chelating protein of egg (267). It is equivalent to lactoferrin in milk, as it has approximately 49% sequence homology with lactoferrin (268). There are two iron-binding sites, and anions such as bicarbonate or carbonate are bound alongside iron. Antimicrobial efficacy must be preceded by iron deficiency and alkaline pH (269). Ovotransferrin antimicrobial peptide (OTAP-92) kills bacteria through a membrane damage mechanism (270). For this reason, Gram-positive bacteria are generally more sensitive to its activity than Gram-negative bacteria. *Micrococcus* and *Bacillus* species are especially susceptible, as are some yeasts (268, 271). Recent research has sought to enhance the antimicrobial efficacy of ovotransferrin by applying it in foods with other antimicrobials. Ko et al. determined that 20 mg of ovotransferrin per ml in brain heart infusion broth completely inhibited the growth of *E. coli* O157:H7, as did ovotransferrin combined with 2 to 2.5 mg EDTA per ml (272). Ovotransferrin is also applied in K-carrageenanbased edible coating for its antimicrobial effect on fresh chicken breast stored at 5°C (273).

Avidin

Avidin is a tetrameric glycoprotein present in egg albumen. Its concentration varies with the hen's age but is approximately 0.05% of the total albumen protein (269). Avidin is stable to heat and a wide pH range. Avidin binds up to four molecules of biotin with high affinity. While its exact biological function is not known, it is thought that avidin is a nonspecific defense mechanism, inhibiting

function of biotinylated enzymes and thereby slowing the growth and spread of microbial contaminants (274, 275). Avidin is capable of inhibiting the growth of biotin-requiring yeast and bacteria (276). Applications of avidin in food models have not been reported. However, an avidin-biotin complex is used in a wide variety of bioanalytical applications (277).

Chitosan

Chitosan is the *N*-deacetylated derivative of chitin. Chitin is a mucopolysaccharide which is a natural component of fungal cell walls and the most abundant polysaccharide found in nature after cellulose (278). It is also a by-product of shellfish processing. Its derivative chitosan contains a series of polymers with different ratios of glucosamine and *N*-acetylglucosamine. Chitosan inhibits growth of foodborne fungi and bacteria (279–281) and is reported as nontoxic, biodegradable, and biocompatible by several researchers (282).

MICs for bacteria and yeasts vary widely depending on the molecular weight of the polymer, degree of acetylation, pH, temperature, and presence of interfering compounds, such as proteins and lipids (280). Nevertheless, when chitosan is combined with other antimicrobials, such as organic acids or antimicrobial polypeptides, the MIC of chitosan can be reduced. Zivanovic et al. (283) reported that chitosan dissolved in acetic acid was bactericidal to *S.* Typhimurium and *L. monocytogenes* strains, although the rate of inactivation was strain and concentration dependent. Combining chitosan with divergicin M35, a class IIA bacteriocin fermented by *Carnobacterium divergens* M35, halved the MICs of antimicrobials for *L. monocytogenes* (284).

Several studies have incorporated chitosans into edible films with antimicrobials for enhanced inhibition of microorganisms. Combining 2 mg of lysozyme with chitosan films significantly increased the size of the zones of inhibition against *E. coli* O157:H7 and *L. monocytogenes* and significantly reduced pathogen

populations in culture medium (285). Incorporating garlic essential oil, sorbic acid, or nisin into chitosan films significantly inhibited growth of *L. monocytogenes*, *Bacillus* spp., *Staphylococcus*, *Salmonella*, and *E. coli* (286). Park et al. (287) found that chitosan-based coatings containing potassium sorbate and applied to strawberries produced significant reductions in *Rhizopus* and *Cladosporium* populations compared with controls. Vandyousefi and Bhargava (288) applied an emulsion containing cinnamon oil and chitosan to shelled eggs to determine its effect on internal quality and shelf life. The study demonstrated that coatings with cinnamon oil-chitosan ratios of 9:91 and 12:88 could preserve the internal quality of eggs and prolong their shelf life under refrigeration conditions.

CONCLUSIONS

Chemical preservatives and natural food antimicrobials continue to be important tools for preserving food against microbiological spoilage and the growth of pathogens. The general trend is moving towards clean labels, with consumers demanding foods with fewer synthetic additives or foods with natural ingredients that are deemed more environmentally friendly. The natural antimicrobials will likely continue to grow in popularity. Additional research is needed to determine the levels of natural antimicrobials required for successful inhibition of foodborne pathogens, their mechanisms of action, and their safety. Novel applications of existing antimicrobials, including encapsulation, incorporation into edible polymers, and use of combinations of antimicrobials capable of synergistic inhibition of foodborne microorganisms, are being investigated. Major challenges include demonstrating the efficacy of antimicrobial compounds in food products at concentrations that do not have adverse sensory effects, as well as controlling the cost of these interventions.

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Michael Leonidas Chikindas

28
Biological Control of Food-Challenging Microorganisms

Before we dive into the intriguing and challenging world of biological (i.e., natural) preservatives, it is important to first understand why these antimicrobials are emerging as a novel approach for the control of undesired microorganisms in food products.

Over the course of the evolution of the food industry, food preservation has evolved from simple methods such as drying, salting, and using sugars and/or natural acidification to more sophisticated technologies. It is not in the scope of this chapter to discuss the terminology and legal classification of food preservatives, as they are defined by various regulatory agencies. However, the term "natural" has emerged as a way to distinguish substances of an unmodified origin (as determined by the FDA) in food formulation from "artificial" substances, i.e., those of a chemical or synthetic origin. That is why, according to the FDA Code of Federal Regulations Title 21 (revised as of 1 April 2017; https://www.ecfr.gov/cgi-bin/text-idx?SID=bb4e846ae19064cc1b87826409b86470&mc=true&node=se21.2.101_122&rgn=div8), "… common salt, sugars, vinegars, spices or oils extracted from spices…" are excluded from the list of chemical preservatives. In addition, FDA approval in 1988 for the use of nisin as a preservative in cheese spreads established a legal precedent for the further application of biological preservatives in food products (1). Therefore, naturally derived and unmodified proteinaceous antimicrobials (such as bacteriocins) and more complexly organized multimolecular structures (such as bacteriophages) belong to the group of natural preservatives, provided that they can control undesired microorganisms in food products. In this chapter, the terms "biological" and "natural" are used interchangeably.

It is also important to mention that the rapidly growing interest in biopreservatives is driven largely by our constantly increasing knowledge of possible negative health effects of some of the chemical preservatives and by the consumers' awareness of these negative effects.

BIOPRESERVATION WITH LIVE MICROORGANISMS

One way to categorize microorganisms is by the major metabolic products resulting from the utilization of various carbohydrates. A broad group of Gram-positive, non-spore-forming cocci or rod-shaped microorganisms producing lactic acid as the end product of glucose fermentation are defined as lactic acid bacteria (LAB). Some reports on LAB of importance for both beginners and fundamental and applied science investigators have been compiled by Wackett (2). LAB include several genera: *Lactococcus*, *Lactobacillus*, *Enterococcus*, *Streptococcus*, *Pediococcus*, *Carnobacterium*, *Leuconostoc*, *Oenococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella*. Numerous strains of microorganisms taxonomically related to the first six genera have been known for centuries to be safe and are commonly used as starter cultures in food fermentation. It is important to mention that while some strains are safe for humans and are utilized in a variety of food products as starter cultures, live preservatives, or both, there are plentiful reports on potential human pathogens which belong to the LAB (e.g., references 3 and 4). Strikingly, most reported cases of lactobacillus-related bacteremia (including cases caused by probiotic strains) are seen in the elderly or infants, age groups with either a declining or incompletely developed immune system. Therefore, safety is neither a genusnor species-specific feature—it is something that must be determined for each individual strain.

Many LAB produce one or a combination of several substances with antimicrobial properties, including lactic acid, hydrogen peroxide, bacteriocins, and diacetyl, with LAB-produced bacteriocins and diacetyl being more active against Gram-negative than Gram-positive pathogens (5). Moreover, a combination

of diacetyl with reuterin (a broad-spectrum antimicrobial produced by some isolates of *Lactobacillus reuteri*) can result in increased antimicrobial activity (6).

Weak organic acids have been recognized for their antimicrobial properties for more than a century and are used as antimicrobial agents in many food and personal care and hygiene products. Lactic acid produced by LAB during the fermentation of food directly contributes to the lowering of the pH, which becomes a significant factor in the control of food safety and quality. For example, a desirable acidification of the food product, provided by a fermenting LAB, is an effective replacement for nitrites in cured meat (reviewed in reference 7). The acidic pH of LAB-fermented food products is reported as inhibiting numerous foodborne pathogens, such as *Clostridium botulinum*, *Listeria monocytogenes*, *Escherichia coli*, etc. (for reviews, see references 8 and 9).

Bacteriocinogenic strains of LAB are capable of inhibiting the growth of foodborne pathogens and spoilage microorganisms, mostly Gram-positive species, thus increasing the safety and shelf life of food products (for example, see reference 10). When used as starter cultures or live preservatives, bacteriocinogenic microorganisms may be categorized as GRAS (generally recognized as safe), provided that they have a history of safety and use by the food industry prior to the 1958 Food Additives Amendment (as outlined in 21CFR170, FDA Code of Federal Regulations, Title 21, volume 3, revised 1 April 2017; https://www.ecfr.gov/cgi-bin/text-idx?SID=58ae784cf65acac9f530a3fbec916fbe&mc=true&node=pt21.3.170&rgn=div5). According to amendment 21CFR170, the bacteriocinogenic starter, the live preservative, or a (partially) purified bacteriocin may have the manufacturer's self-affirmed GRAS status or a GRAS status affirmed as a result of evaluation by an independent surveyor. However, in any of these cases, the FDA may request justification.

It is important to mention that while some strains of LAB may have GRAS status, it does not mean that a self-proclaimed or FDA-affirmed status may be expanded to the entire species or genus. Regrettably, this is a rather common mistake, observed in numerous publications.

The existing FDA regulations clearly state that natural antimicrobials used as food preservatives and live preservatives should have GRAS status affirmed for specific application(s). Additionally, the Food Safety and Inspection Service of the U.S. Department of Agriculture (USDA FSIS) evaluates and approves the efficiency and safety of novel food preservatives, including live, GRAS preservatives and their products. The use of novel bacteriocins requires an integrated review by both the FSIS and the FDA before any applications are possible (11, 12).

Some of the more common commercially available bacteriocin-producing food-grade microorganisms are listed in Table 28.1 (13). When a food preservation strategy utilizing live preservatives is being designed, numerous essential variables must be taken into consideration. These variables are determined by the food environment and include water activity, pH, temperature, atmospheric composition, fat content, presence of nutrients and inhibitors, other microorganisms, etc., and are comprehensively reviewed by Gálvez and coauthors (Fig. 28.1) (14).

BIOPRESERVATION WITH NATURALLY DERIVED ANTIMICROBIALS

Every living creature produces antimicrobial substances to protect itself from invading microorganisms. In order to present the entirety of biopreservatives as a cohesive picture, we have divided these antimicrobials into several groups, as depicted in Fig. 28.2, with some examples given for each subgroup. This figure is intended as an approximation to guide the reader through the chapter and is not meant to suggest an attempt at classification.

Nonproteinaceous Antimicrobials of Plant Origin: Challenges Are Many

In addition to the nonproteinaceous antimicrobials mentioned in Fig. 28.2, plants, like other eukaryotic organisms, produce antimicrobial proteins (AMPs). Plant-derived antimicrobials have been thoroughly reviewed by Cowan (15) and more recently by other investigators (e.g., reference 16), and there are also studies with some emphasis on plant antimicrobials of proteinaceous origin (17). Many

identified plant-derived crude extracts and purified substances have been studied and reported as safe and active against foodborne pathogens such as *L. monocytogenes*, even in the challenging food processing and formulated-food environments. For instance, *Psoraleae semen* L. (Bogolji) and *Sophorae radix* L. (Gosam) antimicrobial extracts still possessed their antilisterial activity after exposure to pH 4.0, NaCl concentrations up to 6%, and high-temperature treatment (80°C and 100°C for 10 min). Interestingly, these antimicrobial activities were observed in the water-insoluble fractions of the studied plant extracts (18).

Table 28.1 Examples of commercially available bacteriocin-producing food-grade microorganisms[a]

Product name	Microorganism(s)	Bacteriocin(s)	Manufacturer	Application
BioSafe	*Lactococcus lactis* subsp. *lactis* BS-10	Nisin A	Chr. Hansen	Cottage, feta, and ripened cheeses, prevention of late blowing and off flavors due to clostridia
HOLDBAC (formerly Bio Profit [Valio]; same species but different strains)	*Propionibacterium freudenreichii* subsp. *shermanii* DSM 706 and *Lactobacillus rhamnosus* DSM 7061	Undefined bacteriocins; see U.S. patent application US 20150150298 A1 (4 June 2015)	DuPont Nutrition Biosciences ApS	Inhibition of mold and psychrotrophs in cottage cheese
Bactoferm F-LC	*Pediococcus acidilactici*, *Lactobacillus curvatus*, and *Staphylococcus xylosus*	*L. curvatus* produces sakacin A, and *P. acidilactici* is likely to produce pediocin PA-1/AcH	Chr. Hansen	Control of *L. monocytogenes* and as a meat starter
ALCMix1	*Lactobacillus plantarum* and *Staphylococcus carnosus*	Produce plantaricin and carnocin bacteriocins, respectively	Danisco DuPont	Antilisterial cultures for fermented sausages and cooked ham
Bactoferm B-SF-43	*Leuconostoc carnosum*	Leucocin	Chr. Hansen	Control of listeria in vacuumand modified-atmospherestored meat products
Bactoferm B-2	*Lactobacillus sakei*	Sakacin	Chr. Hansen	Control of listeria in vacuumand modified-atmospherestored meat products
Bactoferm B-FM	*Staphylococcus xylosus* and *L. sakei*	Sakacin	Chr. Hansen	Control of listeria in vacuumand modified-atmosphere-fresh meat products

[a] Table is from reference 13.

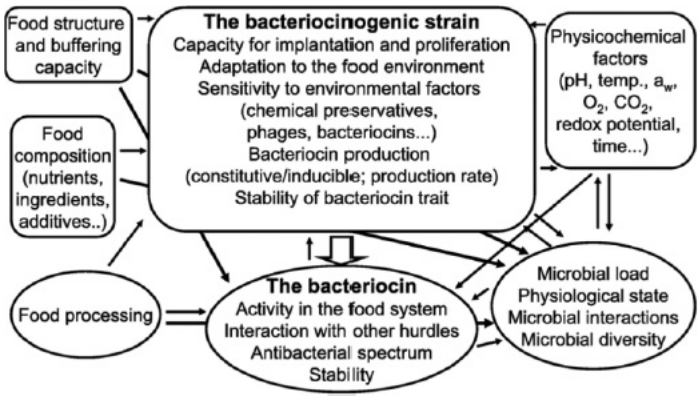


Figure 28.1 Environmental factors essential for biopreservation by bacteriocinogenic GRAS microorganisms. Image from reference 14.

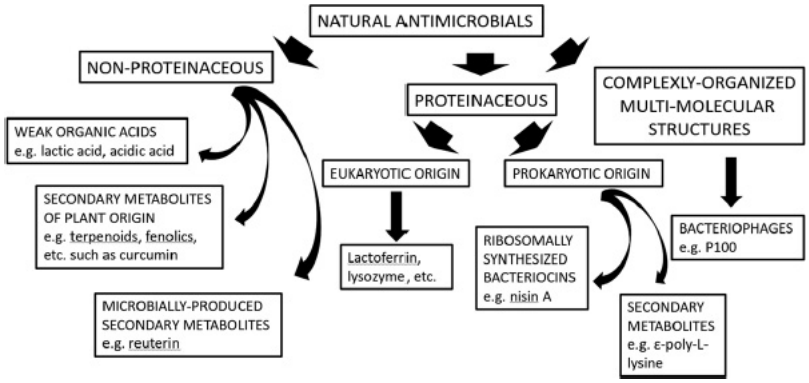


Figure 28.2 Natural antimicrobials of interest for food preservation.

Plant-derived nonproteinaceous antimicrobial substances are often described as having multiple mechanisms of action against targeted microorganisms. Additionally, one of the major challenges for food application of many of these substances (extracts) is their limited water solubility (19). This challenge has been addressed by many investigators with suggested approaches for micro- or nanoencapsulation of plant-derived antimicrobials. While microencapsulations may be useful in the preservation and enhancement of antimicrobial activity (20), nanoencapsulation seems to be more effective in delivering the substances' antimicrobial function (21). In fact, nanotechnology is currently under intense investigation for use in many food-related applications (22–24).

In their study (21), Topuz and coauthors utilized highpressure homogenization to produce a nanoemulsion of anise oil. This formulation delivered 2-log-better inhibition of the common foodborne pathogens *E. coli* O157:H7 and *L. monocytogenes* Scott A than pure anise oil. Additionally, nanoformulated anise oil produced 1-log-better inhibition of these pathogens than the coarse emulsion. In a different study, plant-derived terpenes and d-limonene were nanoencapsulated and evaluated for antimicrobial activity against three model microorganisms representing Gram-positive bacteria (*Lactobacillus delbrueckii*), Gram-negative bacteria (*E. coli*), and yeasts (*Saccharomyces cerevisiae*). The objective of this study was to effectively control a broad range of microorganisms in juices without significant negative impact on the food product's qualities (25). The formulations' MICs and minimum bactericidal concentrations (MBCs) were determined, and in allcases, the nanoencapsulated for In their study (21), Topuz and coauthors utilized highpressure homogenization to produce a nanoemulsion of anise oil. This formulation delivered 2-log-better inhibition of the common foodborne pathogens *E. coli* O157:H7 and *L. monocytogenes* Scott A than pure anise oil. Additionally, nanoformulated anise oil produced 1-log-better inhibition of these pathogens than the coarse emulsion. In a different study, plant-derived terpenes and d-limonene were nanoencapsulated and evaluated for antimicrobial activity against three model microorganisms representing Gram-

positive bacteria (*Lactobacillus delbrueckii*), Gram-negative bacteria (*E. coli*), and yeasts (*Saccharomyces cerevisiae*). The objective of this study was to effectively control a broad range of microorganisms in juices without significant negative impact on the food product's qualities (25). The formulations' MICs and minimum bactericidal concentrations (MBCs) were determined, and in allcases, the nanoencapsulated formulations were found to have MICs and MBCs lower than or similar to those of nonencapsulated preparations. Also, high-pressure-homogenized emulsions of the studied formulations at 10 g/liter effectively inhibited 10^3 CFU/ml of *L. delbrueckii* in orange and pear juices for 16 days at 32°C. It should be mentioned that while MICs of nanoformulated plant-derived antimicrobials such as essential oils are often reported as being 10 to 20 times lower than those of pure (nonencapsulated) substances, their performance in food product models is still far from being acceptable for any food application. In a recent study, chitosan-benzoic acid nano-gel-encapsulated *Rosmarinus officinalis* essential oils were evaluated for their ability to control *Salmonella enterica* serovar Typhimurium-challenged beef cutlets. After 12 days of incubation, 2 mg of nanoformulated essential oils per g of beef cutlet produced an approximately 2-log reduction of the pathogen compared to untreated meat, which is approximately 1 log better than the activity delivered by 100 mg of the nonformulated (pure) essential oil (26).

In addition to the above-mentioned challenges with the effectiveness of plant-derived antimicrobials (alone and nanoformulated) in food model systems, their safety should be seriously considered, especially because nano-formulations, while delivering better bioavailability for many substances, also raise concerns about potential health risks due to their ability to easily penetrate various biological barriers (27).

Biopreservatives of a Proteinaceous Nature

As depicted in Fig. 28.2, proteinaceous biopreservatives are a diverse group of substances of both prokaryotic and eukaryotic origins. The natural source and proteinaceous structure of these substances are why these biopreservatives are particularly favored by consumers concerned with the health and safety of food products. Indeed, proteins are normally subjected to digestion by the proteolytic system of the human gastrointestinal tract (28), and while some of them, such as lactoferrin and its derivative lactoferricin, can cause various responses in eukaryotic hosts, these effects are generally reported as positive (for example, see reference 29; also see the excellent review in reference 30). The proteinaceous food biopreservatives included in this chapter are presented in Table 28.2.

Table 28.2 Proteinaceous food biopreservatives examples

Name	Dosage (mg/kg)	Microbial targets	Food applications	Comments
Polylysine	100–1,000	Gram-positive and -negative bacteria, yeast, molds	Sliced fish, rice, noodles, cooked vegetables (Japan)	FDA GRAS notice GRN no. 135, with intended use as a antimicrobial agent in cooked rice and sushi rice at lev from 5 to 50 parts per million (https://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices&id=135; last accessed 24 January 2018)
Lactoferrin	1,000	Gram-positive and -negative bacteria	Infant formulas	FDA GRAS notice GRN no. 465, with intended use as a ingredient in infant formulas (https://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices&id=465; last accessed 24 January 2018)
Lysozyme	125–250	Gram-positive bacteria	Used in milk, wine and in casings for frankfurters	FDA GRAS notice GRN no. 191, with intended use as a ingredient in infant formulas (https://www.accessdata.fda.gov/scripts/fdcc/index.cf set=GRASNotices&id=191; last accessed 24 January 2018)
Nisin A	5–200	Gram-positive bacteria	Canned foods, dairy products, cooked meats, poultry	FDA GRAS notice GRN no. 65, with intended use on casings for frankfurters and on cooked meat and poult products as an antimicrobial agent (https://www.accessdata.fda.gov/scripts/fdcc/index.cf set=GRASNotices&id=65; last accessed 24 January 20

Polylysine: a Broad-Range Antimicrobial Polypeptide

ε-Poly-l-lysine (polylysine) is a secondary metabolite isolated from the soil microorganism *Streptomyces albulus* subsp. *lysinololymerus* strain 346 (31). It is composed of 25 to 35 l-lysine residues and has a net positive charge. In this molecule (Fig. 28.3), l-lysine residues are connected via isopeptide bonds between ε-amino and *R*-carboxyl groups (32). Polylysine is produced with the assistance of a membrane-associated nonribosomal peptide synthetase, and the producer cell is partially protected from polylysine's antimicrobial activity by an aminopeptidase, a product of the *pld* gene (33). Polylysine is particularly attractive as a food preservative due to its broad range of antimicrobial activity, covering Gram-positive and Gram-negative bacteria as well as yeast and molds (34). Equally compelling is its safety: in a rodent oral toxicity study, it caused no adverse effects or mortality when delivered at a dose of 5 g/kg of body weight (35).

The mechanism of antimicrobial action for polylysine has been studied and modeled using *E. coli* (36). Polylysine initially acts on the cellular membrane. The primary interaction between polylysine and L-glycero-D-mannoheptose of the membrane lipopolysaccharides is driven by electrostatic forces leading to the detergent-like removal of lipopolysaccharides. It is still unknown whether polylysine is active in periplasmic space or whether it simply transitions to the inner membrane. When the inner membrane is reached, a so-called "carpet-like interaction" (reviewed in reference 37) takes place in which polylysine interacts with the membrane's leaflets, gradually removing them and causing thinning of the inner membrane. Once inside the cell, polylysine has multiple mechanisms of action causing downregulation of the expression of virulence genes, as well as negative effects on the SOS response and the regulation of oxidative stress, etc. (38).

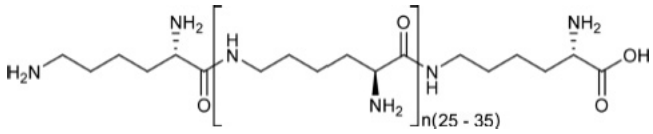


Figure 28.3 Chemical structure of polylysine.

Metabolomic analysis of polylysine-treated *S. cerevisiae* revealed several different, concentration-dependent mechanisms of antimicrobial action (39). At high levels, close to the MBC, polylysine caused multiple, clearly identifiable damages to the yeast membrane. When used at 40% of the MBC, polylysine did not cause the death of the yeast population; however, the yeast's life cycle was much slower than that of untreated cells. Finally, when used at 10% of the MBC, polylysine did not have any negative effects on yeast viability, but significant negative changes in cellular biochemistry and physiology (e.g., inhibition of glycolysis) were observed, most likely in relation to disruption of the membrane's integrity.

The antimicrobial activity of polylysine against the foodborne pathogens *Salmonella* Typhimurium, *L. monocytogenes*, and *E. coli* O157:H7 was evaluated in laboratory nutrient medium (40) and in several food models (41). In the latter experiments, tryptic soy broth was supplemented with 10% (wt/wt) of fat-free or whole milk or 10% (wt/wt) extracts of beef, bologna, rice, and vegetables. These experiments were designed as a preliminary evaluation of the possible effects of various food formulations on polylysine's antimicrobial activity. These experiments were conducted at 12°C, mimicking temperature-abused storage of refrigerated foods. Interestingly, after 6 days of incubation, 0.2% polylysine caused, on average, a 7-log reduction of the viable cell count of *E. coli* O157:H7 in food model systems and an approximately 2-log reduction of the pathogen when incubated in tryptic soy broth. Comparable results were achieved against *Salmonella* Typhimurium; however, these required 0.04% polylysine. An approximately 1-log reduction in the number of the pathogen's viable cells was observed when the cells were incubated in tryptic soy broth. Finally, similar results were obtained with *L. monocytogenes*: 0.02% polylysine was effective in delivering an approximately 8- to 9-log reduction of the pathogen in every food-mimicking system, while there was a 3-log reduction in the viable cells that had been grown for 6 days in the presence of the antimicrobial in broth alone. It is important to mention that the observed inhibition of allthree foodborne pathogens developed gradually over the 6-day storage period. This interesting study produced several conclusions, two of which are very important: (i) food composition may positively influence the preservative's activity and ability to control foodborne pathogens, and (ii) some preservatives may work well during extended storage time but should be intelligently formulated with other antimicrobials capable of more immediate action against foodborne pathogens.

Lactoferrin: a Milk-Derived Multifunctional Protein with Antimicrobial Activity

Eukaryotic iron-binding glycoproteins of approximately 80 kDa are found in various biological fluids, such as milk, saliva, vaginal secretions, tears, etc., and are known as lactoferrins (42). The amino acid sequence of bovine lactoferrin is 69% identical to that of lactoferrin of human origin, while porcine lactoferrin is 72.6% identical to bovine lactoferrin and shares 70.7% sequence identity with human lactoferrin (43).

Lactoferrins possess activity against a broad range of human pathogens, including Gram-negative (e.g., *E. coli* and *Salmonella*) and Gram-positive (e.g., *Streptococcus* spp., *L. monocytogenes*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*) bacteria and yeasts (e.g., *Candida albicans*). Several modes of action, most likely initiated during the antimicrobial's interaction with the cellular membrane, have been reported for lactoferrins against various microorganisms, including several expected interactions with iron, lytic activity, interference with adhesion, inhibition of multiple cellular functions, etc. (44). Lactoferrins are also active against enveloped and nonenveloped viruses (reviewed by Jenssen and Hancock [45]).

Food-grade lactoferrin is produced from fresh cow's milk or from by-products of dairy fermentation, such as milk whey. Some examples include the commercial products Bioferrin (Glanbia; Kilkenny,

Ireland), Vivinal (FrieslandCampina; Amersfoort, Netherlands), and lactoferrin from Ferrin-Tech (Phoenix, AZ), Synlait (Rakaia, New Zealand), and Fonterra Group (Auckland, New Zealand). Possible food applications of lactoferrin have been reviewed by Duran and Kahve (46).

An iron-free formulation (apo-lactoferrin) resisted pH 4.0 during a 5-minute treatment at 90 and 100°C (47). However, lactoferrin's activity is reduced by various factors, including divalent cations (Fe^{2+}, Mg^{2+}, and Ca^{2+}). These and other activity-inhibiting challenges have been addressed by Naidu, who immobilized lactoferrin on food-grade polysaccharides (such as carrageenan) (48). The resulting patented technology produced highly active lactoferrin capable of inhibiting various foodborne pathogens, including *E. coli* O157:H7, even on meat surfaces and in iron-rich environments. This technology has received FDA-affirmed GRAS status for use as an antimicrobial spray (processing aid) on beef carcasses (FDA GRAS notice GRN no. 130; https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=130).

Interestingly, while noting the structural instability of lactoferrin treated for 5 min at pH 3.0 and 100°C, Abe and coauthors observed increased antimicrobial activity (47). Lactoferrin-derived peptides with increased antimicrobial activity can also result from proteolytic digestion (49). It is important to mention that proteolytic digestion of bovine lactoferrin can produce a diverse group of peptides with antimicrobial activity: LF1-11 (the N-terminal part of lactoferrin), lactoferrampin (lactoferrin residues 268 to 284), and lactoferricin (lactoferrin residues 17 to 41). These derivatives have been reviewed by Sinha et al. (50). Remarkably, lactoferricins of a bovine origin were reported as having higher antimicrobial activity than lactoferricins of human, goat, or rodent origin (51). The antimicrobial action of bovine lactoferrin hydrolysate is likely to be attributed to its function on the cellular membrane, as was shown with the foodborne pathogen *E. coli* O157:H7 (52). Similar to lactoferrin, the hydrolysate's antimicrobial activity is negatively influenced by numerous factors, including divalent cations, pH, temperature, food product constituents (e.g., low activity in ultra-high-temperature-pasteurized milk, as reported by Murdock and Matthews [53]).

Lysozyme: One of the Cohort of Antimicrobial Enzymes

Antimicrobial enzymes are a diverse group of proteinaceous substances and include many representatives with activity against both food spoilage and infectious microorganisms. These include antifungal chitinases; broad-antimicrobial-spectrum glucose oxidases, inhibiting *S. aureus*, *Clostridium perfringens*, and *S. enterica* serovar Infantis; and lactoperoxidases with documented bacteriostatic or bactericidal activity (depending on the microorganism and environment) against *P. aeruginosa*, *E. coli*, *Salmonella* Typhimurium, etc. (for a review, see reference 54).

Lysozymes are a relatively conserved group of cationic antimicrobial enzymes produced by virtually alleukaryotic organisms and capable of killing a broad range of Gram-positive and Gram-negative bacteria by hydrolyzing peptidoglycan in the cell wall (55). There are three major groups of lysozymes: chicken type, goose type, and invertebrate type. These groups differ biochemically, enzymatically, and structurally. Egg white lysozyme is suitable for food processing technologies, as it can survive 100°C treatment for up to 30 min at acidic pH (but not at neutral or basic pH, which are not so common for food products).

Lysozyme is generally active against Gram-positive bacteria and not particularly active against Gram-negative microorganisms, which present an additional barrier, the outer membrane. However, when used in combination with a chelating agent, such as EDTA, lysozyme is recorded as having improved activity against Gram-positive pathogens while also killing Gram-negative foodborne microorganisms and most spoilage bacteria. While a substantial amount of data on lysozyme's antimicrobial activity (alone and in various combinations in laboratory media and food systems) is available, these different studies are difficult to compare because of the many variables which have a significant influence on the outcome of these studies.

Hughey et al. investigated the antimicrobial activity of lysozyme (alone at 100 mg/kg or combined with 5 mM EDTA) against *L. monocytogenes* in Camembert cheese and reported no complete inhibition of the foodborne pathogen under the studied conditions with an initial inoculation of 104 CFU/g of

cheese (56). The reported 1-log reduction in the viable cell count of the pathogen is not sufficient for adoption of this treatment as a preservation method. Some food products may even act as "pathogen protectants." For instance, lysozyme is ineffective against *L. monocytogenes* in milk. However, the pathogen can be "sensitized" to lysozyme by sublethal heat treatment at 62.5°C for 15 s (57), which is likely to produce temporary changes in the cellular membrane, making it susceptible to the antimicrobial's action (58).

Sinigaglia and coworkers studied the effect of lysozyme combined with EDTA on waterborne mozzarella spoilage coliforms and *Pseudomonas* (59). The authors reported effective inhibition of both groups of microorganisms during a 7-day period of storage at 4°C, with no significant inhibition of lactic acid bacteria. Gill and Holley evaluated the combined action of lysozyme, EDTA, and the AMP nisin (discussed below) against spoilage and foodborne microorganisms in meat products (ham and bologna) that were subjected to treatment with combinations of these antimicrobials by dipping (60) or coating (61). In a different study, these investigators explored the activity of selected combinations of antimicrobials relevant to the food industry against spoilage and pathogenic microorganisms grown in laboratory media (62). Interestingly, dipping ham in a formulation with 500 mg/kg lysozyme-nisin (1:3) and 500 mg/kg EDTA effectively controlled *Salmonella* Typhimurium for 3 weeks and both *Brochothrix thermosphacta* (a common meat spoilage microorganism) and *E. coli* O157:H7 for 4 weeks of storage at 4°C. Under the same experimental conditions, the Gram-positive pathogen *L. monocytogenes* was effectively suppressed for only 2 weeks.

These microorganisms were effectively inhibited in the same food systems when the antimicrobial treatment was delivered in a gel formulation. However, the results were strikingly different for the experiments conducted with the microorganisms grown in nutrient broth: the antimicrobial formulations were significantly less active than in the challenged food systems. This important observation indicates the necessity to thoroughly investigate novel food preservatives in real food systems to avoid false-positive and false-negative interpretations of their activities.

Bacteriocins: Food Preservatives, Not Antibiotics

Bacteriocins' place in the world of AMPs: definition and classification

AMPs are naturally produced by allliving organisms: the skin of the African clawed frog (*Xenopus laevis*) is rich with magainins, the salivary glands of humans generate histatins, white blood cells produce defensins, etc. (for a review, see reference 63). In eukaryotes, AMPs are believed to be an ancient functional group of molecules, often serving as one of the first lines of defense against various microbial intruders. The mechanism of antimicrobial action by AMPs is determined by their secondary structure: they can form α-helices, β-sheets, or extended/random-coil structures (reviewed in reference 64). Like eukaryotes, bacteria also produce various AMPs, such as nonribosomally synthesized polylysine (described above) and bacteriocins, which are generally defined as "ribosomally synthesized antimicrobial peptides produced by one bacterium that are active against other bacteria, either in the same species (narrow spectrum), or across genera (broad spectrum) and, as with host defense peptides, cell signaling mechanisms can also be involved" (65).

The ability of virtually allbacteria to produce bacteriocins was first postulated by Tagg in 1992 (66) and then solidified with more recent evidence (67). The apparent simplicity of isolating new bacteriocin-producing microorganisms has resulted in numerous reports on "novel producers" and "novel bacteriocins," many of which have not provided sufficient evidence to confirm these claims. Indeed, the initial tests are very simple and straightforward: a potential bacteriocin producer's colony grown on solid nutrient medium is overlaid with soft agar containing the targeted microorganism. Alternatively, the supernatant of the tested isolate can be added to a well in solid nutrient medium seeded with the targeted strain. The appearance of an inhibition zone with sharp edges and sensitivity of this activity to proteolytic enzyme(s) are supposedly considered evidence of antimicrobial activity that can be attributed to proteinaceous substances. However, this is absolutely insufficient and results in premature claims regarding the microorganism's and antimicrobial's novelty. In fact, the strain must

also be taxonomically identified and the nature of the proteinaceous substance should be elucidated to the point of confirming both its ribosomal nature of synthesis and sequence identity.

It is important to mention that the definition of bacteriocins has changed several times since it was first proposed in the 1970s, especially with a need to separate the rather different bacteriocins of Gram-negative bacteria from those synthesized by Gram-positive microorganisms. This resulted in even further subdivision, with a separate classification proposed for bacteriocins produced by LAB as well (68), many of which are considered important for various food applications (starter cultures, live preservatives, flavor producers, etc.). According to the proposed classification, bacteriocins from LAB are divided into three major classes: class I contains small (less than 10 kDa) posttranslationally modified proteins, class II contains small but unmodified proteins which may still require processing to remove the leader region during the molecule's translocation across the membrane, and class III contains molecules larger than 10 kDa with various mechanisms of action. These classes are then further divided into subclasses. For the purpose of this chapter, it is important to mention that nisin A belongs to subclass Ia, the lanthipeptides, which contains unusual posttranslationally generated amino acids, such as lanthionine or methyl-lanthionine. Another bacteriocin of importance in current food applications is pediocin PA-1 (also known as pediocin AcH), which belongs to class IIa, or pediocinlike bacteriocins, also referred to as antilisterial bacteriocins or YGNGVXC motif bacteriocins (for reviews of class IIa bacteriocins, see references 69 and 70).

Genetic organization of a "generic" bacteriocin operon, with nisin A and pediocin PA-1 as examples

Genes with different functions and in different numbers are involved in the production of these classes and subclasses of bacteriocins. However, a "generic" bacteriocin operon may consist of several genes whose products are essential for a bacteriocin's production and the normal physiology (healthy status) of a bacteriocin-producing cell (Fig. 28.4). These genes encode (i) a prebacteriocin, (ii) an immunity protein protecting the producer cell from its own product, (iii) possibly an accessory protein, which is engaged in the bacteriocin's translocation across the membrane, (iv) an energy-dependent peptidase-ABC transporter, and (v) possibly signal transduction-regulated production of a bacteriocin, which may involve multiple genes.

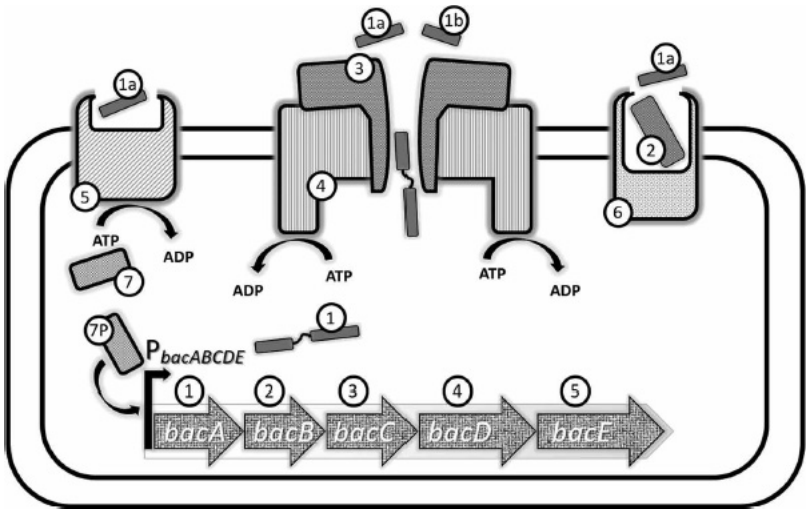
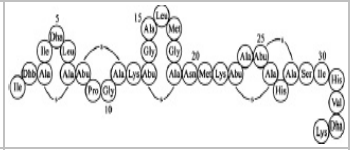
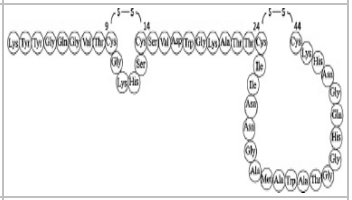
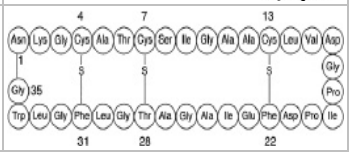


Figure 28.4 Generic bacteriocin operon. The operon's structural gene *bacA* codes for a prebacteriocin (numbered 1). The bacteriocin processing and transport are carried out with assistance of the products of *bacC*, coding for the accessory protein (3), and *bacD*, coding for the ABC transporter (4). The bacteriocin producer cell's immunity to its own product is ensured by the *bacB* gene product, the immunity protein (2). The bacteriocin translocation machinery is engaged in the energy-dependent process, resulting in the cleavage of the bacteriocin's leader region (1b) and release of the mature bacteriocin (1a). The immunity protein (2) protects the bacteriocin recognition site (6) by preventing the bacteriocin (1a) from interacting with this site. The *bacE* gene codes for the histidine protein kinase (5), which is activated by the mature bacteriocin molecule working as a pheromone. The phosphorylated response regulator (7P) interacts with the bacteriocin operon's promoter region, triggering transcription.

Interestingly, when produced, unprocessed prebacteriocins have no or very low antimicrobial activity (71, 72), which may also serve as a mechanism for protection of the producer cell from damage by its own bacteriocin within the cell membrane. Moreover, overexpression of a bacteriocin's processing/translocation machinery may lead to a higher extracellular production of this AMP, indicating that perhaps, under normal physiological conditions, not all of the ribosomally produced bacteriocin molecules leave the cell, and some of them are proteolytically digested inside the producer cell (71).

Processing and translocation of a bacteriocin across the membrane are guided by products of two genes and eventually result in formation of a mature (processed) bacteriocin molecule in the extracellular environment. One of these proteins is an ATP-dependent ABC transporter (encoded by *nisT* in the case of nisin [73] and *pedD* in the case of pediocin PA-1), an ATP-binding cassette translocator which belongs to the hemolysin B (HlyB) type of proteins (74). Also, class IIa bacteriocins' ABC transporters contain an N-terminal domain which is involved in the proteolytic cleavage of the N-terminal leader region from prebacteriocin molecules. The accessory protein is encoded by *pedC* (in the case of pediocin PA-1) and *lcnD* (in the case of lactococcin A) (75) and is similar to the *E. coli* hemolysin A secretion protein HlyD (76). It is involved in bacteriocin translocation across the membrane and is likely needed to ensure that the correct disulfide bridges are formed in the mature pediocin PA-1 molecule (77). In the case of nisin, the serine protease coded by *nisP* is involved in cleavage of the N-terminal leader region of nisin, and the propeptide's posttranslational modification is conducted with the assistance of two membrane-associated proteins encoded by *nisB* and *nisC* (78).

Table 28.3 Bacteriocins: AMPs with varying charges, structures, and targets

Name	Class and/or type	Source	Overall charge	Target	Sequence
Nisin A	Class Ia, lantibiotic	*Lactococcus lactis*	Positive	Lipid II	
Pediocin PA-1	Class IIa	*Pediococcus acidilactici*	Positive	Phosphotransferase system	
Subtilosin A	Cyclopeptide, Sactibiotic	*B. subtilis*	Negative	Under Investigation	

Bacteriocin immunity genes and their products are very specific and provide the producer cell with immunity against its own or very similar bacteriocins (79). For instance, *nisI*, *nisF*, *nisE*, and *nisG* gene products confer immunity to the bacteriocin nisin A. Specifically, *nisI* codes for an extracellular lipoprotein whose C terminus protects the membrane's lipid II, thus providing immunity to the cell's own product (80). Additional genes (*nisEFG*) code for an ABC transporter (for a review, see reference 81). All four gene products are required for the effective protection of the producer cell against antimicrobial action by nisin (82).

It is also important to mention that by default, when we or other authors discuss "nisin," we generally mean nisin A, which is one of a family of nisins consisting of several highly similar lantibiotics (e.g., nisin A, Z, F, Q, etc.; for a review, see reference 83).

Pediocin-like operons contain immunity genes coding for α-helical immunity proteins ranging in size from 9.7 to 12.7 kDa, with the C-terminal region reported as recognizing the corresponding bacteriocin's C terminus, although this physical interaction has not yet been shown (for reviews, see references 84 and 85).

The production of nisin is self-regulated by the bacteriocin's presence in the environment, where it serves as a pheromone, interacting with the producer cell's membrane-localized sensor kinase, a constituent of the socalled EnvZ-OmpR-type two-component regulation system. The intracellular response regulator (second component) is activated by phosphorylation, which occurs with a phosphor transfer from the sensor kinase's transmitter domain. Finally, the activated response regulator's output domain interacts with the promoter regions (P_{nisA} and P_{nisF}), triggering transcription (for a review, see reference 86). Some of the pediocin-like operons were reported as being regulated by a two-component signal transduction system (87, 88); however, this has not been reported for pediocin PA-1 (89).

Bacteriocins' mode of action as multifunctional AMPs

Many studied bacteriocins have multiple functions (in addition to being antibacterial) and often have more than one mechanism of antimicrobial action (for a review, see reference 13). At sub-MIC concentrations, bacteriocins are likely to serve as environmental signals that repel possible intruders in the bacteriocin producer's ecological niche, also preventing these intruding bacteria from settling in the environment—both activities target inhibition of microbial quorum sensing, which is essential for biofilm formation (reviewed in reference 90).

In sensitive cells, lethal interactions with bacteriocins start at the membrane, where both the charge and the AMP's hydrophobicity may be essential for the initial contact between the antimicrobial and

the cell membrane. It is important to mention that while many bacteriocins have a net positive charge (including nisin and pediocin PA-1), not all of them are cationic (Table 28.3). This is particularly important because some authors still suggest a generalized definition of bacteriocins, which includes a statement on their cationic nature. A good example of an anionic bacteriocin is subtilosin A, a cyclopeptide produced by *Bacillus subtilis* which has three aspartates and glutamates and just one lysine in its sequence (91). This bacteriocin belongs to the group of sactibiotics (for a review, see reference 92), also classified by some authors as class V bacteriocins, and has recently received a lot of attention as a potential alternative to conventional antimicrobials for various applications or as antibacterials which are studied as a complementary treatment together with conventional antibiotics (93).

The next stage of interaction may involve a cell membrane-located recognition site, such as the lipid II anchor in the case of nisin (94), and a mannose-specific phosphotransferase system as a “receptor” for pediocin-like bacteriocins (95). These are not receptors in the true sense; a receptor-like function has been reported so far with only one membrane-located protein in *Streptococcus pyogenes*, which is recognized by a two-component bacteriocin from *Streptococcus mutans*, designated lantibiotic Smb (96). In fact, the existence of specific bacteriocin recognition sites for some bacteriocins (without diminishing the role of the primary electrostatic interaction) was proposed in the early 1990s (97).

The next phase of the bacteriocin-cell membrane interaction involves pore formation. Wedge-like and barrel-stave models were proposed during the late 1990s (98) and have been thoroughly studied over the years; these were recently reviewed, with specific examples for three classes of bacteriocins and with some emphasis on food applications (for example, see reference 65). Bacteriocin-induced pore formation causes multiple devastating damages to the targeted cell, starting with dissipation of one (99, 100) or both (101) components of the proton motive force (transmembrane potential and transmembrane pH gradient). The bacteriocin-induced transient pores allow leakage of various cellular constituents, although some bacteriocins may form specific channels, for instance, causing leakage of potassium ions (102). Also, depending on the specific bacteriocin-target interaction, ATP can leak outside the cell, as in the case of nisin (103), or it can be predominantly hydrolyzed intracellularly (104).

In addition to being active against vegetative cells, some bacteriocins have been reported to kill microbial spores. The accumulated knowledge on bacteriocinspore interactions is rather limited, and the molecular mechanisms of these processes have not been elucidated. However, this topic was recently critically reviewed (105), with several significant conclusions. So far, of all the studied bacteriocins, only enterocin AS-48 was reported as having sporicidal activity against *Alicyclobacillus acidoterrestris*, as confirmed by electron microscopy, which showed pronounced damage to the endospore structure (106). Nisin activity against spores is likely to be species dependent: it promotes germination of spores of the foodborne pathogen *C. botulinum* (107) but inhibits germination of the spores of a spoilage microorganism, *Bacillus sporothermodurans* (108). Also, allof the reviewed bacteriocins required germination to start acting against the tested spores. This was thoroughly studied for nisin, and Gut and coworkers, using several mutated and truncated nisin derivatives, showed that pore formation is crucial for nisin’s action against germinating spores of *Bacillus anthracis* (109, 110; reviewed in reference 105). It should be mentioned that while gastrointestinal anthrax resulting from ingestion of *B. anthracis* is rather rare, it is reported as causing mortality in about 40% of the treated patients (for a review, see reference 111).

As was justly emphasized by Egan and coworkers in their comprehensive review (105), there is very limited information on bacteriocin action against spores of food spoilage microorganisms and foodborne pathogens. While the advance in control of spore-forming food-related bacteria is of great importance, the slow progress in these studies is likely due to the limited number of reliable methodological approaches available for mechanistic elucidation of bacteriocin-spore interactions, and it is hoped that the development of novel methods (for example, see reference 112) will accelerate progress in this field of research.

Resistance to bacteriocins

Numerous sophisticated approaches are utilized by microorganisms to survive in competitive environments. These can be divided into two groups of survival approaches: adaptations and mutations. Microbial adaptation to the presence of a bacteriocin in the environment can take various forms: biofilm-associated microorganisms will likely be more tolerant to bacteriocin action, spores are likely to survive the bacteriocin's presence in the environment, and vegetative cells can adapt by modifying their membrane composition. Positive mutations leading to bacteriocin resistance lead to various changes in the cellular membrane and intracellular processes, making the cell resistant to bacteriocin action. It is noticeable that some authors loosely use the term "resistance," which should be attributed only to mutation-determined changes, while the term "tolerance" is more appropriate for adaptation-driven changes.

Bacteriocin resistance should be considered a positive quality for microorganisms which are expected to stay alive and to perform normally in various environments while protected by the action of bacteriocins (e.g., food-fermenting microorganisms and healthy microbiota of the gastrointestinal tract). However, resistance can create some challenges in food products preserved with bacteriocins. Microbial resistance to nisin (along with other lantibiotics) (113) and pediocin PA-1 (89) was recently reviewed. In the case of nisin resistance, several major cellular components were highlighted as contributing to resistance. For instance, the DltB protein is required for d-alanine incorporation into the monomers of the microbial wall's teichoic acid, which can be modified with positively charged d-alanine (114). In turn, this results in a more positively charged membrane, which repels nisin molecules, as in the case of *Lactococcus lactis* with overexpression of the *dlt* operon (115). d-Alanination of lipoteichoic acid also causes a decrease in microbial sensitivity to nisin (116). Nisin-resistant bacteria may produce nisin-specific proteolytic enzymes, such as a nisin resistance protein in lactococci or nisinase in bacilli, enterococci, and some other microorganisms.

Broad-range lantibiotic resistance approaches utilized by various microorganisms are compiled in Table 1 of reference 113. Similar to nisin resistance, d-alanination of lipoteichoic acid ultimately leads to a more positively charged membrane, and this was found to be a factor in increasing resistance to class IIa bacteriocins (including pediocin PA-1) (117). Based on this group's previous studies (118), class IIa bacteriocin resistance in the foodborne pathogen *L. monocytogenes* can also be attributed to the higher fluidity of the cell membrane than that of the membranes of sensitive cells, which is caused by higher levels of unsaturated phosphatidylglycerol.

Microbial resistance to food preservatives should be considered a serious challenge. However, bacteriocin resistance has not yet been reported to be associated with any outbreaks. Moreover, a study has shown that the multiple-stress exposure approach in food preservation may allow the sensitization of bacteriocin-resistant foodborne pathogen (119). In this study, nisin-resistant *L. monocytogenes* was treated with sublethal heat (55°C) and nisin and was found to regain its sensitivity to the bacteriocin.

Utilization of bacteriocins as food preservatives with multiple forms of delivery

The rapidly growing number of studies on bacteriocin activity against pathogens indicates a growing interest among researchers and industries in these antimicrobial substances for various applications expanding beyond food preservation (e.g., personal care products, new antimicrobials for health care). To avoid confusion, it is important to make clear that bacteriocins are different from conventional antibiotics (see Table 28.4, which is adapted from reference 120).

Bacteriocins act as food preservatives in three major forms of delivery. For convenience, some examples have been compiled in the tables, instead of being presented in a narrative manner. The first category contains partially purified formulations, mostly derived from milk fermentation by the producing microorganism. Many of the commercially available food-grade formulations of nisin contain 2.5 to 5% (depending on the manufacturer) nisin, with the rest being NaCl and insoluble milk constituents. While over the last half-century numerous bacteriocins were investigated for their ability to control foodborne pathogens and spoilage microorganisms in model and food systems, nisin is the only GRAS bacteriocin used as a partially purified formulation on an industrial scale around the world. Still, in addition to nisin, several other bacteriocins have been studied in model and food systems and showed promising results in their ability to control undesired microorganisms (Table 28.5). The

second category contains the crude products of fermentation of food-grade substrates by bacteriocinproducing LAB with GRAS status (e.g., MicroGARD [121]). Finally, the third category is an assortment of bacteriocin-producing protective cultures (Table 28.1).

Table 28.4 Bacteriocins are not antibiotics[a]

Characteristic	Bacteriocins	Antibiotics
Application	Food	Clinical
Synthesis	Ribosomal	Mostly secondary metabolites
Activity	Mostly narrow spectrum, few broad spectrum	Varying spectrum of activity, mostly broad
Host cell immunity	Yes, different mechanisms	No
Mechanism of target cell resistance or tolerance	Mostly various adaptations or mutations causing changes in the cellular membrane	Various modifications or mutations affecting different sites depending on the mechanism of action
Interaction requirements and phases	Sometimes docking or recognition sites, possibly in combination with the primary electrostatic interaction with the membrane	Mostly specific targets in the cell
Mode of action	Mostly at the microbial membrane, resulting in pore formation; some may have multiple concentration-dependent mechanisms of action: quorum sensing inhibition, bactericidal, lytic	Cell membrane or intracellular target
Toxicity/side effects	None known at the concentrations used	Yes

[a] Adapted from reference 120.

Table 28.5 Bacteriocins' activity in food products (recent publications)

Bacteriocin	Origin	Target	Food product	Achieved result	Reference
Nisin	*Lactococcus lactis* subsp. *lactis*	*L. monocytogenes*	Red-pepper seasoned cod roe	*L. monocytogenes* was undetectable in samples after 28 days of storage at 15°C (600 μg/g Nisaplin treatment); lower concentrations showed strain-specific activity.	148
Nisin	*Lactococcus lactis* subsp. *lactis*	Total microbial population	Yogurt	Total microbial population was lower in nisin-containing yogurt than in controls over a 40-day period at 10–15°C, with nearly a 1-log difference on day 35. Interestingly, growth in the nisin-treated yogurt peaked at 25 days, while growth in the control continued to increase.	149
Pediocin PA-1	*Pediococcus pentosaceus* NCDC 273	Various	Raw buffalo (*Bubalus bubalis*) milk	Significant reduction in the total count of viable bacteria, *S. aureus*, and lactic acid-producing bacteria. Treatment was less effective against coliform bacteria, and no effect was seen against yeasts and molds.	150
Enterocin AS-48	*Enterococcus faecalis* A-48-32	*B. cereus* LW1, S. *aureus* CECT 976	Liquid egg	Notable inhibition of both pathogens, after incubation at 4°C and 96 h and 28°C and 48 h. For *B. cereus*, 5 μg/ml was able to reduce the pathogen count to undetectable levels, while 15 μg/ml was required for *S. aureus*.	151
Enterocin AS-48	*E. faecalis* UGRA10	*L. monocytogenes*	Raw and smoked fish	Reduction in listeria with respect to the untreated control by 3.13 and 2.8 CFU/cm^2 in raw hake and salmon, respectively, after 7 days at 4°C. For smoked salmon, a 4.25-log CFU/cm^2 reduction was observed after 30 days at 4°C.	152
Enterocin MR10A	*Enterococcus faecalis* N1-33/N41-51	*B. cereus*	Steamed rice	After inoculation and storage at 25°C for 48 h, a 4.0% N41-51 culture supernatant showed a 6-log reduction in CFU/g compared to the untreated sample, and a 4.0% N1-33 culture supernatant showed an approx 1-log reduction.	153

Bacteriocin	Origin	Target	Food product	Achieved result	Reference
Enterocin	*Enterococcus faecalis* L3B1K3	*L. monocytogenes* ATCC 7466	Fresh cheese	Semipurified enterocin was effective in inhibiting *L. monocytogenes* during incubation at 4°C for 72 h; treatment with 134 μg/g of cheese caused a 10-fold reduction, while 536 μg/g reduced *Listeria* counts below detectable limits.	154
Paracin C	*Lactobacillus paracasei* CICC 20241	*A. acidoterrestris*	Apple juice	30 μg/ml was effective against 14 of 19 tested strains, with lethality between 85 and 99% after 24 h. Treatment of *A. acidoterrestris* DSM 3922 spores did not reduce viable-cell counts but did increase sensitivity to heat treatment.	155
Sakacin P, sakacin X	*L. curvatus* MBSa2	*L. monocytogenes* Scott A	Salami	2-log and 1.5-log reductions in viable-cell counts after 10 and 20 days, respectively	156
Aureocin A70	*S. aureus* A70	*L. monocytogenes* L12	Skim milk	5.51-log reduction in *L. monocytogenes* cell counts compared to the control after 7 days of storage at 4°C	157

Major approaches for the improvement of bacteriocin efficacy in food applications

As was already mentioned, in the food environment, the activities of various bacteriocins can be challenged by numerous physical, chemical, and biological factors. Therefore, significant attention is given to the improvement of bacteriocin performance in real foods. These efforts can be divided into several categories, which may ultimately be used either alone or in combination. First, improvement of bacteriocin efficacy is addressed by enhancing its stability against proteolytic digestion (122) and/or enhancing and broadening antimicrobial action resulting from directed mutagenesis (123, 124). Second, possible synergies between bacteriocins and food-grade antimicrobials and/or physical stressors (e.g., temperature, pressure, and irradiation) interacting with different targets is considered a valid approach (for example, see reference 125) (Table 28.6). Finally, the effectiveness of antimicrobial action delivered by bacteriocins can be finely tuned with their controlled delivery. For instance, in a model study with nisin, Balasubramanian and coworkers showed that the most effective inhibition of a targeted model microorganism occurs in the case of bimodal release of the antimicrobial into the environment (126). In this case, the prompt release of a large quantity of the antimicrobial from the carrier contributes to the effective inhibition of the targeted microorganisms. The second phase of slow release over the extended period of time delivers much smaller quantities of the bacteriocins into the environment, contributing to efficient control of possible survivors. For reviews on controlled delivery and antimicrobial packaging, see for instance references 127 and 128.

Table 28.6 Increased activity of bacteriocins when combined with synergistically acting stressors[a]

Bacteriocin	Other stressor(s)	Effect	Reference
Nisin A	Bacteriophage mixtures LM-103 and LMP-102 (5 × 107 PFU/ml)	Reduced *L. monocytogenes* by 5 log on honeydew melon slices stored at 10°C for 7 days compared to control (nisin alone; 400 IU) 3-log reduction	158
Nisin A	Endolysin LysH5	Synergistic action in pasteurized milk at 37°C against *S. aureus*	159
Nisin and/or enterocin AS-48	HIPEF	Bacteriocins were more active when used sequentially after HIPEF, delivering 6-log reduction of S. *aureus*	160
Nisin A	Reuterin and lactoperoxidase	Three antimicrobials synergized in controlling *L. monocytogenes* and *S. aureus* in the semisolid dairy product cuajada, delivering approx. 4-log reduction of foodborne pathogen after 12 days of incubation at 10°C compared to combinations of any two antimicrobials	161
Nisin A	Lactoferrin	Combination of nisin (250 IU/ml) and lactoferrin (500 μg/ml) inhibited *E. coli* O157:H7; combination of nisin (10 IU/ml) and lactoferrin (250 μg/ml) inhibited *L. monocytogenes*	162
Nisin A	Cinnamaldehyde	Nisin in combination with the cinnamon bark's essential oil synergistically acted against 13 foodborne isolates of *S. aureus* when tested in milk	163
Nisin A	Thymol and lactobionic acid	Synergistic action against *L. monocytogenes* reported in reduced-fat and whole milk with nisin used at 250 and 500 IU/ml, respectively. Thymol and lactobionic acid were used at 2 and 10 mg/ml, respectively.	164
Pediocin PA-1	Sodium diacetate	Pediocin PA-1 at 5,000 AU/ml synergized with 0.5% diacetate at 25°C in delivering 7-log reduction of *L. monocytogenes* in turkey slurries	165
Pediocin PA-1	Hydrostatic pressure and high temperature	Combination of pressure (345 MPa), temperature (50°C), and bacteriocin acts synergistically on *S. aureus*, *L. monocytogenes*, *E. coli* O157:H7, *Lactobacillus sakei*, *Leuconostoc mesenteroides*	166
Pediocin PA-1 sakacin P, or curvacin A	Pleurocidin Synergistic action of each of the three class IIa bacteriocins with the fish AMP against the lab strain *E. coli* ATCC 14763	167	

[a] HIPEF, high-intensity pulsed electric field. AU, arbitrary units.

Bacteriophages: Selectively Acting Natural Killers

Bacteriophages are complex, highly organized, multimolecular structured viruses of bacteria, which infect bacterial cells and utilize the cells' resources for multiplication. There are numerous reviews on bacteriophages, and some are suggested here, with an emphasis on the use of bacteriophages as rapidly developing and promising therapeutic antimicrobial agents (e.g., references 129 and 130; the latter provides a good overview of the history of bacteriophage discovery, classification, and research efforts). Whether these strict parasitic predators (bacteriophages and viruses in general) should be considered living creatures is beyond the scope of this chapter, although it is an interesting and controversial topic, and we recommend some studies addressing the issue (131, 132).

Bacteriophages follow their prey and consequently are as ubiquitous in food environments as bacteria themselves. Bacteriophages are just as much of a challenge to the biotech industry, which often relies on microbial fermentation, as they are a threat to the food industry, where microorganisms' role is essential in food processing, safety, etc. (133). On the other hand, bacteriophages targeted at spoilage and infectious microorganisms in the dairy industry are considered a promising tool for improving the safety and quality of fermented dairy products (134). In addition, bacteriophages can contribute to the development of a new generation of protein- and DNA-based vaccines and function as antimicrobials protecting biotechnological fermentation against most common spoilage challenges (135). Bacteriophages are considered a promising aid in the improvement of the health, safety, and quality of animals of agricultural importance and their by-products, helping to control pathogens such as *E. coli* O157:H7, *Salmonella*, *Campylobacter*, *L. monocytogenes*, etc. (for reviews, see references 136 and 137). Clearly, bacteriophages can be utilized as quality- and safety-controlling antimicrobial agents at every step of the food chain, "from the farm to the fork" (138).

Using bacteriophages as food preservatives is somewhat similar to their use as "antibiotic agents" targeted at pathogens in infected human subjects (or animals), although the differences are many and striking. Some of these similarities and differences are summarized in Table 28.7.

The effectiveness of bacteriophages' action as food preservatives is determined by both external factors (environment and targeted pathogen) and the bacteriophage's intrinsic qualities. The latter includes the multiplicity of infection (MOI), which is defined as "the ratio of infectious virions to cells in a given culture" (further reading on the limitations of MOI as a parameter used in microbiology is suggested) (139). It is generally assumed that higher MOIs correspond to enhanced efficacy. MOIs determined in the lab environment differ from those reported in *in vivo* studies. They also vary across multiple studies, making the results confusing and difficult to compare, perhaps because MOI by definition assumes a homogeneously infected cell population, which is not always achievable in practice. Some reports indicate that while lab studies showed the effective control of *E. coli* O157:H7 with bacteriophages, the *in vivo* results were not as impressive, pointing to ineffectiveness of bacteriophages in ruminants under the chosen experimental conditions (140). Moreover, cocktails of bacteriophages targeting different serotypes of several foodborne pathogens would have to be formulated into an all-inclusive bacteriophage-based food preservative to effectively control a broad range of pathogenic microorganisms that may vary greatly based on the food product in question.

L. monocytogenes was one of the first foodborne pathogens whose control in food systems was attempted with the assistance of bacteriophages. This may have been driven by the fact that while listeria does not cause many outbreaks and the morbidity is not the highest, mortality rates can reach up to 25%, especially in immunocompromised subjects (for reviews on listeria and listeriosis, see references 141 and 142). According to the CDC, there are 12 serotypes of *L. monocytogenes*, of which 1/2a, 1/2b, and 4b are most often isolated from food sources and reported in outbreaks. There are reports on bacteriophages with a broad range of activity against several serotypes of *L. monocytogenes* as well as reports on the particular resistance of some isolates of this pathogen to various bacteriophages. Strydom and Witthuhn reviewed the ability of bacteriophages to control *Listeria* in various food products (see Table 2 in reference 143). The critically reviewed studies indicate that, in general, a single treatment with a high number of bacteriophages usually delivers the best antimicrobial effect. However, in some studies, the pathogen started to regrow after an initial application of the bacteriophage formulation.

There are studies where repeated applications of bacteriophage treatment delivered good results. However, these cannot be called conclusive, as the results vary depending on the experimental scenario. For instance, Guenther and Loessner (144) reported on effective control of *L. monocytogenes* in several cheeses with the broad-host-range bacteriophage A511. When used at 3×10^8 PFU/cm^2, this bacteriophage caused a 2.5-log reduction in viable cell count over 21 days of ripening of Camembert cheese, and additional bacteriophage application did not deliver any further reduction in the numbers of listeria (144). The same authors reported that in a different experiment with red-smear cheese, the second application of bacteriophage delayed the outgrowth of *Listeria* but did not eliminate the pathogen. The bacteriophage treatment was effective when used at a high number (1×10^9 PFU/cm^2) and against the lowest initial number of *Listeria* cells (10^1 to 10^2 CFU/cm^2): this resulted in elimination of *Listeria* below the detection limit, compared to the 6-log growth of the pathogen in untreated cheese. Interestingly, Guenther and Loessner point at several promising studies where bacteriophages were used in combination with other, synergistically acting preservatives, such as nisin (mentioned above [Table 28.6]) and lauric arginate (144).

Table 28.7 Bacteriophages as an aid in infectious diseases and food safety: commonalities and differences in food and infection scenarios

Challenge	Infection	Food
Targeted microorganism	Often starts with a specific infectious agent; may lead to multimicrobial and sometimes secondary infections with different microorganisms	Usually specific microorganisms, whose possible presence is determined by the available nutrients and environmental conditions of the food system
Physical factors pH	Varying pH, mostly neutral or basic, but acidic in some healthy organs (e.g., stomach, vaginal environment)	Mostly below pH 7.0
Temp	Normal temp is approx 37°C; elevated temperature may occur in the case of infection	Ambient temperature is appropriate for certain products. Refrigeration (4°C) or freezing (approx. –10 to –20°C) temperature is required for food products as indicated on the label. Temperature-abused storage (often reported in the range of 10–14°C) is documented in numerous cases of spoilage and in outbreaks due to foodborne pathogens.
Environment Physiological and biochemical parameters	Stable in healthy subjects, with normal biochemical and physiological processes; changing in infected organisms	Stable in microbiologically unchallenged products; changing with outgrowth of spoilage microorganisms and/or foodborne pathogens
Microbiology	Healthy microorganisms are challenged and often suppressed or even replaced by infectious bacteria	Presence of food-related microorganisms is determined by the type of food product. There are products where microorganisms should be suppressed to provide the desired shelf life. No live bacteria should be present in canned foods.
Treatment Goal	Eradicate pathogens, if possible, with little to no damage to the host and the healthy microbiota (e.g., lysis of pathogens can "assist" with release of various toxins)	Eradicate foodborne pathogens before the food product is manufactured and prevent outgrowth of pathogens and spoilage bacteria during the product's transit and storage up until the point of consumption by the consumer.
Challenges	Effective therapy requires a threshold population (10^5 to 10^6 CFU) of actively growing bacteria. Bacteria can "disguise" themselves (e.g., by hiding in macrophages), and the environment can be challenging and cause nonspecific interactions that may prevent bacteriophages from reaching their intended target.	Not likely to be applicable as the only agent for control of "zero tolerance" pathogens. The environment can be challenging, causing nonspecific interactions that may prevent bacteriophages from reaching their intended target. Foodborne pathogens in low numbers can escape the bacteriophages' "radar." Stressed, viable but nonculturable pathogens may allow the absorption of the bacteriophage but may not necessarily result in killing of the intended target.
Overcoming challenges	Using cocktails of bacteriophages targeted at different serotypes and combinations with synergistically acting stressors	Using cocktails of bacteriophages targeted at different serotypes and combination with synergistically acting stressors

On the regulatory side, the FDA has affirmed GRAS status for a P100 bacteriophage preparation against *L. monocytogenes*. The P100 preparation was approved for use on meat and poultry products (FDA GRAS notice GRN no. 218; https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=218&sort=GRN_No&order=DESC&startrow=1&type=basic&search=218) as well as in normally aged and ripened cheeses (FDA GRAS notice GRN no. 198; https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=198&sort=GRN_No&order=DESC&startrow=1&type=basic&search=198). It has shown promising results in reducing *Listeria* in cooked turkey and roast beef, especially when combined with chemical preservatives such as potassium lactate and sodium diacetate (145). The authors evaluated the bacteriophage preparation against *Listeria*-challenged products stored at refrigeration temperature (4°C) and at abused-storage temperature (10°C). Interestingly, the bacteriophage performed the best in the abused-storage scenario, delivering a 7.5-log/cm^2 reduction of *Listeria* in cooked turkey and a 7.2-log/cm^2 reduction in roast beef.

Several formulations of multiple bacteriophages active against foodborne pathogens have been designed, have received approval from the regulatory agencies, and are currently marketed for various applications in food processing and preservation (for a review, see reference 146). Of particular interest are those active against *Shigella* (ShigaShield; FDA GRAS notice 672; https://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices&id=672&sort=GRN_No&order=DESC&startrow=1&type=basic&search=672) and *Salmonella* (SalmoFresh; FDA GRAS notice 435; https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=435&sort=GRN_No&order=DESC&startrow=1&type=basic&search=Salmonella).

CONCLUSION

Meaningful fundamental study and exploration of possible food applications for natural/biological preservatives are still in their infancy, as it has been less than a century since their initiation. Unlike chemical preservatives, biopreservatives require sophisticated novel approaches in their formulations, controlled delivery, and combinations with synergistically acting stressors—all aimed at achieving the desired effectiveness and reproducibility in real food systems. An understanding of the role of health-promoting microorganisms in food and in human microbiota naturally includes an understanding of the need for more selective action of food preservatives, aimed specifically at spoilage and pathogenic microorganisms. The rapidly growing breadth of experimentally generated evidence indicates that this goal may be achieved with an increasing number of biopreservatives such as those discussed in this chapter (147).

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29
Bacteriophages for Biological Control of Foodborne Pathogens

NEED FOR BIOLOGICAL CONTROL OF FOODBORNE PATHOGENS

Food safety is challenged by globalization and international trade as well as changes in lifestyle and consumer demands for a more natural and preservative-free diet. The latter trend has sparked the development of minimally processed foods that are low in salt and sugar yet safe, appetizing, and with long shelf lives. However, when foods are produced with limited physical preservation technologies and few chemical preservatives, the bacterial counts may increase and the food environment may not prevent growth of pathogenic and spoilage bacteria. While food spoilage results in significant economic loss for food producers, food poisoning has an even wider societal influence due to its effects on human health, food export, and economy. In primary production, the prophylactic and therapeutic use of antibiotics selects for antibiotic-resistant bacteria and contributes to the emergence of multidrug-resistant bacteria, resulting in declining efficacy of antibiotics used to treat bacterial infections in humans. Thus, novel trends in consumer demands and the global threat of antibiotic-resistant bacteria have generated the need for natural preservation techniques, both to reduce the use of preservatives in food production and to provide alternatives to aid safe food production. Bacteriophages, the natural killers of bacteria, may provide alternative biological solutions for control of foodborne pathogens covering the entire food chain.

BENEFITS OF USING PHAGES FOR BIOLOGICAL CONTROL OF FOODBORNE PATHOGENS

Bacteriophages are obligate parasite viruses that are specific to bacteria, thus being harmless to humans, animals, and plants. Phages do not have a metabolism of their own; rather, they use the bacterial host machinery to propagate, a process that leads to the killing of the infected bacteria. Because phages are dependent on bacteria to propagate, they are mainly found in environments that encompass diverse and abundant bacterial communities. Recent research has demonstrated that both the human and animal guts not only contain a highly diverse bacterial microbiota but also hold a rich and diverse virome of phages and other viruses (1). Therefore, it is no surprise that phages are also abundant and ubiquitous in foods and can be isolated from raw products such as beef and chicken (2), processed foods (3), fermented products like cheese and yogurt (4), and seafood (3, 5). This suggests that we daily consume not only bacteria but also extensive amounts of their phages.

Since their discovery a century ago, phages have been used to prevent and treat bacterial infections. Early work on the therapeutic use of bacteriophages in animals and humans was performed by French scientist and codiscoverer of phages Félix d'Hérelle. In his first paper, he observed that phage presence correlated with disease clearance in patients with dysentery (6). Moreover, he performed a study using rabbits, showing that phages provided protection from *Shigella* infection. Later, d'Hérelle treated cholera and other enteric human diseases using phages.

As described in this chapter, phages have proven to be promising for biological control of foodborne pathogens. In general, phages are particularly beneficial for developing natural and specific food control agents as novel antimicrobials due to their inherent characteristics (Table 29.1). Importantly, phages are self-replicating and self-limiting; thus, at low dosages, phages will multiply as long as a bacterial host is present, thereby enhancing the overall antimicrobial activity. In addition, the action of phages does not have any considerable impact on the physical, chemical, and overall organoleptic properties of the food. Because phages are abundant in the environment, they are easy to isolate and

usually persist well in foods. Phages are highly specific and leave the remaining microbiota untouched, another property that favors phages over conventional methods that may affect the beneficial microbiota of the food. Furthermore, phages have low inherent toxicity and are already present in foods as well as the human and animal gut. Finally, phages can be used along the entire food chain, including phage therapy for reduction of pathogen colonization of animals in primary production and phage biocontrol during food production.

Table 29.1 Main advantages of using bacteriophages for food safety [a]

History of safe use Ubiquitous in nature, including food ecosystems Natural commensals of humans and animals Extensive clinical use in Eastern Europe	Highly active and specifc No adverse effects on the intestinal microbiota Innocuous to mammalian cells Autoreplicative	Versatile use along the food chain Phage therapy: use in preharvest processes such as primary production Phage biocontrol: use at harvest during slaughter and fresh produce Phage biopreservation: use in RTE products	Source of potent antimicrobials Endolysins and other peptidoglycan hydrolases Depolymerases and other carbohydratedegrading enzymes

[a] Modified from reference 120.

In this chapter, we explain the principles and mechanisms behind the use of phages for biological control of foodborne pathogens, as well as the rationale and outcome of using phages for therapy and biocontrol, including the challenges and limitations of such applications. In terms of future prospects, we discuss the technical and regulatory challenges of widespread industrial use of phages for biological control of foodborne pathogens.

NATURE OF BACTERIOPHAGES

Phage Diversity and Taxonomy

Phages are the most abundant microorganisms on earth, outnumbering bacteria by a factor of 10, with an estimated 10^{31} phages in the biosphere (7). Most phages identified (approximately 96%) belong to the order *Caudovirales* and harbor double-stranded DNA genomes inside a polyhedral capsid attached to a tail. Both capsid and tail structures commonly consist of proteins forming the highly ordered and complex architecture of the phage particle. The tailed phages of *Caudovirales* encompass three major families (*Siphoviridae*, *Myoviridae*, and *Podoviridae*) displaying different tail morphologies. Phages of the family *Podoviridae* have short, noncontractile tails, while *Siphoviridae* phages carry long, flexible but noncontractile tails, and contractile tails are the unique characteristic of *Myoviridae* phages. Thus, phages belonging to the same family are morphologically related, yet they may not show any similarity at the DNA level. With the recent advances in DNA sequencing technologies, the number of phage genome sequences in the databases has increased tremendously, yet often without any morphological information. This has prompted a new classification scheme based on genome sequence and proteome analyses to classify phages (8). Fortunately, genome- and proteome-based classifications are in accordance with the morphology-based classification and allowed further classification of phages into species, genera, and subfamilies by comparative analyses (9). Importantly, the enormous abundance and diversity of phages in the biosphere provide an almost unlimited source of diverse phages for applicative purposes, including controlling foodborne pathogenic bacteria.

Phage Life Cycles

Being obligatory parasites, phages rely on their host bacteria for propagation. While strictly virulent phages cause cell lysis when new phage particles are released from the infected bacteria (lytic life cycle), temperate phages also have the ability to maintain their genomes in the bacteria in a dormant

form without causing cell death (lysogenic life cycle) (Fig. 29.1). Temperate phages can either integrate their genetic material into the bacterial chromosome and become prophages or maintain the genome as a plasmid in the cytoplasm. In both situations, the phage genome replicates and is passed on to the daughter cells during growth of the bacteria. However, unfavorable conditions for the bacteria may trigger dormant temperate phage to enter the lytic life cycle, thus propagating and releasing progeny phages by cell lysis. Temperate phages need specific genes for commencing and maintaining the lysogenic life cycle, such as integrases and repressors. By comparative bioinformatics analyses of phage genomes, genes involved in the lysogenic cycle of phages can be identified, thus allowing phages to be classified as temperate. In phage applications, it is important to avoid temperate phages due to their ability to perform specialized transduction by packaging host DNA flanking the site of integration. Furthermore, temperate phages may carry genes that can phenotypically alter the host bacterium.

Importantly, virulent phages with a strictly lytic life cycle always resulting in bacterial death are promising natural agents for biological control. In the lytic life cycle, the phage binds specifically to a bacterial surface receptor, injects its genome into the cytoplasm of the bacterial host, and converts the bacteria into a phage-producing cell. This includes directing bacterial resources to phage genome replication and synthesis of structural phage proteins, which then allow packaging of the phage genome into the assembled head followed by assembly of newly completed phage particles. Last, phage-encoded lytic enzymes rupture the cells from inside and promote bacterial lysis and release of progeny phages.

Even though the major steps of the phage life cycles have been known for decades, the understanding of the specific interactions and mechanisms that allow phages to hijack the bacterial metabolism is limited. For a long time, it was assumed that phages primarily control their hosts by eliminating host transcription and degrading host mRNA, based on the example of phage T4 (10). However, recent work has demonstrated large variations in the mechanisms by which phages interact with the infected bacteria. For example, phages infecting *Pseudomonas* spp. have been shown to influence host cell transcription, RNA degradation, cellular motility, and metabolism (11–13). In addition, some phages degrade the genomes and plasmids of their hosts to recycle the nucleotides for replication of the phage genome, whereas others leave the host DNA untouched. Recently, a feature important for phage applications was described, involving the propagation of two virulent phages that do not degrade host plasmid DNA, thus leading to release of the plasmid upon host lysis, which then can cause horizontal gene transfer by transformation (14). Additionally, certain virulent phages may promote generalized transduction by packaging parts of the host's bacterial genome (15). Due to the vast diversity of phages, it is expected that each phage may utilize a unique mechanism of host takeover.

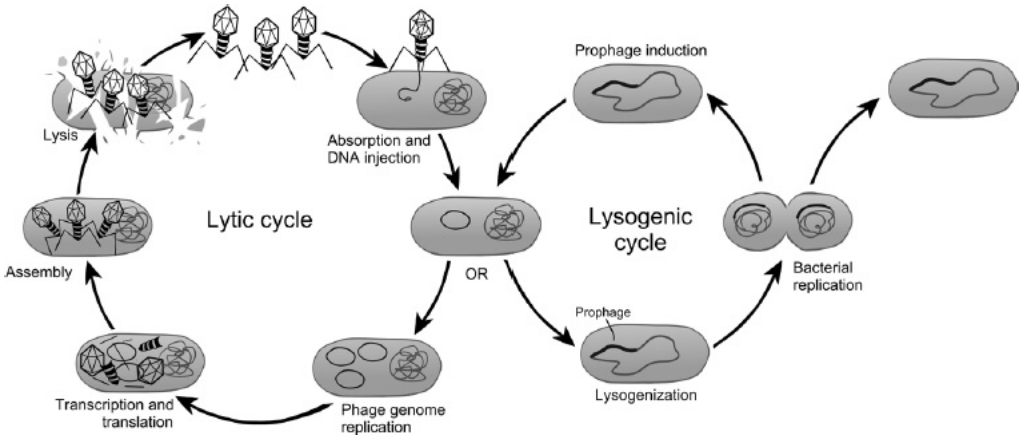


Figure 29.1 Life cycles of phages.

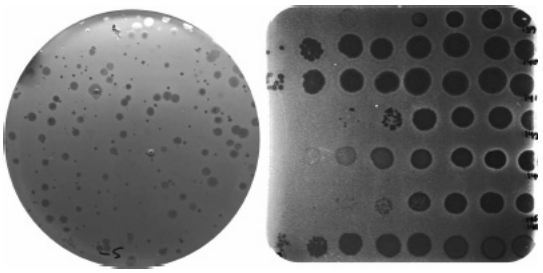


Figure 29.2 Plaque morphologies. (Left) Dilution of a wastewater sample that harbors phages showing different plaque morphologies. (Right) Ten-fold dilutions of purified phage stocks showing consistent plaque morphologies (right).

The killing of bacteria through lytic infections can be observed in different ways in the laboratory: by lysis of liquid cultures containing phages, by reduction of the number of CFU of the target bacteria in food models, or by phages forming zones of clearance (plaques) on bacterial lawns (Fig. 29.2). While plaque formation is useful for isolating and counting phages, reduction of the number of CFU in food models allows determination of the phage's killing efficiency in food biocontrol applications.

Phage Receptors and Host Range

The vast diversity in bacterial cell surface structures allows phages to recognize and adsorb to their particular host by binding to a specific surface component. Such receptors can be any surface component of the bacteria, including carbohydrate moieties, comprising lipopolysaccharides (LPSs), capsular polysaccharides, teichoic acids, and outer membrane proteins, as well as protruding protein structures, such as flagella and pili (16, 17). For some phages, adsorption is a two-step process in which a phage reversibly interacts with a primary receptor and then irreversibly binds to a second receptor to inject its genome. Phage adsorption may also be influenced by the accessibility and spatial distribution of the receptor on the cell surface, as well as environmental conditions such as availability of divalent cations or tryptophan for activation of adsorption. Examples of receptors of selected phages infecting foodborne pathogens are listed in Table 29.2. In some Gram-negative bacteria, such as *Salmonella*, numerous phage receptors have been identified and include flagellar proteins and outer membrane transporters, whereas core carbohydrates and the variable O antigen of the LPS have been shown to influence the initial phage binding serving as the primary adsorption (for a review, see reference 18). Thus, the large diversity of receptors reflects the enormous diversity of phages. For Gram-positive bacteria, the majority of identified receptors are associated with either peptidoglycan or teichoic acid structures of the cell wall. For example, phages infecting *Listeria monocytogenes* bind to the peptidoglycan, the glucosaminyl and rhamnosyl components of ribitol teichoic acid, or the glucosaminyl of ribitol teichoic acid (19). Finally, some phages may use more than one receptor, as exemplified by *Escherichia coli* phage T4, which can use both outer membrane porins (OmpC and OmpF) and the terminal glucose of the *E. coli* B-type LPS (20).

For tailed phages belonging to *Caudovirales*, the initial adsorption is mediated by receptor-binding proteins (RBPs) located at the distal part of the phage tail. These RBPs form long fibers or stubby spike-like structures that bind specifically to the bacterial receptor. Due to the specific interactions of the binding domains, amino acid substitutions in RBPs may drastically change the affi nity. In phage genomes, genes encoding RBPs are prone to diversification through mutations, duplications, and intra- and interspecies recombinations, leading to mosaic RBPs with new binding affi nities reflected by changes in the host ranges (17, 21). One study even showed that accumulation of mutations in RBP genes over the course of several phage replication cycles changed the RBP binding specificity from a protein to a carbohydrate moiety (22). RBP binding to the bacterial receptor typically triggers a conformational change at the distal tail that is transmitted along the tail, allowing the release of the phage genome from the capsid into the bacterial cytoplasm. Thus, the specificity of the phage binding to its receptor ensures that the phage injects genetic material only into a bacterium that has the potential to support propagation.

The host range of a phage defines the breadth of bacteria that a phage is able to infect and kill during propagation. Only when the phage is able to complete the lytic life cycle and produce new phage particles can the infected bacterium be regarded as a host (23). As only limited bacterial hosts support phage propagation, phages are usually specific to certain bacterial strains, species, or more rarely genus. While this specificity allows directed killing of pathogenic bacteria using phages, it also defines the coverage of the phage solution. Thus, detailed knowledge of the host range is a prerequisite for designing phage solutions to target specific bacteria. Recently, bioinformatics tools able to predict bacterial hosts at the genus level have been developed (24, 25). Still, biological data defining the host range are required for application purposes, as these programs do not take the natural diversity of bacterial strains of the same genus and species into account. Experimental host range data can be generated by determining the ability of a phage to form single plaques on bacterial lawns encompassing diverse strains of bacterial collections.

Table 29.2 Examples of phage receptors of selected phages infecting foodborne pathogens

Bacterium and phage	Receptor	Reference
S. enterica SPN2T	Flagellar proteins FliC and FljB	121
iEPS5	Flagellar protein FliK	122
vB_SenMS16	Outer membrane protein OmpC	46
SPC35	Vitamin B_{12} receptor BtuB	123
ST27	Efflux pump component TolC	124
ES18	Ferrichrome transporter FhuA	125
SPN1S	O antigen of the LPS	121
SSU5	Core carbohydrates of the LPS	126
E. coli O157		
v10	O157 antigen of the LPS	127
C. jejuni		
F336	MeO*P*N attached to Gal*f*Nac of capsular polysaccharide	26
F379	Motile flagellum	128
L. monocytogenes		
A511	Peptidoglycan	19
A118	Glucosaminyl and rhamnosyl components of ribitol teichoic acid	19
A500	Glucosaminyl residues in teichoic acid	19

Phage Resistance

For phage applications, it is important to consider both intrinsic phage resistance mechanisms already found in the natural bacterial populations and the selection or emergence of resistant subpopulations when phage solutions are applied. During exposure to high phage titers, bacteria most commonly develop phage resistance by modulating the availability of receptors or preventing phage access. These first-line defenses include receptor mutations, loss of receptors, or even synthesis of components that physically mask receptor access, thus preventing phage adsorption and infection. One example is *Campylobacter jejuni* phage F336, where the host gains resistance by preventing adsorption, either by losing the receptor or by introducing certain modifications to the capsule that indirectly prevent access to the receptor (26–28). This strategy of developing resistance also influences susceptibility to other different phages (29). However, such receptor changes become dominant in the bacterial population only if the environment allows bacterial growth. In a food environment that does not support growth of the pathogenic bacteria, phage-resistant variants cannot be selected for and will not become the dominant population. On the other hand, the application of phages targeting enteric bacteria residing in the animal gut may favor selection of phage-resistant receptor mutant populations.

In most applied studies, the underlying mechanisms of phage resistance development were not investigated in detail, and therefore, most of our understanding of phage resistance development is based on studies of well-characterized phage-bacterium model systems. From these studies it is clear that, depending on the receptors, mutations arising from phage exposure and resulting in phage resistance may influence the biology and pathogenicity of the bacteria. *Salmonella enterica* serotype Typhimurium may become resistant to several phages, but at the expense of reducing its virulence (30). It was shown that phase variation of the *opvAB* operon generates two subpopulations with different LPS O-antigen lengths. When exposed to phage, the virulent phage-sensitive subpopulation with long O antigens diminishes, while the avirulent phage-resistant subpopulation with short O antigens survives. Thus, while acquisition of phage resistance results in reduced virulence, phase variation permits recovery of virulent subpopulation when phage challenge ceases (30). However, phages may also counteract receptor changes and regain or acquire the ability to adsorb to novel receptors, thereby maintaining or even changing or expanding their host range. It has been proposed that phage genes involved in host recognition are among the fastest-evolving phage genes due to selection pressure during phage-bacterium coevolution (31). For example, phage T7 can evolve to infect *E. coli* cells with different LPSs upon successive propagations on *E. coli* strains expressing such LPS structures. The resulting T7 phage derivatives were found to carry mutations in the gene encoding the tail fiber protein responsible for interaction with LPS (32). Such experiments demonstrate the possibility to evolve and select for phages that recognize different receptor variants by mutations in the RBPs.

When phages are used for biological control of foodborne pathogenic bacteria, phage resistance development can be minimized by using phage cocktails. Phage cocktails consist of two or more phages, ideally targeting different receptors. The use of such cocktails decreases the likelihood of the development of resistance, as bacteria attacked by multiple phages will be killed before acquisition of resistance. Therefore, knowledge of phage receptors can aid in the design of efficient phage solutions that avoid phage resistance development.

Due to the coevolution and coexistence of bacteria and phages in all ecological niches, it is plausible to expect that bacteria have evolved many different and so far-unidentified ways to counteract the killing effect of phages but also that phages have developed responses to such mechanisms. Internal phage resistance mechanisms may include the ability to degrade the phage genome using host-encoded nucleases. Such restriction-modification defense systems modify the bacterial DNA to ensure self-discrimination and degrade only the invading nonmodified phage genomes to prevent the phage takeover during infection. However, there are examples of phages adapting to restriction-modification systems by modifying their own DNA, thus protecting the phage genome from degradation by host restriction enzymes (33).

Another internal phage defense mechanism is the CRISPR-Cas (clustered regularly interspaced short palindromic repeats [CRISPR] and CRISPR-associated proteins), which functions as an adaptive bacterial immunity system targeting foreign nucleic acids (34). The CRISPRCas loci are generally composed of an array of direct repeats interspaced by nonrepetitive diverse spacers and flanked by a varying number of different *cas* genes. In short, invading phage DNA is digested by a Cas nuclease in a sequence-specific manner. This requires the phage genome to contain a DNA sequence (protospacer) that matches one of the diverse spacers of the CRISPR array in the bacterial genome. The bacteria may evolve the CRISPR array by acquiring novel spacers upon initial phage infection, thus gaining resistance to future infections of the phages carrying the same protospacer sequence, working as an adaptive immunity (35). Still, phages have also developed countermeasures to CRISPR-Cas-mediated phage resistance mechanisms. Mutations in the protospacer sequences allow them to avoid spacer-mediated recognition of their genomes. More recently, phage anti-CRISPR proteins were discovered and shown to prevent the action of the CRISPR-Cas in different ways (36).

Bacteria may also encode abortive infection mechanisms in which an infected cell dies before phage replication for the benefit of the remaining sister cells in the population (31). Bioinformatics analyses have revealed clusters of defense islands abundantly found in many different bacteria, suggesting the presence of numerous novel phage resistance mechanisms that may shape phage-host interactions

(37). A number of these internal phage resistance mechanisms have been experimentally shown to affect phage replication (38–40). For phage application development, it is important to recognize that bacterial populations found in food and farm animals may be highly diverse, with differences in phage sensitivity. To ensure the isolation of phages able to infect a majority of a natural microbial population, a panel of defined highly resistant mutants can be employed as isolation hosts. In practice, this has not yet been done systematically, and to date, molecular studies of overcoming bacterial resistance have been generated from single-phage–single-host combinations, with an understanding of their broader presence in microbial systems gained through comparative bioinformatics.

CONSIDERATIONS PRIOR TO PHAGE APPLICATION FOR FOOD SAFETY

Bacterial Densities, Volume, and Environmental Conditions Affect Outcomes

When the concentration of phages to be used for a given application is being determined, it is important to consider the level of bacterial contamination, the volume, and the environmental conditions (41). Phages must first be physically able to collide with the bacteria in order to infect. Because phages are not able to move actively and are dependent on diffusion to find their hosts, microstructures of the inert surfaces may affect or even prevent diffusion. Also, the available fluid will impact the volume the phages must diffuse through to encounter the target bacteria. On the other hand, if limited fluid is available, phage diffusion may be hampered. Moreover, the target bacteria must be physically accessible in the food matrix, and the higher the reduction of bacteria aimed at, the more phages might be needed. Finally, using standard assumptions of mass-action interactions and moderate phage adsorption constants, Hagens and Loessner calculated that it takes around 1,000 years for one phage and one bacterium to meet within 1 ml of fluid (41). Thus, not only are the numbers of phages and bacteria important, but also, the overall volume will also influence the outcome of the phage application. In practice, phage applications are tested under the relevant conditions at different multiplicities of infection (MOIs; defined as the number of phage particles per bacterial cell) in order to determine the actual dose needed to obtain the desired efficiency of the phage application. However, at very high MOIs (>100), cells may lyse without phage propagation due to the changes in the membrane potential caused by excessive yet unspecific interaction of phages with the cell surface. This phenomenon is known as nonspecific killing, or “lysis from without,” and is usually associated with cell wall-degrading enzymes located at the tail tip of many bacteriophages (42).

In primary production, farm animals used for food production may be colonized with zoonotic bacteria at very high numbers, thus increasing the likelihood of phage-bacterium encounters when phages are applied therapeutically. In addition, phage treatment may lead to significant phage propagation in the gut, allowing progeny phages to infect new target cells and contribute to reductions in the bacterial counts. When a phage application to be used during slaughter is being designed, the distribution and number of target bacteria should be carefully considered, as this may vary between animals and different technologies of the slaughter process. In food matrices, the environment can be very diverse and very specific depending on the food product. Moreover, high-quality production of the raw material as well as good hygienic practices throughout the production chain often leads to low levels of contamination by the pathogenic bacteria. Thus, to target a low number of bacteria on a solid surface requires high numbers of phages, simply to ensure that phages will encounter a bacterial host. It is also important to consider whether the bacteria are able to grow in the food environment. In addition, application on solid food matrices limits the phage diffusion, and thus, high numbers of phages will be required to achieve infections and significant reductions in the bacterial counts. Finally, environmental factors may influence the success of different stages of the lytic life cycle, and these must be considered when phages are used to control foodborne pathogens.

Bacteriophages Are Safe for Humans and Animals

Since phages are abundant in the environment, humans and animals consume phages on a daily basis from food and water. As phages are specific to bacteria, they do not actively interact with human and animal cells, but nonetheless they may be perceived as foreign particles and stimulate the human and animal immune systems. However, this does not affect the safety but rather the efficiency of the phage application, and to date, there is no evidence that phages exert harmful or allergenic effects on humans or animals. On the contrary, the use of phage therapy to treat infections over the past century has provided a large body of evidence showing that phages are harmless for humans and animals. However, applying phage to kill bacterial cells within the human body may cause adverse effects, not because of the phages themselves, but as a side effect from the release of large amounts of cellular material, such as LPS, during lysis. This has been observed in only a few clinical applications with high bacterial loads and therefore cannot be directly compared to the effects of most other food safety applications. Oral toxicity studies have shown that high doses of *L. monocytogenes* phages fed to albino rats had no measurable impact on rodent behavior or physical appearance (43). Also, a study using oral administration of *E. coli* phages to mice and human volunteers showed no adverse effects on the test subjects (44, 45). In summary, phages as entities are well tolerated by humans and animals and are part of their own natural microbiota, and they are therefore considered safe.

Historically, phage preparations used for therapy in humans were made from crude phage extracts, which often contained immunogenic and pyrogenic materials from the lysed bacterial cells. Modern methods of large-scale phage production require the removal of such cell debris, LPS, or endotoxins originating from the host bacteria used for production. This may be achieved by downstream purification of the phage preparation to remove cell debris and toxin contamination from the propagating bacterial host. In addition, it has been suggested that nonpathogenic surrogate host bacteria can be used for large-scale production to eliminate the hazards associated with large culture volumes of pathogenic bacteria. This may also require less stringent downstream purification procedures if the surrogate host does not produce the soluble toxins which may be associated with a pathogenic strain.

For safety reasons, temperate phages should be avoided in biological control of foodborne pathogens, as they may alter the phenotype of the bacterium by expressing genes during the prophage state. It is known that prophages may carry and express moron genes that are not involved in phage propagation but provide beneficial traits to their host by encoding virulence or antimicrobial resistance factors. Moreover, following induction, temperate phages may transfer fragments of host DNA by specialized transduction into nontargeted bacteria (possibly to other related species) by packing bacterial DNA flanking the prophage integration site into the progeny phage capsids. Phage genome sequencing and bioinformatics analyses can reveal unwanted genes or genes associated with a lysogenic lifestyle. Virulent phages that perform generalized transduction should be avoided to prevent the spread of genes encoding toxins, antibiotic resistance factors, or virulence factors. These phenomena can be tested in the laboratory by conducting transduction experiments (46).

There is a particular concern that extensive use of phages may also select for bacterial strains with altered phenotypes, as has been observed with extensive use of antibiotics. In practice, by investigating the phage-resistant strains and determining the mechanisms and frequencies of phage resistance, it is possible to evaluate the consequences of phage resistance for colonization and virulence of pathogenic bacteria. In addition, using predefined phage cocktails globally and continuously in a manner similar to antibiotics may select for bacterial populations that become permanently resistant to such specific phage cocktails. However, comprehensive studies of phage infection patterns suggest that new infectious phages will nevertheless be available (47). Compared to antibiotics, the diversity and numbers of phages are immense, suggesting that phage resistance may not be a concern at all, even in the long run (48).

Designing Phage Applications for Biological Control

When novel biological applications for the control of foodborne pathogens using phages are being designed, both safety and efficacy should be considered, and any phage that is used must meet several criteria. For biological control of pathogenic bacteria, only virulent phages should be used, as

temperate phages can lysogenize and not kill the host or might cause horizontal gene transfer between susceptible strains. Ideally, these virulent phages should be produced using a nonpathogenic host, and they should show no signs of being able to perform transduction. The complete genome of the phage should be available and devoid of any lysogeny-, pathogenicity or antibiotic resistance-associated genes as well as proteins associated with allergic reactions. In addition, oral feeding studies should demonstrate no adverse effects. Ideally, these phages should degrade the host DNA during infection to minimize the risk of transfer of host genes following the lysis of the target bacteria (14).

While these criteria are important for human and environmental safety, the following considerations are necessary to ensure the efficiency of the phage solution. Phages should have as broad a host range as possible in order to cover the potential diversity of strains of the target bacterium. In reality, covering the strain diversity of even one target bacterial species is challenging and remains a major obstacle for phage application efficiency. Phage cocktails consisting of multiple phages can be designed to tackle this limitation and increase the coverage of the application. Importantly, such phage cocktails can delay the emergence of phage-resistant populations. Phages in the cocktails should be dependent on different receptors for infection, since accumulating mutations to counteract phage infection for several receptors will be cumbersome for the target bacteria. Finally, for phage solutions to be commercially feasible, phages must be able to be produced on a large scale and be sufficiently stable over the storage and application period.

BIOCONTROL OF FOODBORNE PATHOGENS IN PRIMARY PRODUCTION

Rationale for Applying Phages in Primary Production

The habitats of zoonotic foodborne microbial pathogens include animals used for food production, but many of these bacteria do not result in clinical symptoms in livestock, thus allowing undetected contamination of foods. Furthermore, intensive animal breeding practices, global animal transportation, and the introduction of new animals to production facilities facilitate spread between individual animals within herds or flocks, consequently increasing the risk of food contamination throughout the farm-to-fork chain. Therefore, the elimination or reduction of zoonotic foodborne bacterial pathogens, early in the farm-to-fork chain, is regarded as a cost-effective approach with a high potential to reduce the risk of human infections. A study assessing the efficiency of controlling *Campylobacter* in broiler meats estimated that applying phage therapy to *Campylobacter*-positive flocks throughout the Netherlands could result in a 63% reduction in the risk of human campylobacteriosis (49). Moreover, control of foodborne pathogens in production animals can reduce the shedding and incidence of new infections in herds or flocks, thus contributing to good production practices in livestock. In addition, management of both pathogens and zoonotic bacteria in primary production and aquaculture is dependent on the use of antimicrobials, contributing to the current trend of increasing prevalence of antibiotic-resistant bacteria. Collectively, these issues drive the search for novel approaches to the management of zoonotic foodborne bacteria during primary production, including application of phages.

Administration of phages to production animals for prophylaxis or therapeutic purposes is not a novel concept and was quickly initiated after the discovery of phages. A number of works have compiled therapeutic uses of phages for diseases of livestock (50–52) as well as of aquatic animals (53–58), but here, we focus on the accumulating studies demonstrating that phage therapy in production animals is an effective measure against several foodborne pathogens belonging to different genera.

Outcome of Applying Phages in Primary Production

Several works have investigated phage therapy in ruminants, swine, and poultry, with the primary focus being on *E. coli* O157:H7, *Salmonella enterica* subsp. *enterica* serotypes, or both *Salmonella* and *Campylobacter*. A summary of studies of these approaches and their outcomes is listed in Table 29.3. In most of these studies, the use of phages for reduction of specific pathogens was promising.

However, the outcome of phage therapy is dependent on the successful encounters between phage particles and the target bacteria in the animal, as well as their individual success in killing the target bacteria. When administered orally via feed or drinking water, phages should be able to tolerate the acidic conditions of the digestive tract and maintain their viability in the presence of digestive enzymes and other digestive compounds (e.g., bile) before reaching the intestinal tract. Hence, the stability of phages in feed material or drinking water, adequate dosage, number of doses (single doses versus continuous administration), and the route of administration are important factors affecting the success of encounters between phages and their target pathogen.

Administration of Phages in Primary Production

In order to allow phages to bypass the deteriorative environment of the stomach without rapid loss of viability, a number of different approaches have been used. In some work, researchers coadministered antacid buffers (e.g., calcium carbonate or sodium bicarbonate) along with phage cocktails to neutralize stomach acid (59–62). Although this approach was useful for experimental studies, its application in livestock production with thousands of animals would be challenging. In chickens, a comparison of phage administration via drinking water and aerosol spray was performed (63). Borie and colleagues showed that a prophylactic single-dose coarse spray treatment of chickens 24 h prior to bacterial challenge can also lead to significant reductions in *Salmonella* Enteritidis loads (63). In sheep and cattle, rectal phage administrations were tried as an alternative, due to the fact that the lymphoid follicle-rich recto-anal junction (RAJ) mucosa is a significant colonization site for *E. coli* O157:H7 (64). To reduce the colonization levels in experimentally inoculated Holstein steers, phages SH1 and KH1 were coadministered directly to the RAJ mucosa, while the phages were also continuously administered via drinking water throughout the experiment (65). Although they observed significant reductions in all five treated steers, a total clearance was seen in only a single calf. Later, it was found that oral treatment with a cocktail of four phages resulted in lower O157:H7 counts than RAJ mucosa application, although the differences were not significant (66). Researchers concluded that RAJ application alone might not be sufficient to ensure encounters of phages with *E. coli* O157:H7, since the bacterium can also be found at the proximal regions of the intestines, such as cecum, colon, and rarely duodenum (67).

In aquaculture, phage therapy is currently not feasible in settings such as grow-out ponds or sea cages due to the large volume of water. However, it remains an attractive alternative particularly for hatcheries that can establish high phage titers in hatching and larval growth tanks (68). In addition, pretreatment of live feed with phages, targeted and controlled phage-releasing feed adjuvants, injection of phage preparations, and immersion of fishes in phage solutions offer an array of different administration approaches that can be considered according to their practicality and the particular aquaculture practice (56).

Microencapsulation-based delivery techniques are another method to protect phages from adverse acidic conditions. In this approach, phages are packaged into miniature capsules (300 nm to 1 mm) to protect against acids and can be liberated at controlled rates under specific conditions (e.g., in the intestine). Ma and colleagues encapsulated the broad-host-range *Salmonella* phage Felix O1 in the natural polysaccharides alginate and chitosan and evaluated the phage stability *in vitro* in simulated gastric fluid of pig (69). They found that the encapsulated phages survived significantly better than free particles under low-pH conditions and in the presence of pepsin or bile salts and were almost completely released from the microcapsules in simulated intestinal fluid with a pH of 6.8. Later, others encapsulated four different *E. coli* O157:H7 phages in methacrylate polymer and fed them to steers either orally in gelatin capsules or in feed (70). Although fecal samples yielded active phages in acceptable levels, the shedding of the bacterium was not significantly reduced. In another work, Wall and colleagues investigated whether oral administration of a microencapsulated phage cocktail could effectively reduce *Salmonella* counts in market-weight pigs (71). The pigs were kept in contaminated holding pens and allowed to comingle with *Salmonella*-negative mock-treated pigs in order to imitate the preslaughter transport- and lairage-associated *Salmonella* colonization of the pigs. Although significant reductions in cecal colonization were observed in *Salmonella*-positive animals, colonization

of *Salmonella*-negative and mock-treated control pigs was not prevented. Colom and colleagues encapsulated three *Salmonella* phages in cationic liposomes to improve protection in the gizzard and retention time in the intestines of chickens (72). They found that liposome-encapsulated phages were markedly more stable to acid and retained in the intestine significantly better than nonencapsulated phages. Consequently, the oral administration of a liposome-encapsulated phage cocktail significantly reduced *Salmonella* colonization of chickens, with activity persisting more than 15 days postadministration. Additionally, their approach of encapsulating phages into liposomes allowed storage of the preparation for at least 3 months without a significant loss of activity and increased protection of phages against lyophilization. Thus, microencapsulation is a very promising approach to protect phages to be administered to live animals; however, other technologies may be developed.

Table 29.3 Outcomes of phage therapy against foodborne pathogens in the primary production

Pathogen and animal	Bacterial inoculum (CFU)	Phage	Phage dosage (PFU)	Application method	Application time[a]	Reduction
E. coli O157:H7						
Lamb	10^8	DC22	10^{13}	Oral	Day 2 p.i.	Undetectable[b]
Ewe	10^{10}	CEV1	10^{11}	Oral	Day 3 p.i.	2–3 log
Ewe	10^{10}	KH1	10^{11}	Oral	Day 0	9–10 log
Steer	10^6	Cocktail (KH1 and SH1)	10^{10}	Rectal	Days 0, 1, 2, and 4 p.i.	1–4.6 log
Sheep	10^{10}	8-phage cocktail	10^9	Oral	Day 2 p.i.	4 log
Steer	10^9	Cocktail (wV8, rV5, wV7, and wV11)	10^{11}	Oral	Days—2, 0, 2, 6, and 9 p.i.	3.5–5 log
Steer	10^{10}	Cocktail (wV8, rV5, wV7, and wV11)	10^{11}	Oral, rectal, and oral and rectal	Days—2, 0, 2, 6, and 9 p.i.	5–7 log
Cattle	10^{10}	Cocktail (e11/2 and e4/1c)	10^{11}	Oral	Days 1, 2, and 3 p.i.	Undetectable
Steer	10^{11}	Encapsulated cocktail (wV8, rV5, wV7, and wV11)	10^9	Oral	Days—1, 1, 3, 6, and 8 p.i.	4–5 log
Ewe	10^{10}	Cocktail (CEV1 and CEV2)	10^{11}	Oral	Day 3 p.i.	3–4 log
Cattle hide	10^5–10^6	Cocktail (e11/2 and e4/1c)	10^9–10^{10}	Spraying on Hide	1 h p.i.	1.5 log
Cattle	Natural Load	Cocktail (Finalyse)	10^{10}	Spraying on Hide	Upon animal Arrival	From 57.6 to 51.8% in Prevalence
Salmonella enterica subsp. *enterica* serotypes						
2-day-old chickens	10^8	ϕAB2	10^5	Oral	Day 0	2 log (by 13th day)
Newly hatched chicks	10^4	Various: single and cocktails	Various	Oral, feed or Water	Various	0.3–1.3 orders of magnitude
3-week-old pigs	10^8	Felix O1	10^{10}	Oral and Intramuscular	3 h p.i.	1–3 log
1-day-old broilers	10^8	Cocktail (CNPSA1, CNPSA3 and CNPSA4)	10^{11}	Oral	Day 7 p.i.	3.5 orders of magnitude
3-day-old chicks	10^5	Cocktail (S2a, S9 and S11)	10^6	Oral	Days 0, 1, 2, 5, 6, and 7 p.i.	6-fold in cecum
36-day-old chickens	10^8	ϕ10, ϕ25 or ϕ151	10^9 or 10^{11}	Oral	Day 2 p.i.	2.2–4.2 log

Pathogen and animal	Bacterial inoculum (CFU)	Phage	Phage dosage (PFU)	Application method	Application time[a]	Reduction
1-day-old chicks	10^3	Cocktails (CB4Ø and WT45Ø)	10^8	Oral	1 h p.i.	30–55% in recovery rate
10-day-old chickens	10^6	Cocktail (BP1 to -3)	10^8	Coarse spray or drinking water	24 h prior to Inoculum	1.5 log
3- to 4-week-old pigs or market-weight pigs	10^8 or 10^9	Microencapsulated cocktail of 15 phages with Felix O1	10^9	Oral	Day 0 and every 2 h p.i. for 6 h	2–3 log or 1.5 log
Pigs	10^9	Microencapsulated cocktail of 14 phages	10^{11}	Feed or oral	For 5 days p.i. or day 5 p.i.	1 or 2 log
Pigs	10^5, 2 days	Cocktail (F3-8)	10^3, 10^5, 10^7, and 10^9	Oral	Day 1 p.i.	1–1.5 log
Campylobacter spp. 10- or 32-day-old chickens	10^5	Phage NCTC12669 or NCTC12671 singly or consecutively	10^9–10^{10}	Oral	Days—3 to 6 p.i. or days 5 to 10 p.i.	2 to 1 log or 3 to 1 log
Broiler chickens	10^3 to 10^8	CP8 and CP34	10^5, 10^7, and 10^9	Oral	Day 5 or 7 p.i.	0.5 to 5 log
Broiler chickens	10^8	CP220	10^5, 10^7, and 10^9	Oral	Day 5 p.i.	2 log
31-day-old chickens	~10^5, 10^6, and 10^7	Cocktail (ϕCcoIBB35, ϕCcoIBB37 and ϕCcoIBB12)	10^6 or 10^7 in Feed	Oral or feed		2 log
6-day-old chicks	10^4	Single (NCTC12673) or cocktail (NCTC12673, NCTC12674, NCTC12678 and NCTC12672)	10^7	Oral	Day 3 p.i.	Max, 2.8 log
Field flocks	Various	Cocktail (NCTC12673, NCTC12674, NCTC12678 and NCTC12672)	~10^6–10^8	Drinking water	Various	Up to 3.2 log

Pathogen and animal	Bacterial inoculum (CFU)	Phage	Phage dosage (PFU)	Application method	Application time[a]	Reduction
Various-age chickens	10^7–10^9	Single (CP14) and cocktail (CP14 with CP81 or CP68) consecutively	10^8–10^{10}	Oral	Day 5 or 7 p.i.	Up to 3.5 log
Vibrio parahaemolyticus Brine shrimp	Various	Vpms1	Various	Tank water	Various	~10% decrease in mortality
Vibrio harveyi Shrimp	10^5	Single or cocktail (VHM1, VHM2, and VHS1)	10^9	Tank water	Day 0	3–5 log

[a] p.i., postinoculation.

[b] Undetectable, to undetectable levels.

Emergence of Phage-Resistant Mutants in Livestock

A concern regarding phage therapy in livestock is selection for phage-resistant mutants of the target bacteria, which may dominate the population after phage exposure, as the target bacteria are metabolically active under *in vivo* conditions. As mentioned previously, phages and their hosts continuously coevolve in a variety of ecological niches, a concept termed “arms race evolution.” In this situation, both phage-resistant mutants and phages are excreted with the feces, continuously circulating in the farm environment, and may potentially affect the activity of the phage preparation used against the target pathogen at primary production. At the same time, phage-resistant bacteria may lose fitness in colonization or virulence and might be outcompeted by their sensitive-phenotype counterparts (59, 73, 74), although this might not always be the case (27, 75, 76). Therefore, determining the molecular basis of the phage resistance posttherapy, as well as its persistence and consequences for the fitness (both in colonization of the animals and pathogenicity in humans) of the target bacteria must be further researched.

Researchers have compared the frequency of phageresistant mutants of three *Salmonella* serotypes in chickens (60). In brief, they observed that the number of phage-resistant colonies posttreatment was at least doubled in comparison to those in control groups and that higher phage concentration selected for greater proportions of resistant mutants. However, upon consecutive subculturing *in vitro*, these resistant mutants reverted back to being phage sensitive. Moreover, when obtained resistant mutants were readministered to the birds, neither colonization levels nor the percentage of the emerging resistant mutants differed noticeably from those in the control group (60).

As mentioned above, in addition to expanding the host range, phage cocktails that are composed of individual phages using different receptors offer a promising strategy to control emergence of phage-resistant mutants. Fischer and colleagues demonstrated that the application of a phage cocktail against *C. jejuni* in commercial broilers can both moderate (from 43% to 24%) and delay the emergence of phage resistance in comparison to application of a single phage (62). On the other hand, the use of phage cocktails might have unintended consequences, as phages in a cocktail may exchange genomic material, possibly through superinfection of the same host, resulting in the emergence of novel hybrid phages (77). However, this phenomenon was observed in continuous cultures in the laboratory and not in the gut of the animals. Another approach to increase the efficacy of phage therapy in primary production includes the rotation of different phage cocktails composed of different phages. This approach allows targeting of phage-resistant mutants that have emerged from the use of the previous

phage cocktails and are currently applied in aquaculture practices (56, 78). Proving such efficacy in large livestock farms would be challenging due to large numbers of animals; however, in aquaculture practices, tracing the efficacy can be feasible, and thus, cocktail rotation appears to be an applicable strategy (56, 78).

Collectively, phage therapy in primary production appears to be a promising approach, yet current data indicate that single-phage or phage cocktail administration will not be enough to ensure production of pathogen-free animals. Instead, phage therapy can currently be considered as an additional mitigation strategy for management of foodborne pathogens. However, there are currently no phage preparations approved for therapeutic use in livestock. The challenges facing regulatory acceptance are discussed later in this chapter. Moreover, in the real-life setting, ensuring administration of standard dosages to all animals remains a major challenge, particularly in intensive animal breeding and aquaculture. Finally, the application of phages at an epidemiological end point, such as on foods, would prevent phages and phage resistant mutants from circulating in the farm environment, and such applications have attracted considerable interest, as described below.

BIOCONTROL OF FOODBORNE PATHOGENS AT SLAUGHTER

Rationale for Applying Phages at Slaughter

As explained above, studies on phage therapy in live animals vary considerably depending on the animal, administration route, and dosage, as well as the phage(s) used, resulting in different efficiencies of the phage application. The efficacies of phage-based applications after primary production are expected to be superior to those of phage therapy in live animals, as certain untraceable dynamics, such as the immune response of the animal and the ever-changing microenvironment of phages in the gut or farm environment, can be avoided. Cattle hides and chicken feathers have long been known to play a role in contaminating carcasses during the slaughtering processes. Hence, phage preparations applied on hides or feathers prior to slaughter may reduce the pathogen loads preslaughter.

Outcome of Applying Phages at Slaughter

An early work demonstrated that the application of phages to cattle hides was a promising approach to reduce pathogen numbers. One hour after application of the phage preparation to hides, Coffey and colleagues observed a significant reduction of 1.5 log CFU/cm^2 in *E. coli* O157:H7 counts (79). The first regulatory approval for the use of phages on live animals via hide or feather spray/mist application was issued by the U.S. Department of Agriculture (USDA) in 2007. Since then, there have been several commercial *E. coli* O157:H7 and *Salmonella* phage preparations on the market for use on cattle hides and poultry feathers prior to slaughter. One recent study evaluated the efficiency of a phage preparation in reducing the contamination levels of *E. coli* O157:H7 on cattle hides and carcasses (80). After applying phage spray at the lairage area of two different commercial beef processing plants, Arthur and colleagues observed ~6% and 0.5% reductions in the prevalence of *E. coli* O157:H7 on hides and carcasses, respectively. As the reduction was not significant, researchers concluded that the application of phages to cattle hides before going through the lairage environment may not improve food safety (80).

As with many phage-based biocontrol approaches, the outcome of phage application on hides and feathers is also dependent on ensuring successful encounter of the phages with target bacteria. In the lairage environment, however, increasing amounts of organic matter accumulate on the hides of the animals. Perhaps due to this fact, the application of phages to hides upon arrival of the animals at the slaughterhouse remains insufficient, and their application at the exit of lairage, just before slaughtering, could increase the efficiency. So far no reports have evaluated preslaughter efficacy of phage application on feathers of poultry mimicking real-life application. However, due to the factors mentioned above, the outcome of such applications might not be favorable. Collectively, preslaughter application of phages on animal hides and feathers has a limited degree of success.

BIOCONTROL OF FOODBORNE PATHOGENS IN FOODS

Rationale of Applying Phages in Foods

The contamination of food materials with foodborne pathogens can occur in primary production, throughout transportation, and during or after processing of foods. Therefore, application of phages for biocontrol of specific foodborne bacteria should be considered according to how the food item is contaminated with the pathogen (i.e., surface contamination or in deeper fractions) and take the food processing steps into account. The application of phages at an epidemiological end point, such as in food processing plants or slaughterhouses, is advantageous for limiting the circulation of phages and avoiding a constant selection of phage-resistant strains.

However, applying phages to control foodborne microbial pathogens in foods is a complex and challenging task. The design and efficiency of phage solutions for food safety are highly influenced by the food environment during processing and storage. The microenvironment of food is primarily characterized by intrinsic factors, such as available free water and pH, as well as extrinsic factors, such as temperature, oxygen level, and the relative humidity of the environment. Moreover, these conditions might change throughout the processing of foods. While these factors are known to influence the growth of bacteria, they may also affect the activity of the phages and the efficiency of the phage application. There seem to be no general rules as to under which conditions a phage will be most efficient, since this may be dependent on the phage and influenced by a multitude of environmental variables. Thus, it is necessary to evaluate phages for their killing efficiency and stability under relevant conditions encountered during food processing. The environmental variables of the food processing cannot be mimicked by *in vitro* experiments in laboratory media, and thus, such experiments do not always reflect the efficiency of phage biocontrol on the foods. Therefore, determining the efficiency of phage killing on an actual food model with relevant processing conditions is required.

Nonetheless, experiments in laboratory media can be used to compare different phages with respect to their biology and stability. Since phage infection and killing are usually most efficient when the bacteria are at a metabolically active state, the ability of the target pathogen to grow in the food might also influence the outcome of phage biocontrol. Therefore, it is important to understand how the food environment influences the growth of the target pathogen as well. Even though phages are robust, some conditions such as high temperature or low pH may inactivate phages, and foods that go through or harbor such deteriorative environments may not be suitable for phage application.

For identifying phage application sites in the food processing plant, the process design and the processing technology of the food item must be considered. The application of phages should be considered at a processing step that allows optimal phage distribution and survival. Furthermore, the relevant mode of application should be considered to ensure phage-host encounters in or on the food matrix and according to the nature of food contamination. Since many food products, including fresh produce and meat, are surface contaminated, spraying of food products or dipping them into phage solutions may be an effective mode of application, whereas in other foods, phages may be directly added to the raw material (e.g., milk, brine, or fruit juice). As mentioned before, foods are often contaminated with pathogens at low levels, and the application technology as well as the concentration of applied phages should be carefully considered to increase the likelihood of phage-bacterium encounters.

For all these reasons, literature on the efficiency of phage biocontrol covers a broad variety of food items inoculated with relevant foodborne pathogens and kept under various conditions after exposure to different modes of phage applications. Table 29.4 displays a summary of these studies and outcomes of phage biocontrol. Due to the large variance of the enlisted biocontrol studies with regard to the food item, phage or phage cocktail investigated, experimental conditions, and mode of applications, it is hard to draw general conclusions. However, there are strong tendencies towards better phage biocontrol efficiencies when initial bacterial counts are higher, phages are applied in higher numbers (i.e., a high MOI is used), and the environmental temperature is above refrigeration temperature

(Table 29.4). In the following paragraphs, we summarize and discuss the outcome of phage use for biocontrol of pathogens in meat and processed meat products, in milk and dairy products, and in fresh produce.

Outcome of Phage Biocontrol of Pathogens in Meat and Meat Products

In fresh meat from production animals, poultry, and fish, the pH, water content, and rich nutrients support growth of a variety of bacteria. The slaughtering, cooling, storage, cutting, and packaging practices may vary between different slaughterhouses according to needs and regulatory frameworks. Nonetheless, the safety of raw meats is primarily dependent on hygienic production, as well as the chilling of the carcasses to refrigeration temperatures (4 to 8°C) both to delay microbial spoilage and to suppress the growth of pathogenic bacteria. In addition, modified atmosphere packaging of raw meat is known to delay spoilage, yet it does not have a major influence on growth of most of the foodborne pathogenic bacteria relevant for fresh meat.

Contamination of raw meat with enteric zoonotic pathogens such as *Salmonella*, *Campylobacter*, and pathogenic *E. coli* begins at the abattoir upon removal of the inedible parts (e.g., hides and feathers) and offal. Moreover, meats can also get cross-contaminated with ubiquitous pathogens, such as *L. monocytogenes*, due to their contact with conveyors, personnel, abattoir utensils, or other inert surfaces. Since contamination is at the surface of the meat, phages offer a pathogen-specific approach that has the potential for biocontrol without influencing the overall sensory qualities of the meat. Upon slaughter, efficient chilling of the carcasses is important for food safety, and consequently, the activity of phages that are applied immediately on the surface of the carcasses or at the final fresh meat product will be challenged by rapid drops in temperature and surface humidity. Hence, several scientific works have evaluated the application of phage solutions on meat surfaces under a variety of storage conditions (4 to 37°C) that mimic meat production, refrigeration, or abused-refrigeration temperatures associated with cooling and transportation of fresh meats and products thereof (Table 29.4).

O'Flynn and colleagues showed that seven of nine steak pieces challenged with 2 log CFU of *E. coli* O157:H7 underwent total elimination of the pathogen 1 h after phage cocktail treatment (81). Significant pathogen reduction was observed during storage at 37°C, a temperature that allowed active host metabolism but is not relevant for storage of fresh meat. On the other hand, Carter and colleagues evaluated the effect of phage-bacterium contact time on the surface of meat at 4°C, over 24 h of incubation (82). They showed significant reductions in the bacterial counts 5 min after phage cocktail application. While 5 min at 4°C is not an optimum period for successful completion of any known lytic phage cycle, this finding indicates that phages do encounter and infect their hosts on the meat surface, regardless of the temperature, and perhaps the phage life cycle is completed later, when conditions allow host metabolism to proceed (i.e., on plating media).

Table 29.4 Outcomes of phage biocontrol against foodborne pathogens on foods

Food	Bacterial inoculum (CFU)	Phage	MOI	Application method	Condition(s)	Reduction
E. coli O157:H7						
Steak meat	2 log	Cocktail (e4/1c, e11/2, pp01)	10^6	Spotting	37°C	4–5 log
Broccoli, tomato, spinach, ground meat	2.5–4 log	ECP-100 cocktail (ECML-4, ECML-117, ECML-134)	10^4–10^6	Spray	10°C	94–100%
Iceberg lettuce Cantaloupe	3.7 log 4.5 log	ECP-100 cocktail (ECML-4, ECML-117, ECML-134)	10^2–10^4	Spray Spotting	4°C, 20°C	~2–3 log
Spinach, romaine lettuce	4–6 log	BEC8 cocktail (38, 39, 41, CEV2, AR1, 42, ECA1, ECB7)	1, 10, 10^2	Spotting	4°C, 8°C, 23°C, 37°C	1–3 log
Beef steak, ground beef, romaine lettuce	3 log	EcoShield cocktail (ECML-4, ECML-117, ECML-134)	10^2, 10^3, 10^4	Spray	4°C	94–98%
Beef	~3–5 log	Single DT6 or cocktail (DT1, DT6)	10^2–10^5	Spotting	5°C, 24°C	0.3–2.6 log
Spinach, lettuce	4.5 log	EcoShield cocktail (ECML-4, ECML-117, ECML-134)	10^2	Spray	4°C, 10°C, modified atmosphere	1.2–4.3 log
Raw beef	Various (2–4 log)	FAHEc1	10–10^4	Spotting	37°C	0.5–2.6 log
Raw and cooked beef	0.6–4 log	FAHEc1	10–10^4	Spotting	5°C, 24°C	0.2–4.6 log
Iceberg lettuce	2–3 log	EcoShield cocktail (ECML-4, ECML-117, ECML-134)	10^2–10^5	Immersion with different durations, spray	4°C	1 log to undetectabl [a]
Ground beef, spinach, cheese	5–7 log	Cocktail (FFH1, FFH2, FFH3)	1	Spotting	4°C, 24°C, 46°C, 63°C	0.1–3.3 log

Food	Bacterial inoculum (CFU)	Phage	MOI	Application method	Condition(s)	Reduction
Milk	3 log	PhaxI	10^3	Addition to milk	37°C	Undetectab
RTE raw meatball	0.5–1.8 log	M8AEC16	2.4–5.6 log	During manufacturing	4°C, 22°C	0.7–2.1 log
Edible cabbage	3 log	HY01	10^5, 10^6	Spotting	37°C	~2.5 log
Green bell peppers, spinach	6.6 log	OSY-SP	~10^2	Immersion	4°C, 25°C	0.9–3.5 log
Beef, pork, chicken meat	5 log	BPECO19	10^3, 10^4, 10^5	Spotting	4°C, 37°C	0.5 log to Undetectab
Beef	3 log	Chitosan coating film embedded with liposome-encapsulated phage	Not Applicable	Wrapping with chitosan coating film	25°C	4.8 log
Salmonella enterica subsp. *enterica* serotypes						
Melon and apple Slices	~4 log	SCPLX-1 cocktail (4 phages)	5, 10, 20	Spotting	5°C, 10°C, 20°C	2.5–3.5 log
Cheddar cheese (whey or curd)	4 log	SJ2	10^4	Addition to milk	Various	3–4.4 log
Chicken frankfurter	2 log	Felix O1 or its Variant	10^4	Spotting	22°C	1.8–2.1 log
Chicken skin	2–3 log	P22 or 29C	1–10^7	Spotting or Spray	4°C	2 log to Undetectab
Sprout seeds, Mustard	2–3 log	Cocktail (A, B)	10^4	Soaking	25°C	1.5 log
Chicken carcass	0–3 log	PHL4 or cocktail (72 phages)	Various (1 to 10^{10})	Spray; immersion	37°C	2 log
Chicken carcass	0–2 log	WHR	10^7–10^8	Spray	37°C	2 log to Undetectab
Raw and cooked Beef	2–4 log	P7	10, 10^4	Spotting	5°C, 24°C	2.8–5.9 log
Alfalfa sprout seeds	7 log	Single or cocktail (SSP5, SSP6)	70	Immersion	25°C	~1 log
Tomato rhizospheres	6 log	Cocktail (F01, P01, P102, P700, P800)	10^{-3}, 1	Extending to the stem base	15°C	0.8 log

Food	Bacterial inoculum (CFU)	Phage	MOI	Application method	Condition(s)	Reduction
Sprouting mung bean and alfalfa seeds	6 log	Cocktail (F01, P01, P102, P700, P800, FL41)	10^{-1}, 10^{2}	Immersion	20°C, 25°C, 30°C	3.4 log
Pig skin	3, 4, 6 log	PC1 cocktail (ϕSH17, ϕSH18, ϕSH19, Felix O1)	10^{-2}–10^{4}	Spotting	4°C	2 log to Undetectab
Hot dogs, cooked/sliced turkey breast, seafood, chocolate milk, egg yolk	3 log	Felix O1, FO1-E2	10^{5}	Various	8°C, 15°C	5 log to Undetectab
Chicken skin	3–4 log	wksl3	10^{3}–10^{4}	Spray	8°C	2.4 log
Pig skin, chicken breast, fresh egg, lettuce	~6 log	Cocktail (UAB_ Phi20, UAB_Phi78, UaB_Phi87)	10^{3}, 10^{4}	Various	4°C, 25°C, 33°C	0.9–4 log
Liquid egg, energy drink, various milks, apple juice, chicken (breast, minced)	4, 5 log	P22	10^{5}, 10^{7}, 10^{8}	Various	4°C	0.8 log to Undetectab
Cooked chicken Meat	3 log	Single or cocktail (ϕSP-1, ϕSP-3)	10, 10^{2}	Spotting	4°C, 28°C, 37°C	1–3.4 log
Chicken breast and Skin	3 log	SalmoFresh cocktail (6 phages)	10^{5}, 10^{6}	Spotting	4°C	1.4–3.3 log
Romaine lettuce	~1.5 log	Salmonelex (now PhageGuard S)	~10^{7}	Immersion	10°C	~1 log
Chicken breast, pasteurized whole milk, Chinese cabbage	4 log	Single and cocktail (PA13076, PC2184)	10^{2}, 10^{3}, 10^{4}	Various	5°C, 25°C	1.5–4 log
Ground pork, liquid egg	7 log	SJ_2	10	Spotting and addition to liquid	4°C, 21°C	~0.5–1.5 lo

Food	Bacterial inoculum (CFU)	Phage	MOI	Application method	Condition(s)	Reduction
Cockles	5 or 6 log or naturally contaminated	Single or cocktail (phSE-2, phSE-5)	10^{-1}, 1, 10, 10^2	Inoculation	16°C	~0.7–2 log
Chicken breast	3 log	SalmoFresh cocktail (6 phages)	10^6 or more	Spotting or Immersion	4°C, 25°C, aerobic or modified atmosphere	0.7–1.2 log
Whole and fresh-cut cucumbers	5 log	SalmoFresh cocktail (6 phages)	~105	Spray	10°C, 22°C	~0.4–2 log
Chicken skin, egg Shell	8.5 log	ZCSE1	10^{-1}	Spotting	4°C	2 log to Undetectab
Sprouting alfalfa seeds	3.5 log	SI1	~10^2	Immersion	22°C	2.5 log
Ground chicken	4 log	Salmonelex (now PhageGuard S)	10^3	Spotting and then grounding	4°C	~0.3–1 log
RTE cooked duck meat	3–4 log	fmb-p1	10^4, 10^6	Spotting	4°C, 25°C	4.5 log
Beef, pork, chicken, and turkey	7, 7.3 log	Salmonelex (now PhageGuard S)	Unknown	Addition during tumbling process	4°C	0.1–1.1 log
Campylobacter spp.						
Chicken skin	2, 4, 6 log	Phi2	10^{-2}–10^5	Spotting	4°C, –20°C	1.1–4.1 log
Chicken skin	4 log	NCTC12673	10^2	Spotting	4°C	~1.2 log
Roast beef slices	2, 4 log	Cj6	10, 10^4	Spotting	5°C, 24°C	1.5–2 log
Chicken meat	~3.2 log	NCTC12684, CP81	Not specified	Spotting	4°C, 37°C	None
Chicken liver stomachates	3, 5 log	ϕ3, ϕ15	10^3, 10^5	Spotting	4°C	0.2–0.7 log
Chicken neck skin	4 log	Single and cocktail of various	10^3	Spotting	5°C, modified Atmosphere	None–0.7 log
C. sakazakii						
Infant formula	2 log	Single (ESP 1-3, ESP 732-1)	10^5, 10^6, 10^7	Addition to reconstituted formula	12°C, 24°C, 37°C	2 log to undetectabl

Food	Bacterial inoculum (CFU)	Phage	MOI	Application method	Condition(s)	Reduction
Infant formula	2 log	GR5	10^5	Addition to reconstituted formula	37°C	2 log to undetectabl
Infant formula	4 log	Cocktail (leB, leE, leN)	~10^4	Addition to reconstituted formula	37°C	4 log to undetectabl
Y. enterocolitica						
Pork	3.5 log	PY100	102, 104	Spotting	4°C	1.5–2 log
Raw minced pork, RTE pork loin, milk	3 log	fHe-Yen9-01	10^5	Various	4°C, 26°C	4.5 log to undetectabl
Shigella spp.						
RTE spiced chicken and braised duck	4 log	Single or cocktail (SF-A2, SD-11, SS-92)	10^4	Spotting	4°C	2 log to undetectabl
RTE deli corned beef, smoked salmon, cooked chicken breast, lettuce, melon, yogurt	~3 log	ShigaShield cocktail (5 phages)	10^2, 10^3, 10^4	Spray or addition to yogurt	Room temp	0.2–1.6 log
Raw and cooked chicken breast	4 log	vB_SflS-ISF001	10^4	Spotting	4°C	1.5–2 log
Vibrio spp.						
Live oysters, oyster flesh	6 log	pVp-1	~10	Immersion, spotting	18°C or room temp on ice	~5–6 log
Oysters at depuration	5, 6, 7 log	VPp1	10^{-1}, 1, 10	In depuration Tank	12°C, 16°C	0.5–2.8 log
L. monocytogenes						
Red meat	6 log	LH7	Not specified	Immersion	4°C, vacuum-packaged	~0.4 log
Fresh-cut apple and melon	5.5 log	Cocktails: LM-103 (12 phages), LMP-102 (6 phages)	~10^2	Spray	10°C	0.4–4.6 log
Surface-ripened red-smear soft cheese (Munster)	1.2 log	P100	10^5, 10^6	In the washing/smearing solution	14–6°C	3.5 log to undetectabl

Food	Bacterial inoculum (CFU)	Phage	MOI	Application method	Condition(s)	Reduction
RTE hot dog, cooked/sliced turkey breast, smoked salmon, mixed seafood, chocolate milk, mozzarella cheese, lettuce, and cabbage	3 log	Single (A511, P100)	10^3, 10^4, 10^5	Various	6°C, 20°C	0.1–7.6 log
Sliced cooked ham	3 log	Listex P100	10^4	Spotting	10°C, vacuum packaged	1 log
Fresh catfish Fillet	4.3 log	Listex P100	Various	Spotting Spray	4°C, 10°C, 22°C	None–2.3 log
Raw salmon fillet	2, 3, 4.5 Log	Listex P100	Various	Spotting	4°C, 22°C	1.8–3.5 log
Queso fresco Cheese	~4 log	Listex P100	~10^4	Spotting	4°C, vacuum Packaged	~4 log
RTE chicken breast Roll	2, 4, 5 log	FWLLm1	~10^2	Spotting	5°C, 30°C	1.5–2.5 log
White mold and red-smear soft cheeses	1, 2, 3 log	A511	10^5, 10^6, 10^7	In the washing/smearing solution	6°C, 12°C	1–3 log
Roast beef, cooked turkey	3 log	Listex P100	10^4	Spotting	4°C, 10°C	1.5–2.1 log
Melon, pear, and apple slices and juices	~3, 5, 8 log	Listex P100	~10^3	Spotting and addition to juice	10°C	0.6-2 log an none–8 log
Minas Frescal and Coalho soft cheeses	5–6 log	Listex P100	10^2–10^3	Spotting	10°C	0.9–2.5 log
Lettuce, hard cheese, smoked salmon, apple slices, frozen entrée	3–4 log	ListShield cocktail (6 phages)	10^2, 10^3, 10^4, 10^5	Spray	4°C, freezer	0.7–2.2 log
Dry-cured ham	2, 3, 4 log	Listex P100	10^2–10^6	Spotting	4°C, 10°C, 20°C	2.5 log to undetectabl
Milk	5, 7 log	Single (LMP1, LMP7)	10, 10^2	Addition to Milk	4°C, 10°C	~0.5–3.3 lo

Food	Bacterial inoculum (CFU)	Phage	MOI	Application method	Condition(s)	Reduction
Sashimi	6 log	Listex P100	10^6	Not specified	6°C, 12°C	5 log to undetectabl
S. aureus						
Raw and heat treated milk/whey	6 log	K	10^2	Addition to Milk	37°C	Undetectab
Milk, acidic and enzymatic curd	6 log	Cocktail (ϕ88, ϕ35)	~10^2	Addition to milk	25°C, 30°C, 37°C	Undetectab
UHT-treated, pasteurized whole, semiskimmed, and whole raw milks	2 log	Single or cocktail (ϕH5, ϕA72)	10^{-1}, 1, 10^2, 10^3–10^4	Addition to Milk	4°C, 22°C, 37°C	1–4.5 log
Milk	Various (2.8–6 log)	Cocktail (ϕIPLA35, ϕIPLA88)	Various (10^{-2}–5)	Addition to Milk	Various (17–37°C)	6 log to undetectabl
During manufacturing of fresh- and hard-type cheeses	6 log	Cocktail (ϕIPLA35, ϕIPLA88)	1	Addition to milk	4°C, 11°C, 25°C, 32°C	4.6 to undetectabl
During manufacturing of Cheddar cheese	6 log	Cocktails (Team1, P68, LH1-MUT and ϕ812, 44AHJD, ϕ2)	10^2–10^3	Addition to Milk	4°C, 32–35°C	Undetectab
Fermented soybean product (cheonggukjang)	2, 3 log	JBP901	10^6, 10^7	Spotting	37°C	2–2.5 log
Fermented soybean product (cheonggukjang)	1, 3 log	Single (BCP1-1, BCP8-2)	10^5, 10^7	Spotting	37°C	2 log to undetectabl
Fermented soybean product (cheonggukjang)	3 log	JBP901	10^5	Spotting	37°C	2 log to undetectabl

[a] Undetectable, to undetectable levels.

Although this is an important finding, there are also examples in which storage at refrigeration temperature influenced the outcome of phage biocontrol. When Big-wood and colleagues applied phage P7 or CJ6 on 2-cm² raw beef samples inoculated with *Salmonella* or *Campylobacter* at low densities (<100 CFU/cm²), significant reductions were observed only when the samples were stored at 24°C, not at 5°C (83). The adverse effect of low storage temperature on overall pathogen reduction by phage biocontrol on meat has been observed in several other studies of different phage-pathogen combinations (Table 29.4). However, this phenomenon should not be considered to reflect a general rule, as the outcome of phage application will still be influenced by densities of bacteria and phage and

their successful encounters in the food matrix. When Tomat and colleagues applied a cocktail of phages (DT1 and DT6) at an MOI of ~10^5 on beef samples inoculated with *E. coli* O157:H7, they observed a significant reduction (~1.4 log CFU) in 3 h only when samples were stored at 24°C, not at 5°C (84). On the other hand, when a single phage (DT6) was used at a much lower MOI (~10^2), storage at 5°C rather than 24°C led to significant pathogen reduction (~0.4 log CFU) in 3 h, demonstrating the multifactorial nature of the phage biocontrol outcome. Moreover, the mode of host recognition can also be influential in the outcome of phage biocontrol. Zampara and coworkers showed that the ability of phages to reduce *Campylobacter* on refrigerated chicken skin was dependent on the receptor used by the phages (85).

When variables such as storage temperature or bacterial inoculum density are kept constant, higher phage concentrations (i.e., higher MOIs) often lead to higher efficiency in phage biocontrol. Such phage concentration-dependent results have been observed in several reports that have evaluated the effect of phage MOI when other variables remain unchanged. For example, Hooton and colleagues reported the biocontrol efficiency of phage cocktail PC1 against *Salmonella* on pig skin samples at 4°C with three different concentrations of phage and bacterial inocula, establishing an MOI matrix between 0.01 and 10.000 (86). They found that the use of phage concentrations above an MOI of 10 were necessary for obtaining significant reductions, whereas an MOI of 1 or below led to little or no reduction. Such correlations between higher phage concentrations and more efficient pathogen reductions have also been observed on chicken skin with *C. jejuni* (87), on ready-to-eat (RTE) beef, chicken, and salmon with *Shigella sonnei* (88), and on RTE sausages, sliced turkey breasts, and smoked salmon with *L. monocytogenes* (89, 90).

Meats can be ground or processed further to a variety of meat products. In particular, ground meat and ground-meat products are challenging food items for phage biocontrol due to their increased surface area and their potential to harbor pathogens within deeper fractions, preventing phage access. Nonetheless, several works have shown significant reductions in *E. coli* O157:H7, *Salmonella*, and *Yersinia enterocolitica* counts in ground meats with phage application (91–94). Of note, Carter and colleagues found that following the initial reduction of *E. coli* O157:H7 on the meat surface, when meats were ground and recontaminated with the pathogen, residual phages that remained from the earlier surface application did not show any effect, despite being actively present at approximately identical initial numbers in the ground meat. While this finding emphasizes the adverse effect of substantial surface area changes in the food matrix on phage-host encounters, it also demonstrates that residual active phages do not always exert a continuous effect under these circumstances (82). However, the addition of phages in the liquid fraction during manufacturing of a ground-meat-based RTE food achieved significant reductions in *E. coli* O157:H7 counts, presumably due to the homogeneous access of the phages to deeper fractions of the food item prior to artificial contamination (95).

Meat can also be subjected to a number of traditional preservation techniques, such as heat treatment, smoking, curing, fermentation, and drying, as part of food production (see chapter 6 of this volume for more details). These products are produced with different additives that are beneficial for achieving distinct characteristics of the food item, such as color, texture, flavor, and shelf life. As a result, the microorganisms present in meat products are different from those in fresh meat. Pathogenic bacteria in such meat products may originate from either the raw material or cross-contamination during production. Since these products are often marketed as RTE food items and consumed without additional steps to alleviate the risk of foodborne infections (e.g., heat treatment), phages offer promising potential in the management of contaminating pathogens. Guenther and colleagues evaluated the biocontrol activity of phage FO1-E2 on samples of RTE hot dogs, cold turkey breast cuts, and mixed seafood (shrimp, shellfish, and squid) artificially contaminated with ~3 log CFU/g *Salmonella* (96). After 2 days of storage at 15°C, a reduction of more than 2 log was observed in hot dog and turkey samples, whereas in seafood, the reduction was not as pronounced. However, in the following days, the surviving *Salmonella* continued to grow, yet counts remained 1 to 4 log lower than those in the phage-negative control groups. When a similar experiment was conducted in seafood at 8°C, *Salmonella* was not totally eliminated, yet the reduction was 3 log in the first day without the

regrowth of the bacterium over the 6 days of the experiment (96). In another work, Soffer and colleagues demonstrated the biocontrol potential of a commercial phage cocktail on several different food items previously implicated in foodborne shigellosis (88). The five-phage cocktail had a comprehensive host range, lysing 97% of *Shigella flexneri*, *S. dysenteriae*, *S. sonnei*, and *S. boydii* strains tested. When applied at 10^5, 10^6, and 10^7 log PFU/g on RTE deli corned beef slices, chicken breast strips, and smoked salmon samples that were spiked with 10^3 CFU/g of the test bacterium, the phage cocktail yielded significant yet dose-dependent reductions ranging between 31 and 98% regardless of the technology used in production of the food item (88).

An important food safety problem associated with RTE meat products is *L. monocytogenes* contamination. The ubiquitous nature of the bacterium results in cross-contamination of RTE meat products postproduction, while the ability of *L. monocytogenes* to grow at refrigeration temperatures increases the associated risk, in particular for RTE food items that are approaching their expiration date. Therefore, achieving either total elimination or a significant reduction in CFU counts by phage biocontrol has been the aim in several works (Table 29.4). As observed in numerous other studies, when Guenther and colleagues applied different concentrations of phage A511 on RTE hot dogs, cold turkey breast cuts, smoked salmon, and mixed seafood that were spiked with 103 CFU/g of two different test strains, they observed higher efficiency in biocontrol with the higher concentration (108 PFU/g) of the applied phage (89). In the first day of the 6-day incubation at 6°C, A511 reduced *L. monocytogenes* counts in samples of RTE hot dogs, turkey cuts, smoked salmon, and mixed seafood by approximately 3, 1, 1, and 1.5 log CFU/g, respectively. The results were found to be strain dependent, often leading to regrowth at lower levels than in negative controls. All surviving isolates tested were still phage sensitive, and the authors concluded that the observed regrowth stemmed from cells that escaped contact with phage particles due to either the complexity of the food matrices (i.e., uneven surface) or the ability of the food item to adsorb liquid, both of which incapacitate phage diffusion (89). Overall, scientific studies have demonstrated that the application of phages for biocontrol can reduce pathogenic bacteria on meat surfaces, even under refrigeration, yet testing of specific phages and phage cocktails under conditions that are specific for the food is essential for designing efficient phage biocontrol.

Outcome of Phage Biocontrol in Milk and Dairy Products

Due to its rich lactose content and approximately neutral pH (6.6 to 6.8), milk can support the growth of a broad range of bacteria. Moreover, milk is a very complex medium consisting of proteins such as casein, lacto-globulins, immunoglobulins, and bovine serum albumin, as well as large lipid globules, minerals, vitamins, and various acids. Hygienic milk production begins at the dairy farms upon milking of healthy udders and cooling of the raw milk to refrigeration temperature to minimize bacterial growth. Thereafter, heat treatment ensures microbiological safety. Heat treatment includes various pasteurization techniques (e.g., high temperature for a short time or low temperature for a long time, or equivalent combinations). Regular pasteurization usually achieves an approximately 90% reduction in viable bacterial counts, eliminating the pathogenic bacteria, while ultra-high-temperature (UHT) treatment sterilizes milk from all viable bacteria and spores. In practice, heat processing of milk can show variability depending on how the milk is going to be marketed, according to the technological requirements of the downstream dairy product, the regulations in force in different countries, and cultural consumer preferences.

Raw milk contamination with bacterial foodborne pathogens such as pathogenic *E. coli*, *Salmonella* spp., *Shigella* spp., *Staphylococcus aureus*, *L. monocytogenes*, and *Cronobacter* (formerly *Enterobacter*) *sakazakii* can originate from the udders, farm environment, or the milking, storage, or transportation equipment. This could particularly pose a problem if milk is to be used for production of artisan or traditional cheeses, a process which requires raw milk. Moreover, inert surfaces in dairy plants and inadequate personal hygiene are associated with contaminations of milk and milk products. Bacterial pathogens may also be present due to pasteurization errors or cross-contamination from the production environment. In addition, throughout the further processing of raw or heat-treated milk to dairy products such as cheese and yogurt, slow or inadequate acidification of the curd may also lead to

growth of pathogenic bacteria. Numerous works have evaluated phage biocontrol in both raw and heat-treated whole or skim milk, as well as various cheeses and other dairy products (Table 29.4).

Garcia and colleagues evaluated the biocontrol activity of a phage cocktail (ϕH5 and ϕA72) against a mastitic *S. aureus* strain in pasteurized whole-fat milk and raw semiskimmed and raw whole-fat milks (97). Interestingly, by the end of incubation (8 to 10 h) at 37°C, the reduction observed in pasteurized milk was higher (3.6 log CFU) than that observed in raw milk samples (2.2 log and 1 log CFU, respectively). Although allof the observed reductions were statistically significant, the lower activity observed in raw whole-fat milk coupled with the decrease in the phage titer led the authors to conclude that the fat globules and the milk proteins might weaken phage activity (97). Indeed, a similar inhibitory effect of raw milk on staphylococcal phage K was observed by O'Flaherty and colleagues (98). Their results indicate that a combinatory effect of both milk fat and milk proteins and, in particular, heat-labile soluble immunoglobulins is responsible for inactivation of phages. When heat-treated milk was used by other researchers for evaluating phage biocontrol against *Salmonella*, *L. monocytogenes*, and *Y. enterocolitica*, promising results were consistently observed (89, 91, 94, 99). In addition, in heat-treated milk, phages may be valuable for the management of bacterial pathogen growth resulting from cold chain failures, a high-risk condition for dairy plants. In their work, Garcia and colleagues mimicked such a breakdown of a cold chain by shifting the incubation temperature of whole-fat UHT-treated milk samples spiked with *S. aureus* and phage cocktail from refrigeration to 22°C (97). Upon the shift after 18 h of refrigeration at 4°C, phages started to propagate, leading to a bacterial reduction of approximately 4.5 log CFU. Such a delayed yet beneficial activity may be favorable especially when heat-treated milk is cross-contaminated or is to be processed into curd for producing various dairy products that may not suppress the growth of bacterial pathogens.

Enzymatic- or acidic-curd production from milk is a widely used technology for many dairy products. In cheese production, the addition of calf rennin (chymosin) to milk cleaves casein, which then can form bonds with Ca^{2+} ions to produce the enzymatic curd. Acidiccurd formation relies on acidification of milk by the production of lactic acid, which then decreases the pH, leading to aggregation of casein molecules. Yogurt and fresh-cheese technologies are primarily based on acidiccurd formation, whereas in hard-type cheese technology, following (or in parallel with) formation of enzymatic curd, acidification of whey is often necessary for achieving the distinct sensory qualities of the cheese product. For acidification purposes, starter cultures are often used, although in certain products the natural flora of raw milk may also be of benefit. Nonetheless, the temperature used during different production technologies and slow or inadequate acidification may allow growth of contaminating bacterial pathogens. Therefore, Garcia and colleagues evaluated the ability of two phage cocktails in biocontrol of *S. aureus* throughout production of both curd types from pasteurized milk (100). Tested phages did not tolerate acidic curd as the pH dropped below 6, but nevertheless, they drastically reduced the *S. aureus* counts to undetectable levels in less than 1 h and 4 h of enzymatic and acidic curdling, respectively.

In another work, the addition of the same phage cocktail to the milk in production of fresh and hard-type cheeses demonstrated promising biocontrol activities (101). In just 6 h after curdling of fresh cheese, *S. aureus* counts became and remained undetectable, whereas at the end of 30 days of ripening of hard-type cheese, the *S. aureus* count dropped from 6 to 1.2 log CFU/g, while the count in the control group increased to ~7.5 log CFU/g (101). Similarly, *Salmonella* Enteritidis counts could be reduced in Cheddar cheese after the addition of phages to either raw or pasteurized milk before curdling, although the activity was superior in the latter, most likely due to heat-labile phage inhibitors (102).

While the above results strongly indicate that phages could be beneficial during production of dairy products against pathogens already present in the milk, several other works have evaluated the biocontrol efficiency of phages during later stages in dairy production, when pathogens contaminate the surface of cheeses. Due to its ubiquitous nature, ability to grow in cold storage, and implication in numerous foodborne outbreaks, *L. monocytogenes* was the focus of these studies. In one work, a surface contamination of 4 log CFU/g of *L. monocytogenes* on pasteurized hard-type cheese was significantly reduced 0.7 log 5 min after treatment with a phage cocktail of 10^8 PFU/g (90). In another

work that simulated the commercial production of surface-ripened Munster-type soft cheeses, a single application of phage P100 at a high concentration (~10^9 PFU/ml) in the washing/smearing solution led to total elimination of the initial 20-CFU/cm^2 surface contamination of *L. monocytogenes*, while in control groups, counts increased up to ~7 log CFU/cm^2 over 21 days of maturation (43). In another type of cheese (queso fresco), which is dependent on acidic curdling, the initial concentration of 3.5 log CFU/cm^2 *L. monocytogenes* was reduced to undetectable levels (<5 CFU/cm^2) after 1 day of storage at 4°C after application of P100, although total elimination was not achieved (103). In addition to the encouraging results observed for the application of phages to the surface of cheeses for biocontrol of *L. monocytogenes*, phages also have a high potential to totally eliminate the bacterium in cheeses that are marketed in brine (e.g., mozzarella) (89).

Finally, *C. sakazakii* is an emerging ubiquitous pathogen that contaminates milk postpasteurization and survives spray-drying production of powdered infant formulas. Due to its high resistance to desiccation and osmotic stress, its wide range of growth temperatures, its thermotolerance, and its ability to form biofilms, powdered infant formulas were primarily implicated in its transmission. Therefore, several research groups evaluated the biocontrol activity of phages against this pathogen, which causes severe clinical infections in immunocompromised individuals and high mortality in infants (Table 29.4). In their pioneering work, Kim and colleagues tested the outcome in two separate phage-host combinations in reconstituted infant formula at 37, 24, and 12°C over 10, 14, and 60 h of incubation, respectively (104). In both phage-host combinations, they demonstrated significant reductions to nondetectable levels after 2 h, with dose-dependent efficiencies. Interestingly, the biocontrol results differed between the two phage-host combinations for different conditions, emphasizing that phage biocontrol outcomes can be influenced by strain-phage combination and their respective intrinsic biology. In a similar setup, Endersen and colleagues evaluated the biocontrol efficiency of a three-phage cocktail in four commercial brands of powdered infant formulas that were spiked with 4 log CFU/ml of *C. sakazakii* and incubated at 37°C (105). They demonstrated total elimination of the test strain in less than 5 h regardless of powdered infant formula brands. Considering that *C. sakazakii* contamination levels are generally much lower (<1 to 100 CFU/100 g) in powdered infant formulas than what was experimentally tested, these studies strongly suggest that phages have high potential for biocontrol of this particular public health concern, although implementation in practice remains a challenge.

Outcome of Phage Biocontrol in Fresh Produce

Fresh produce is a large and very diverse group of foods representing different food environments and food safety problems. In general, microorganisms grow more rapidly in cut vegetables and fruits than in whole products, due to access to the nutrients after the outer barrier of the product has been broken. Vegetables and fresh fruits may be contaminated by pathogens from soil, fertilizers such as manure, and water. In addition, precut RTE vegetables and fruits undergo washing steps in the production facilities, allowing recontamination and cross-contamination of the final product. Shiga-toxin producing *E. coli* strains (including O157:H7), *L. monocytogenes*, *Shigella* spp., and *Salmonella* spp. are frequently implicated in foodborne-illness outbreaks following consumption of contaminated fresh produce. Therefore, these foodborne pathogens have been the main target for phage biocontrol in such food products. However, to implement phage biocontrol in fresh produce in industrial settings, the application of phages must be adjusted for the production line and washing steps, as well as possible inactivation by organic compounds. To date, most food model studies use either spraying, spotting, or soaking of the fresh produce for different durations for application of phages.

Several studies have determined the efficiency of phage biocontrol against *E. coli* O157:H7 on a variety of fresh fruits, vegetables, and sprouts (Table 29.4). Most of these studies were performed using phage cocktails, including commercial products such as ECP-100 or EcoShield, and showed 0.3- to 4.3-log reductions depending on the food and temperature of incubation. For example, the phage cocktail ECP-100 (total 10^8 PFU/cm^2) reduced the number of viable *E. coli* O157:H7 organisms on contaminated (7×10^3 CFU/cm^2) fresh-cut iceberg lettuce by 2 log after 2 days at 4°C (106). Interestingly, the use of same

phage cocktail to reduce *E. coli* O157:H7 on fresh-cut cantaloupe wedges resulted in a larger reduction at 4°C than at 20°C, suggesting that the phage cocktail was more effective at a lower temperature. However, the application method varied, as the phages were sprayed on lettuce and spotted on the melon wedges (106). The application of the EcoShield phage cocktail to leafy greens lowered *E. coli* O157:H7 counts and was more effective when the leafy greens were packaged under modified atmosphere. However, the reduction in *E. coli* O157:H7 counts in modified atmosphere was not significantly different from that observed in atmospheric-air storage (107).

Phages infecting *Salmonella* were used for biocontrol on the surface of fresh produce, such as fruits, lettuce, cucumber, and sprouts, and demonstrated significant reductions in bacterial counts. A cocktail composed of three phages (UAB_Phi20, UAB_Phi78, and UAB_Phi87) significantly reduced *Salmonella* Typhimurium and Enteritidis counts in packaged lettuce by 3.9 and 2.2 log CFU/g, respectively (108). The lettuce was experimentally contaminated by *Salmonella* by immersion in a suspension of the bacterial inoculum (10^5 CFU/ml) for 5 min and subsequently exposed to the phage cocktail at an MOI of 10^4 by immersion for 5 minutes and then incubated at 60 minutes at room temperature, resulting in significant reductions (108). In another work, whole fresh cucumbers were spiked with *Salmonella* Newport (10^5 CFU per cucumber) and sprayed with 10^{10} PFU/ml of a six-phage cocktail, which led to a significant reduction after incubation at both 10 and 22°C (109). Other studies have shown significant reductions of *Salmonella* in sprouts using a single phage or a cocktail of six phages, leading to 2.5-log reduction at 22°C and a 3.4-log reduction at temperatures ranging from 20 to 30°C, respectively (110, 111). Phages have also been used to target *L. monocytogenes* present on the surface of lettuce by spraying the phage cocktail ListShield, resulting in 1.1-log reductions (90). Finally, phages have been used to target *L. monocytogenes* by spraying a cocktail of 12 or 6 phages on fresh-cut apple and melon. On apples, the reduction was below 0.4 log, whereas on melon, phages were able to reduce *Listeria* by 2.2 to 4.6 log, suggesting that the acidity of the apples may have prevented the efficient reductions of the pathogen (112). These experiments demonstrate that food conditions, such as acidity, have a large impact on the outcome of phage biocontrol in fresh produce.

CHALLENGES AND OUTLOOK FOR INDUSTRIAL USE OF PHAGES

Phages are increasingly used for biocontrol of pathogenic bacteria in the food chain, yet many challenges remain for their successful widespread implementation, including adjustment of existing regulatory frameworks and the efficiency of phage products. Moreover, public understanding of the drivers for novel solutions in foodborne pathogen control and acceptance of phage applications in food biocontrol play key roles in making phage biocontrol widely applicable. Hence, education of consumers and food producers on the safety, efficacy, biology, and ubiquity of bacteriophages is important. Many more activities, including communication of the science behind the use of phages, the potential applications, and general knowledge of phages, are already being promoted largely on social media, websites, and in the press. Some of these activities are driven by independent scientists and scientific communities, but the increasing number of phage companies and food consultants also plays an important role in communicating the pros and cons of phage applications.

In this section, our main focus is on the technical and regulatory challenges of widespread industrial use of phages for biocontrol of foodborne pathogens in foods to discuss the future of a broader implementation of such solutions. The major technical challenges of phage biocontrol are primarily associated with their efficiency, which is influenced by a number of biological and technical parameters described throughout this chapter and reviewed by Moye and colleagues (113). In summary, the efficiency of phage biocontrol is dependent on (i) the specific host range of the phage as well as the diversity of the target bacterium; (ii) the probability that the phage will come into contact with the target bacteria in the food or animal gut; (iii) the killing activity of the phage within the food matrix or animal gut; (iv) the mode of application or administration of phage on the food or in the animal; and (v) the emergence of phage-resistant bacterial populations. As explained previously, there are ways to overcome the specificity and activity challenges by designing and testing phage cocktails in settings relevant for the application. Furthermore, application procedures should ensure successful delivery of

the phage to relevant area of the food where the target bacterium is more likely to be found. While isolation of novel phages may be important for developing efficient phage cocktails consisting of mutually complementary phages, the concept of phage training in the laboratory to select for phages that are able to overcome phage-resistant populations or have expanded host ranges may also be used as a tool to improve the phage solutions for the food industry (114). It is also important to develop innovative ways to administer phages to animals.

Widespread applicability of phage biocontrol requires documentation of efficacy and reproducibility, especially in industrial settings using naturally contaminated food items. However, this poses a challenge, in particular for pathogens that are present in low numbers or at a low prevalence, consequently making it difficult to provide such documentation due to the enormous number of samples needed to demonstrate statistically significant reductions. However, such work is in progress for some pathogens and is expected to complement the necessary documentation for supporting efficient implementation of phage solutions that targets low-number and low-prevalence foodborne pathogens as well. Finally, even though phages do not usually entirely eliminate the pathogenic bacteria, they may still render the food safer for consumption by reducing the pathogen numbers by > 1 to 2 log, thus providing significant improvements in food safety and public health due to decreased risk of exposure.

Several commercialized phage preparations have obtained permissions from regulatory bodies in the European Union and United States. In the United States, these phage products are approved as food additives, and the FDA had issued no-objection letters consolidating certain phage biocontrol products on the GRAS (generally recognized as safe) list. To date, the application of GRAS seems to be the general route of approval for phage products intended for biocontrol of foodborne pathogens in foods in the United States. The GRAS designation applies to lytic phages that are not genetically modified, as phages are already inherently present in food. Moreover, USDA guidelines for safe and suitable ingredients for production of meat, poultry, and egg products include the application of phages to animals prior to slaughter using *E. coli* O157:H7 phages on cattle hides, *Salmonella* phages on poultry feathers, and phage preparations targeting *Salmonella* in meat. Even though these guidelines refer to specific phage products, it is anticipated that other phage products meeting the criteria in the directive may be considered compatible. In the European Union, the European Commission issues approvals of phage products for use on foods based on expert evaluations made by the European Food Safety Authorities (EFSA). Similar to the U.S. FDA, the EFSA evaluated the safety and efficacy of a phage product targeting *L. monocytogenes* and stated that “[T]he material should not present human toxicological problems because the bacteriophage P100, used as active principle, is not regarded as harmful to consumers nor to organisms other than *Listeria* spp., and because the fabrication parameters do not include anything obvious that might compromise safety” (115). Thus, regulatory bodies acknowledge the safety of specific phage products, and it is anticipated that documentation of safety of similar phage products will promote the approval of other phage products for use in the food industry.

For phages to be used to their full potential, regulatory pathways must include defined phage cocktails as well as permission to update the cocktails according to the need. Such constant evolution of phage solutions appears to be the key in providing effective phage products when emerging phage resistance is considered. A similar approach has been implemented in both Georgian and Polish phage therapy centers that maintain extensive therapeutic phage collections which are regularly supplemented with new phages. Importantly, this will ensure the expansion of the total host range of the collection and allow phage cocktail adjustments in response to changing bacterial populations. The current legislation does not allow such adjustments, but the urgent need for alternative antimicrobials may promote the needed changes. Recently, an expert opinion proposed that phage therapy should have its own regulatory platform, allowing more flexible approaches for composition and adaptation of phage products (58) that promote phage training as a means to overcome phage-resistant variants.

Over the past decade, several phage companies with product portfolios targeting pathogenic bacteria relevant for the food industry have emerged (113), suggesting that several phage solution options will soon be more broadly available to food manufacturers. Importantly, phages have much more to offer

other than just killing bacteria. Phages also encode antimicrobial enzymes that may be applied and optimized for killing of pathogenic bacteria in the food chain. To date, these enzymes have mainly been developed for clinical use in human medicine, but as manufacturing prices fall, enzymes may also become applicable for biocontrol in the food industry. Even though such enzymes have mainly been developed to kill Grampositive bacteria, current research also focuses on modifying the enzymes to target Gram-negative bacteria (116, 117). Furthermore, synthetic biology and phage engineering are being used to develop phages targeting specific bacteria or genetic elements, such as phages used for targeted delivery of antibacterial genes, such as those encoding RNA-guided nucleases that specifically degrade the DNA of antibiotic-resistant bacteria (118, 119). Thus, the development of novel phage solutions, including phage enzymes and designer phages, will most likely follow a more widespread use of phages for biological control of foodborne pathogens in the future.

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VI
Fermentations and Beneficial Microbes

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30
Starter Cultures

The production of fermented foods and beverages is one of the oldest biotechnological practices, dating back 7,000 years or more (1). For the vast majority of the intervening time, the biological nature of such fermentations was unknown—the production of these foods was an art or craft based on a series of operations which yielded products that were not only better preserved than the original raw material but also organoleptically pleasing. Throughout human history, many different substrates of animal or plant origin have been fermented, including milk, meat, fish, various vegetables, and soybeans.

The manufacture of fermented foods, although rooted in practices developed over many thousands of years, is today a major biotechnological activity which is valued at many billions of dollars. Fermented foods are generally viewed as being healthful in addition to possessing many organoleptic attributes that are attractive to the consumer. Consequently, the market for these products, particularly dairy products, has been experiencing significant growth, and more recently, new markets have been developed in Asia and Africa for Western-type fermented products. Of course, the converse is also true; many fermented oriental products are gaining increasing acceptance in the Western world.

It is remarkable that the success of such a large and diverse activity is critically dependent on the activity of microorganisms, particularly yeasts and the lactic acid bacteria (LAB). The yeasts are primarily involved in the production of alcohol and in raising dough

and are not the subject of discussion in this chapter; here, we focus exclusively on the LAB, which are central to the production of a wide range of fermented milk, meat, vegetable, soy, and other products. These are the microbes for which the term “starter culture” was specifically coined, as they are added deliberately to a substrate to start a given fermentation process.

This chapter provides a detailed examination of the LAB, outlining their key functional properties, particularly those relevant to their role in fermentation. The historical development of starter cultures, their composition, the formats in which they are commercially available, and the particular issues pertaining to the use of starters in a modern, high-throughput industrial setting are also discussed.

In many regards, the impact of bacteriophages (phages) on the starter culture applications in industrial practice represents an unusual ecological scenario. Bacteriophage infection of starters can impede or destroy starter activity, and controlling them has been a constant theme of starter application since the issue was first highlighted in the mid-1930s (2). Recent advances in the analysis of phage-host interactions, employing molecular and genomics approaches, have provided several knowledge-based strategies for dealing with this problem. These are also reviewed in this chapter. Finally, the grand challenges relating to the use of starters in the production of fermented foods are examined, particularly as they relate to strain diversity and the generation of new products with modified flavor, texture, and health attributes.

DEVELOPMENT OF STARTER CULTURES

The start of the golden age of microbiology, coinciding with the work of Pasteur, Koch, and many others, represented the beginning of our understanding of the processes involved in food and beverage fermentation. Early investigations led to the isolation and identification of bacteria that were typically linked with fermented foods, particularly fermented milk products. Initial efforts to

characterize these bacteria demonstrated their ability to produce lactic acid, and it became clear that the number of different bacteria associated with such products was quite limited. The first of these lactic acid-producing bacteria was characterized by Lister in the 1870s, and he named it *Bacterium lactis* (with subsequent redesignation as *Streptococcus lactis* and *Lactococcus lactis* [the name currently in use] as taxonomic methods improved through the ensuing decades).

Subsequently, additional members of the LAB were identified in a range of fermented milks and other foods. This burst of information regarding the association between fermented foodstuffs and a distinct group (from a functional rather than taxonomic perspective) of bacteria coincided with the rapid transition in the manufacture of some fermented products, especially cheeses, from a small-scale local activity to a full-scale industrial process. It is generally accepted that the first Cheddar cheese-manufacturing factory, using milk from multiple suppliers, was founded by Jesse Williams in Rome in upstate New York in the United States in 1851 (3). By 1880 there were many hundreds of such factories. It quickly became clear that such large-scale operations could not reliably depend on the indigenous LAB, or "backslopping," whereby a sample derived from a previous batch of product (this could be whey in the case of cheese) is added to assist in the fermentation process. The fortuitous coincidence of the discovery of LAB with the need to develop processes for cheese manufacture that could cope with the demands of continuous production on an industrial scale led to the development of starter cultures. Initially, these were simply liquid cultures that were subject to serial transfer and scaling up so that sufficient inocula could be developed to start the fermentation. The use of such cultures provided the manufacturer with a far higher degree of reliability and consistency of performance, and consequently a better-quality product. This process also enhanced product safety.

Later, methods were developed to oven-dry milk cultures or to produce frozen preparations in a manner which allowed survival of

the bacteria. These had the advantage of providing material that was suitable for shipping to geographically distant customers. A further major development was the application of lyophilization to the commercial production of LAB; depending on the concentration of the cells in the preparation, this could mean that there was no need to propagate the starter culture in the manufacturing plant. These technologies are now almost universally employed for the preparation of starter cultures, and a relatively small number of commercial enterprises supply the majority of the world's demand. Considerable research has been devoted to developing these technologies to ensure minimal impairment of the activity of the starters when they are subsequently inoculated into a bulk starter tank or the cheese vats in the factory.

As is evident throughout this chapter, continuous research has been devoted to developing high-performing starter cultures, and this progress has been most evident in the cheese starters, and probably most obviously with those used in Cheddar manufacture. The revolution that was enabled by the application of molecular genetics to the LAB since the 1980s provided an approach to understanding functionality of starter cultures at the level of the gene, a process that has been accelerated considerably in the more recent era of "-omics" technologies. This has been particularly true in the key areas of metabolism, flavor production, environmental sensing, stress responses, and bacteriophage-host interactions. The improved understanding that has been gained over recent decades has allowed a knowledge-based approach, rather than the empirical screening and selection approach, to be applied to improving the quality and reliability of starters (notwithstanding the limitations imposed by regulatory barriers, which in different jurisdictions can be more or less of an impediment to the final application of modified strains).

These developments and their impact, which are described below, may in fact not be universally beneficial in that selection based on genomics-derived information could lead to an ever-narrowing selection funnel, yielding a small group of "ideal" strains, which may result in loss of culture diversity and, consequently, product

diversity. Other advances relating to growth, harvesting, tolerance to drying and freezing, and resuscitation have resulted in starter formats that are more suited to the needs of the manufacturer and more reliable in the high-throughput megafactories that are becoming increasingly common. These developments are also discussed.

LACTIC ACID BACTERIA AND FOOD FERMENTATIONS

The LAB are a diverse group of Gram-positive, obligately fermentative microorganisms that produce lactic acid as the main product of sugar metabolism. This group of bacteria is currently classified in the order *Lactobacillales* and comprises species of the genera *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Leuconostoc*, *Oenococcus*, and *Pediococcus*, among others. In general, LAB have complex nutritional requirements and therefore are usually associated with nutrient-rich environments, occupying a diverse range of ecological niches that includes plants and fermenting plant material, milk, and the gastrointestinal and genitourinary tracts of animals and humans. For millennia, these organisms have been employed in various fermentation processes for the production of fermented foods and beverages, such as cheese, yogurt, wine, and sourdough bread. In more recent times, some members have been shown to have health-promoting effects and are used as probiotics, i.e., live microorganisms used as food supplements in order to benefit health (4, 5). The food-related LAB are afforded GRAS (generally recognized as safe) status, as a result of their long history of use in food fermentations.

Members of the LAB group play an essential role in the food processing industry, where they are used as starter or adjunct cultures for the production of fermented dairy, meat, and plant-derived foods. The technological properties associated with the

different species are central to their selection as starters tailored to meeting particular product requirements (Table 30.1).

Various *Lactobacillus* species are employed as starters in the manufacture of fermented meats. These include *Lactobacillus plantarum*, *Lb. pentosus*, *Lb. sakei*, and *Lb. curvatus*. *Pediococcus* species, including *Pediococcus acidilactici* and *P. pentosaceus*, may also be used in meat fermentations. LAB such as *Leuconostoc* and *Lactobacillus* are responsible for the fermentation of cabbage to produce sauerkraut. Starters employed in the production of sourdough often include *Lactobacillus sanfranciscensis*. Finally, *Oenococcus oeni* is a wine-associated LAB responsible for wine malolactic fermentation, in which malic acid is converted to the softer-tasting lactic acid.

Table 30.1. List of major LAB and their primary applications in food fermentations

LAB	Primary application(s)
Lactococcus lactis	Starter culture used in the production of fermented milk products, e.g., cheese, sour cream, lactic butter, and buttermilk
Streptococcus thermophilus	Starter culture used in the production of fermented milk products, e.g., cheese and yogurt
Lactobacillus spp.	Starter and adjunct cultures used in the production of fermented dairy, meat, and plant products, e.g., cheese, yogurt, sauerkraut, kimchi, and fermented sausage
Weissella spp.	Present in "wild" fermentations, e.g., kimchi and sauerkraut production. Not deliberately added as a starter culture due to link to biogenic amine production.
Oenococcus oeni	Malolactic fermentations in wine production
Pediococcus spp.	Meat and plant fermentations
Leuconostoc spp.	Meat and milk fermentations

KEY FUNCTIONAL ACTIVITIES OF LACTIC ACID BACTERIA

The LAB display a number of key functional activities that are particularly relevant to their role as starter cultures in dairy and food fermentations. These include specific metabolic capabilities, such as the ability to ferment the uniquely milk-associated sugar lactose, as well as other traits, such as bacteriophage resistance, which can be essential in ensuring a successful fermentation.

Sugar Metabolism

LAB metabolize sugars or other carbohydrates through a fermentative process whereby energy is derived from the partial oxidation of an organic compound using organic intermediates as electron donors and electron acceptors (6). ATP is produced by substrate-level phosphorylation, without the requirement for a membrane or electron transport system.

From a biochemical perspective, the LAB include both homofermentative and heterofermentative organisms (Fig. 30.1).

1. Homofermentative LAB produce lactic acid as the primary end product of their fermentations and include *Lactococcus*, *Streptococcus thermophilus*, *Pediococcus*, *Enterococcus*, and certain species of *Lactobacillus*, e.g., *Lactobacillus casei*, *Lb. plantarum*, and *Lb. curvatus*.
2. Heterofermentative LAB produce a number of fermentation products in addition to lactic acid, such as carbon dioxide, ethanol, acetic acid, and formic acid, and include *Leuconostoc*, *Oenococcus*, and certain *Lactobacillus* species, e.g., *Lactobacillus brevis*, *Lb. fermentum*, and *Lb. reuteri*.

The first step in the metabolism of sugars is their transport across the cytoplasmic membrane. Many different transporters exist in LAB, which are generally sugar specific and can be categorized as follows:

1. Phosphoenolpyruvate-phosphotransferase systems (PEP-PTS) that couple sugar transport to its phosphorylation
2. Ion-linked sugar exchange mechanisms, which are secondary transport systems where the sugar uptake is driven by an ion gradient
3. Sugar ATPases, primary transport systems that couple ATP hydrolysis with translocation

Sugars then enter either the Embden-Meyerhof-Parnas (EMP) pathway, in the case of homofermentative LAB, or the phosphoketolase pathway, in the case of heterofermentative species.

Due to the economic relevance of lactose fermentation, the metabolism of this disaccharide has been studied extensively in LAB. In *L. lactis*, the lactose metabolism process starts with translocation of the sugar either via a lactose-specific PEP-PTS to yield lactose 6-phosphate or by a permease. Phosphorylation of lactose by PEP occurs as it is transported into the cell via the membrane-bound lactose-specific PTS. Lactose-6-phosphate is hydrolyzed by phospho-β-galactosidase (encoded by *lacG*) into glucose and galactose-6-phosphate (Gal-6-P). Glucose is phosphorylated by glucokinase, when it then enters glycolysis via the EMP pathway and is metabolized to pyruvate, which is converted to l-(+)-lactic acid by lactate dehydrogenase (Fig. 30.1). Two ATP molecules are generated for each glucose molecule processed. Simultaneously, Gal-6-P is further metabolized by enzymes of the tagatose pathway to the glycolytic intermediates glyceraldehyde-3-phosphate and dihydroxyacetone-phosphate.

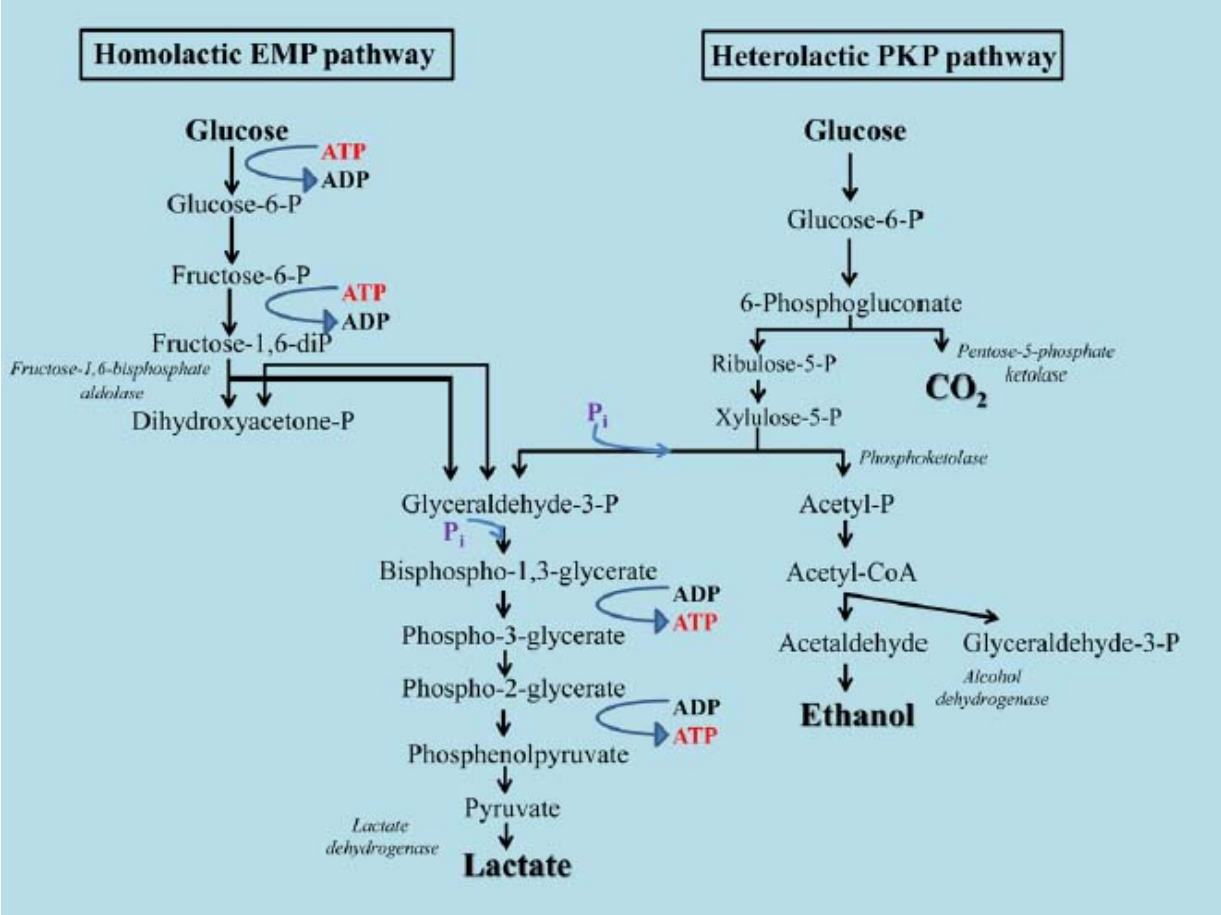


Figure 30.1. Homolactic and heterolactic fermentation pathways for glucose employed by different LAB.

The enzymes for lactose metabolism are generally plasmid-encoded in *L. lactis*. Some lactococci employ a chromosomally encoded lactose permease–β-galactosidase system, depending on *lacZ*, which encodes β-galactosidase. In this case, lactose uptake by a permease is followed by hydrolysis via β-galactosidase, yielding glucose and galactose. Galactose is further metabolized via the Leloir pathway to glucose-1-phosphate, which can, after isomerization to glucose-6-phosphate via a phosphoglucomutase, enter the glycolytic pathway.

With the exception of *L. lactis* and *Lb. casei*, homofermentative LAB do not possess a lactose PTS, and instead, they use a lactose permease system (*lacS*) and an intracellular β-galactosidase (*lacZ*) for metabolism of lactose. Lactose metabolism in the *L. lactis* species also differs from lactose metabolism by other

homofermentative LAB, as in the former, simultaneous catabolism of glucose and galactose occurs. In the majority of other cases, such as in *S. thermophilus* strains, only the glucose moiety is further processed via the EMP pathway, while the galactose moiety is excreted into the growth medium.

In heterofermentative LAB, including some species of *Lactobacillus* and *Leuconostoc*, the phosphoketolase pathway is the major route of glucose metabolism (Fig. 30.1). These bacteria typically produce equimolar amounts of CO_2 plus lactate and ethanol or acetate from the catabolism of glucose. The efficiency of this pathway in terms of ATP generation is about half that of the EMP pathway, as only one ATP is produced per molecule of glucose. Many heterofermentative species also ferment glucose and galactose simultaneously, with galactose being metabolized via the Leloir pathway.

Proteolysis

Members of the LAB are nutritionally fastidious organisms that require an exogenous source of many of the amino acids and peptides necessary for growth (5). For milk-based LAB, the degradation of the main milk protein, casein, through the proteolytic cascade provides the cells with the essential amino acids they require. The first step in the process is initiated by a single cell envelope proteinase (designated CEP or PrtP) which partially degrades the casein into a large number of oligopeptides (Fig. 30.2). CEP enzymes are serine proteases belonging to the subtilisin family and are divided into two main classes based on specificity. Enzymes of the PI class primarily target β-caseins and to a lesser extent κ-casein, while the PIII type are also able to degrade αs1-casein. In general, LAB species possess only one CEP; however, the presence of two enzymes has been described for some lactobacilli. LAB cannot grow in milk in the absence of a functional CEP. However, species that do not exhibit this activity can often grow in milk in the presence of a CEP-positive strain.

In the second step of the process, oligopeptides generated as a result of CEP action are taken into the cell via specific peptide transporters. The main peptide transport system functional in LAB is the oligopeptide permease (Opp) system (Fig. 30.2), identified in *L. lactis*, *Lactobacillus bulgaricus*, *Lb. helveticus*, *Lb. plantarum*, *Lb. sanfranciscensis*, and *Leuconostoc mesenteroides*. The Opp proteins belong to a superfamily of highly conserved ATP-binding cassette transporters that mediate the uptake of casein-derived peptides. This system has been studied extensively in *L. lactis*, where it has been shown to transport peptides up to 18 residues in length. Other active transport systems include a proton motive forcedriven DtpT, which transports di-, tri-, and tetrapeptides containing relatively hydrophobic branched-chain amino acids and which has been identified in *Lb. helveticus*, *Lb. sanfranciscensis*, and *S. thermophilus*. An ATP-driven Dpp (previously referred to as DtpP), with an affinity for more hydrophilic and charged di- and tripeptides, has also been reported in LAB (Fig. 30.2).

After uptake into the cell, the next step in the proteolytic cascade involves degradation of the casein-derived peptides to free amino acids through the combined action of a series of peptidases. These are intracellular enzymes with differing but overlapping specificities that cleave internal peptide bonds of casein-derived peptides. Peptidases can be divided into two types: endopeptidases, which hydrolyze internal peptide bonds, and exopeptidases, which remove amino acids from the end of a peptide chain.

In LAB, the majority of the exopeptidases are aminopeptidases, and their specificity depends on the peptide length and the nature of the N-terminal (or penultimate) amino acid residue. The first peptidases to act are the broad-specificity metallopeptidase PepN, the cysteine peptidase PepC, and the X-prolyl dipeptidyl aminopeptidase PepX (Fig. 30.2). These have been identified in *Lactococcus*, *Lactobacillus*, and *S. thermophilus*. The products generated by these enzymes are then subjected to further cleavage by the tripeptidase PepT and the dipeptidases PepV and PepD. Other enzymes with more targeted substrate specificities are also present.

Examples include PepA, an aminopeptidase acting on acidic residues found in *L. lactis*, *S. thermophilus*, and *Lactobacillus gasseri*; PepS, specific for aromatic residues and found in *P. pentosaceus*, *S. thermophilus*, *Leuconostoc mesenteroides*, *Lb. casei*, and *Lb. sakei*; and PepL, identified only in *Lactobacillus delbrueckii* and displaying high specificity for Leu and Ala residues. The genes encoding the peptide transport systems and the peptidases are typically chromosomally linked, while those encoding the proteases are exclusively plasmid associated.

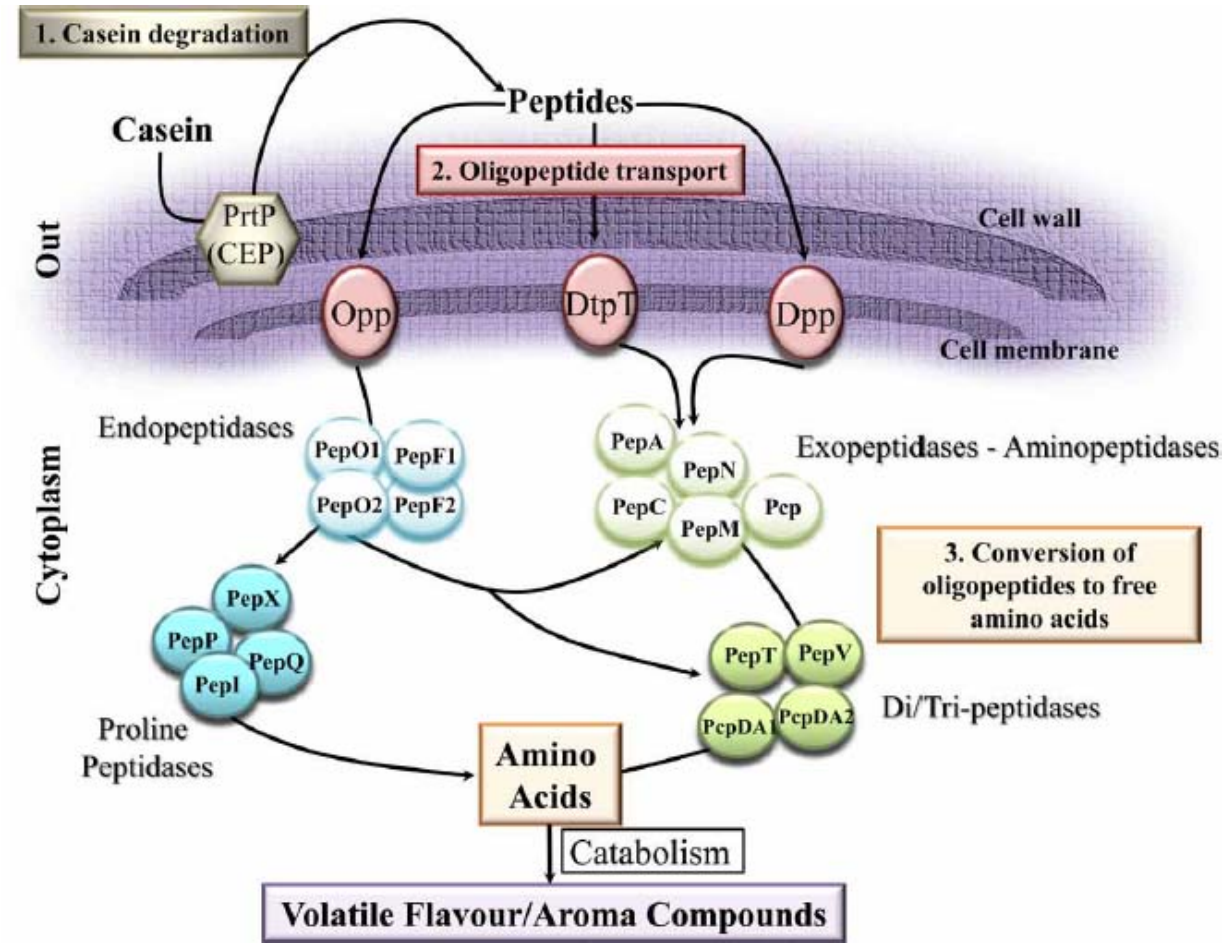


Figure 30.2. Schematic representation of the proteolytic system of *Lactococcus* showing the cell membrane-anchored proteinase PrtP, the membrane-located transport systems (Opp, DtpT, and Dpp), and the array of intracellular peptidases, including endopeptidases, aminopeptidases, di- and tripeptidases, and proline peptidases, that are involved in protein/peptide catabolism and the ensuing release of compounds associated with flavor and aroma.

The subsequent metabolism of free amino acids is an important resource for LAB, and they are used for a number of physiological purposes, such as intracellular pH control, generation of metabolic energy or redox power, and resistance to stress. It is also an industrially important trait, as the end products of amino acid catabolism, such as acids, aldehydes, alcohols, and esters, contribute greatly to defining the flavor and aroma of fermented dairy products. The catabolism of amino acids also has implications with

regard to the safety of fermented foods, as the synthesis of biogenic amines could possibly be a consequence of their activity. In general, the ability of LAB to metabolize amino acids varies greatly among species and is tightly regulated.

Amino Acid Metabolism and Flavor Formation

Flavor development in dairy fermentations is the result of a series of chemical and biochemical processes which occur during the fermentation and ripening of a product (7). Flavor compounds are formed by various biochemical pathways:

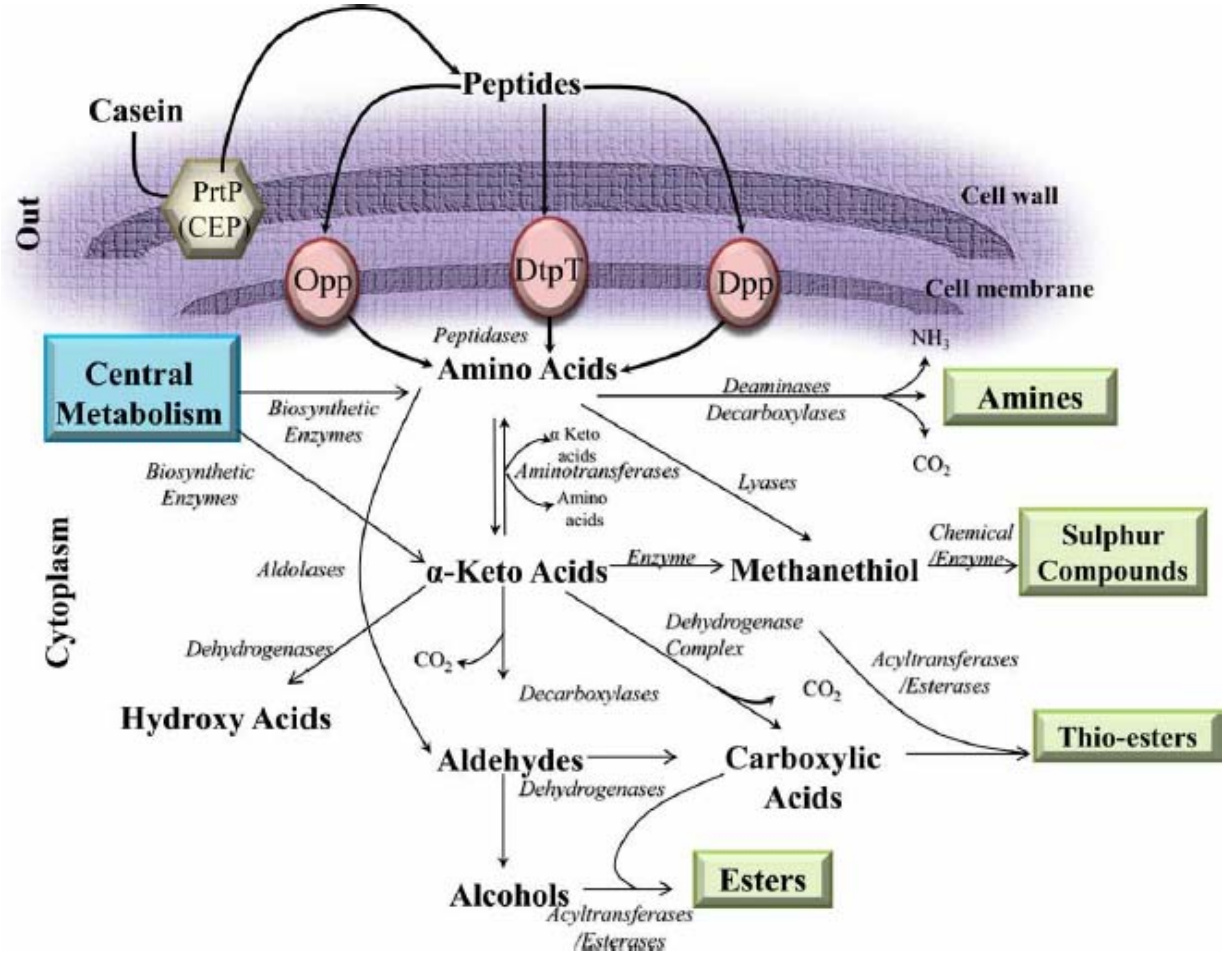


Figure 30.3. Pathways observed in *Lactococcus* through which amino acids are converted to compounds which are significant in the development of flavor in ripened cheeses.

1. Glycolysis, where the conversion of lactose via its intermediate pyruvate to various flavor compounds (diacetyl, acetoin, acetaldehyde, and acetic acid) contributes to typical yogurt or buttery flavors
2. Proteolysis, with free amino acids forming the precursors for many volatile flavor-forming compounds
3. Lipolysis, with the formation of free fatty acids as precursors of flavor compounds such as methyl ketones, alcohols, lactones, and esters
4. Citrate metabolism by certain LAB species, resulting in the production of diacetyl

However, it is proteolysis, and more specifically the catabolism of free amino acids to various alcohols, aldehydes, acids, esters, and sulfur compounds, that is undoubtedly the most important biochemical process for flavor formation in many dairy products but particularly in hard ripened cheeses, such as Cheddar and Dutch types (Fig. 30.3). Certain amino acid groups are the major precursors of volatile compounds, which confer flavor and aroma in fermented dairy products, and the pathways associated with the production of the flavor compounds are highlighted in Fig. 30.3. The amino acid groups involved in volatile compound formation include (i) the branchedchain amino acids valine, isoleucine, and leucine, which are precursors of aroma compounds such as isobutyrate, isovalerate, 3-methylbutanal, 2-methylbutanal, and 2-methylpropanal, found in various cheese types; (ii) the aromatic amino acids tyrosine, tryptophan, and phenylalanine, the products of which are involved in the development of off flavors in certain products; and (iii) the sulfur-containing amino acids (methionine and cysteine), which are linked to the production of potent volatile compounds, including methanethiol, dimethyl sulfide, and dimethyl trisulfide, that are considered essential flavor components in many cheese varieties.

Lipid Metabolism

Lipolysis by LAB is thought to play some, perhaps small, role in the flavor development of certain dairy products, such as Cheddar cheese (8). Free fatty acids (FFA) can contribute to flavor and can be further converted into methyl ketones, lactones, or thioesters, which can also influence the final flavor profile. Starter LAB and the nonstarter microbiota are believed to represent the main sources of lipolytic agents in pasteurized Cheddar cheese. In order to hydrolyze milk fat, starters need to possess active lipolytic and esterolytic enzymes, capable of hydrolyzing FFA and other fat-based substrates. However, it is generally accepted that LAB are weakly lipolytic, and to date, the lipolytic and esterolytic systems of dairy LAB remain poorly characterized. Despite this, the extended presence of LAB starters and adjuncts in Cheddar cheese over the ripening time is thought to play a significant role in the generation of FFAs. Members of the LAB which exhibit lipolytic activity include *L. lactis*, *Lb. casei*, *Lb. fermentum*, *S. thermophilus*, and *Lb. plantarum*.

EPS Production

Many LAB produce extracellular polysaccharides, or exopolysaccharides (EPS), with various chemical compositions and properties (9). Production of EPS by starter cultures can alter the rheological properties of fermented products, leading to typically viscous and “ropy” characteristics. Starter production of EPS can have a significant impact on the properties of certain fermented dairy products, such as yogurt, cheese, milkbased desserts, and sourdough bread. For example, the use of the EPS-producing starters *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* in yogurt production leads to improved consistency, texture, and mouthfeel of the product, whereas in sourdough production, EPS produced by *Lb. sanfranciscensis* facilitates water absorption, softening the gluten content of the dough.

EPSs generally exist in one of two forms: cell-bound EPSs, which closely adhere to the bacterial surface as capsular material, and free

EPSs, which are released into the surrounding medium.

EPSs produced by LAB are often distinguished as either ropy or non-ropy. These long-chain molecules consist of branched, repeating units of sugars or sugar derivatives, are synthesized by highly specific glycosyltransferase enzymes, and can be classified into two groups. Homopolysaccharides consist of one type of monosaccharide (e.g., □-d-glucans, β-d-glucans, or fructans) and are produced by *Leuconostoc* species, some *Streptococcus* species (but not *S. thermophilus*), and certain *Lactobacillus* species (e.g., *Lb. delbrueckii* subsp. *bulgaricus* and *Lb. reuteri*). The majority of EPSs produced by LAB are heteropolysaccharides, composed of different types of monosaccharides, mainly d-glucose, d-galactose, l-rhamnose, and their derivatives. EPSs produced by *L. lactis*, *S. thermophilus*, and many food-related *Lactobacillus* species are of this type.

The genetic determinants for EPS production are typically plasmid encoded in the case of mesophilic starters, while for *S. thermophilus* and thermophilic species of *Lactobacillus*, they are chromosomally encoded functions, and their production is typically more stable. EPS biosynthesis is a complex process, with many enzymes and regulatory proteins involved. It typically begins with sugar transport into the cytoplasm, followed by the synthesis of sugar-1-phosphates, polymerization of the repeating units, and finally export of the EPS. Each of these steps is crucial and can be manipulated to modify the amount and composition of the EPS.

Bacteriophage Resistance

Phage contamination is a significant risk to any industry that relies on fermentation for product manufacture, as it can lead to delays in production, lower product quality, or, in the worst cases, total loss of production. Careful selection of LAB starter cultures known to harbor natural resistance mechanisms that prevent or limit infection by common phages is one control method that can be adopted to minimize the risk. A diverse range of phage resistance

systems have been identified in various LAB species (10), and this is discussed in detail below (see “Bacteriophages”). Furthermore, progress in research on bacteriophages of LAB and their interactions with their hosts and how this knowledge can be utilized to develop more robust starter strains is also discussed in more detail below.

Bacteriocin Production

Bacteriocins are ribosomally synthesized antimicrobial compounds produced by a variety of bacteria that kill closely related bacteria. Bacteriocins have been classified into a number of categories.

1. Class I, the best known of which are the lantibiotics, is defined as small membrane-active peptides (<5 kDa) containing modified amino acids such as lanthionine or β-methyl lanthionine and other dehydrated residues.
2. Class II is the small heat-stable unmodified or minimally modified peptides.
3. Bacteriolysins, formerly referred to as class III bacteriocins, are large heat-labile proteins often with enzymatic activity.

The best-studied LAB bacteriocin is nisin, a lantibiotic produced by *L. lactis* and widely used as a biopreservative in a diverse range of foods (11). It exhibits activity against a broad spectrum of Gram-positive bacteria and is particularly effective against endospores. It acts by disrupting the integrity of the bacterial cell membrane by forming pores. This leads to the dissipation of the membrane potential and the efflux of small metabolites and other cytoplasmic solutes, resulting in the termination of allbiosynthetic processes, leading to cell death. The genetic determinants of nisin production make up an 11-gene cluster, *nisABTCIPRKFEG*, which is typically chromosomal.

Other well-characterized LAB bacteriocins include the two-component class I lantibiotic lacticin 3147, produced by *L. lactis*; the

class II bacteriocins pediocin PA-1 and enterocin A, produced by *P. acidilactici* and *Enterococcus faecalis*, respectively; and the bacteriolysin helveticin J from *Lb. helveticus*. While the production of bacteriocins can be problematic, as it may limit the growth of other useful starter cultures in some fermentations (e.g., in cheese production), bacteriocin-producing strains are routinely incorporated into certain starter culture blends (e.g., those involved in meat fermentations) to limit the proliferation of pathogenic organisms such as *Listeria* (12).

Autolysis

Autolysis can be defined as the spontaneous disintegration of the bacterial cell through the action of the cell's own intracellular peptidoglycan (PG) hydrolases. These enzymes are often referred to as autolysins. Autolysis of cells is the result of the uncontrolled action of PG hydrolases when cell wall assembly and/or repair is inhibited. This action is potentially lethal, and so the activity must be tightly regulated. The enzymes play an important role in cell division, cell wall synthesis, and cell wall turnover. During cheese ripening, these enzymes are crucial, since they allow the cheese microbiota to release their enzyme-rich cytoplasmic content into the cheese matrix during the maturation process, which results in the development of characteristic organoleptic properties. The rate of autolysis is strain dependent and is also influenced by the environment and the cheese-making process.

Autolysins have been characterized in some LAB species. The glycan chains of PG can be cleaved by *N*-acetylglucosaminidases or *N*-acetylmuramidases. *N*-Acetylmuramoyl-l-alanine amidases hydrolyze the bond between the glycan chain and the pentapeptide side chain, while carboxypeptidases and endopeptidases can cleave the different bonds of the peptide side chain. *L. lactis* has four *N*-acetylglucosaminidases, namely AcmA, AcmB, AcmC, and AcmD. AcmA is required for cell separation and is the major autolysin that is responsible for lysis during the stationary phase of growth of *L. lactis*.

Besides the genome-encoded PG hydrolases, phageencoded endolysins can also contribute to autolysis of lactococcal cells (13). Endolysins are generally coexpressed with holins that form pores in the cytoplasmic membrane of the host, thereby abolishing membrane potential and allowing translocation of the endolysin over the membrane. Natural expression of phage lysins can lead to an increase in the release of intracellular peptidases needed for enhanced cheese ripening.

STARTER CULTURE TYPES FOR DIFFERENT PRODUCTS

Starter cultures can be categorized in a number of different ways, often reflecting their composition or specific functional attributes. Some of these classification systems are used by industry practitioners in particular and are more readily applicable to cheese starters. Cultures are generally characterized as either mesophilic cultures, which have an optimum growth temperature in the range of 27 to 33°C and can be non-gas producing or gas producing, or thermophilic cultures, which have an optimum growth temperature in the range of 37 to 50°C. Both types are discussed further below (14).

Mesophilic starters are used in cheeses where the curd cooking or scalding temperature does not exceed approximately 40°C during the early stages of acid production. Such conditions apply to Cheddar, Edam, Camembert, and Gouda cheeses. *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* are the dominant non-gas-producing mesophiles, are typically strong acidifiers, and are used as starter cultures in the manufacture of cheeses such as Cheddar, feta, and cottage cheese. Gas (CO_2)-producing mesophilic cultures include *L. lactis* subsp. *lactis* biovar *diacetylactis* and *Leuconostoc mesenteroides* subsp. *cremoris*. Both species are capable of catabolizing citrate to CO_2 and diacetyl and are used in the

production of Edam, Gouda, and soft mould-ripened cheeses such as Camembert and Brie.

Thermophilic starters are those utilized in the manufacture of cheeses where a higher scalding temperature (>37°C and in some instances 48 to 55°C) is employed during manufacture. This is particularly relevant to Swiss (Emmental and Gruyère) and Italian (Mozzarella, Grana, and Pecorino) cheeses, with the species in question being *S. thermophilus* alone or in combination with thermophilic lactobacilli (*Lb. delbrueckii* subsp. *lactis* and *Lb. helveticus*). *S. thermophilus* is the only *Streptococcus* species employed as starter cultures. This organism is widely exploited in the manufacture of yogurt and Mozzarella cheese, and since the mid-1990s, it has also been used (along with the standard lactococci) in the manufacture of Cheddar cheese. Thermophilic lactobacilli are used as starters in the manufacture of yogurt and Mozzarella cheese (*Lb. delbrueckii* subsp. *bulgaricus*) and are also incorporated as starter adjuncts to promote faster ripening of Cheddar and similar cheeses (e.g., *Lb. casei*), to reduce the incidence of bitterness (e.g., *Lb. helveticus*), and as probiotics in yogurt-type products (*Lb. acidophilus, Lb. rhamnosus*, and *Lb. gasseri*, among others). It should be noted, however, that the distinction between cheeses produced by mesophilic versus thermophilic starters is not as clear as it has been in the past, and an increasing number of cheeses contain both types of starters.

O, D, L, and DL Cultures

Typically, mesophilic cultures used in Cheddar cheese manufacture, where the requirement of the *L. lactis* starter is to produce lactic acid and not to generate gas, are referred to as O cultures. Those that are required to generate the flavor compound diacetyl along with CO_2 gas from the utilization of citrate in the milk, while still utilizing *L. lactis* as the primary producer of lactic acid, are referred to as either D cultures (where the citrate utilizer is *L. lactis* subsp. *lactis* var. diacetylactis), L cultures (where the citrate utilizer is a

Leuconostoc species), or DL cultures (where both are present as flavor and CO_2 producers). The latter starters are used in Dutch cheese manufacture and in the production of sour cream and lactic butter.

Starters may also be subclassified on the basis of the isomer of lactate that is produced as an end product of glycolysis, which is dependent on the type of lactose dehydrogenase (D- or L-lactose dehydrogenase) that is present in the cell or the ability of cells to racemize L-lactate. The isomer D-lactate (e.g., *Lb. delbrueckii* subsp. *bulgaricus*), L-lactate (e.g., *Lactococcus* and *S. thermophilus*), or DL-lactate (e.g., *Lb. helveticus*) will be generated depending on the nature of the lactose dehydrogenase enzyme.

Mixed-Strain and Defined-Strain Starters

The designation of a starter as either a mixed-strain starter (MSS) or a defined-strain starter (DSS) depends on its composition. MSSs are of an undefined composition and are ultimately derived from the original starters that were first developed by commercial starter companies over 100 years ago. Thus, while they frequently made excellent-quality products, they consist of an unknown number of strains (which in the case of Cheddar cheese, for example, would have largely, but not exclusively, been strains of *L. lactis*). As the industrialization and intensification of cheese making increased throughout the 20th century, these MSSs came under increasing pressure and often failed to perform adequately. Issues such as gas production (which in Cheddar is undesirable) and bacteriophage sensitivity prompted New Zealanders, as early as the 1930s, to first move away from such systems. The undefined nature of MSSs made it very difficult to troubleshoot problems, and even when one MSS preparation was replaced with another, one could never know with any degree of assurance that the replacement was in fact significantly or wholly different from the MSS that it was replacing. In other countries, MSSs were employed much later into the 20th century, but in most cases, especially where Cheddar cheese is

manufactured on a large scale, they have largely been replaced by DSSs.

DSSs contain a specified number of well-characterized, scientifically studied strains (usually in combinations of two to six individual strains). For starters such as those used in Cheddar manufacture, the DSS may be as simple as two strains of *L. lactis* whose properties have been thoroughly characterized and whose suitability for cheese making has been demonstrated. Thus, in order to be included in a DSS, individual strains are tested exhaustively to ensure that they grow and acidify the milk at a rate commensurate with the cheese-making process, that they do not produce mutually inhibitory compounds (such as bacteriocins), that they generate the appropriate flavor profile (and this may be, to a greater or lesser degree, a reflection of their propensity for autolysis), that they will not be killed by the scald temperatures used in the cheese-making process, and that they exhibit the appropriate degree of salt tolerance. However, it is in the area of bacteriophage sensitivity that the most rigorous and challenging tests are applied in the selection process. Since phages are virtually impossible to fully eliminate from cheese plants and because severe phage infection of the starter can be devastating to the manufacturing process (and this, after all, was the principal reason why there was a need to move away from the MSS system), it is essential that strains included in DSS systems exhibit a high degree of phage resistance. Thus, a rigorous selection process must be applied. This usually involves collecting phage samples from industrial cheese plants (phages will be present in whey) and using cocktails of factory whey as a selective screen to identify isolates that exhibit resistance.

While there may have been a naive assumption that strains exhibiting total resistance to such phage challenges could be readily isolated, this was proved erroneous once these strains were deployed in cheese plants. Nevertheless, it was apparent that some strains did exhibit a significant degree of robustness (while still not totally resistant), and these could actually operate on a reliable basis in real-world situations. However, it was essential to constantly

monitor the whey drawn from the cheese vats for phage levels. Typically, it is found that phagerobust cultures can tolerate levels of about 10^3 to 10^4 PFU/ml of whey, but any increase above this level is a signal of impending problems and a warning that it would be prudent to replace the phage-sensitive component of the DSS with another well-characterized and phage-unrelated strain. This is the strength of the DSS system—the known composition of the starter culture allows individual strains to be constantly tested for sensitivity to factory phage, and if they start to fail, the offending strain can be removed from the starter and replaced with another strain. Indeed, in order to protect the DSS starters further, factories often rotate two different DSSs in a single day's manufacture so as to reduce the pressure on individual DSS systems. Approaches such as these have been adopted on an almost worldwide basis for Cheddar cheese starters, and similar strategies are also used for many other cheese types that are produced on an industrial basis.

A notable exception is Dutch cheese manufacture, where undefined MSSs are still used almost exclusively. While attempts have been made to develop a defined-strain system for the Dutch cheese industry, allefforts have failed to reproduce the flavor and other quality attributes imparted by the mixed strains. Microbiological analysis of these strains has shown that they are highly complex and that their stability, even in the face of intensification of manufacturing schedules and the consequent increased risk of phage interference is, in part at least, attributable to the dynamic ecological balance that exists between phagesensitive and phage-resistant components—a state of equilibrium that may be maintained by the presence of phage in the actual starter preparations.

Generally, in other cheese fermentations, such as those utilizing thermophilic starters (Italian and Swiss) as well as yogurt fermentation, the industry has moved to defined systems except in more traditional settings, where backslopping (i.e., the use of whey from a previous fermentation as the starter for the next fermentation) is practiced in the traditional manner.

In general, it can be concluded that DSSs have met the demands of modern, large-scale industrial fermentations. They are relatively robust in terms of phage resistance, they are reliable, which ensures predictable manufacturing schedules, and the fact that they can be used over relatively long periods of time ensures product of consistent quality. A limitation may be that the rigorous process of strain selection can result in a limited number of distinctly different strains (especially in terms of flavor development capacity), which can result in a sameness in cheese flavor, irrespective of who the manufacturer is. This issue is being addressed, in part at least, through the increasing use of adjunct starters, which is the topic of the next section.

Secondary and Adjunct Starters

While the starter cultures described above are primarily responsible for acid production in food fermentations, other additional strains, which do not contribute significantly to acid development, are used to perform functions which are essential to the characteristics of the product (secondary strains) or confer additional benefits that that primary starter alone cannot deliver (adjunct starters) (15). Examples of secondary starters include *Propionibacterium shermanii*, which contributes significantly to flavor development in Swiss cheese manufacture (propionic acid derived from lactate) and is also responsible for the formation of eye holes due to CO_2 production. Other examples of secondary starters include the inoculation of molds of the *Penicillium* species (*Penicillium camemberti* in surface-ripened Camembert and Brie cheeses and *Penicillium roqueforti* in various blue cheeses) or even the coating of the surfaces of very heavily ripened cheeses, such as Munster and Limburger, with bacteria to create a smear. In allof these cases, the primary starter is responsible for acid production during manufacture, but the flavor is developed to a complete or significant extent by the secondary starter, and their presence is essential to the formation of the product's final characteristics.

Adjunct starters are not essential to the formation of the product's final properties but instead contribute some additional functionality which enhances the product. Adjunct cultures are being met with increasing favor by cheese makers, in particular to enhance flavor or to promote accelerated flavor development. The use of adjuncts in Cheddar cheese involves the addition of usually, but not exclusively, particular *Lactobacillus* species (*Lb. helveticus*, *Lb. casei, Lb. paracasei*, *Lb. rhamnosus*, and *Lb. johnsonii*) along with the primary starter. The adjunct is added in high cell numbers but is not the agent of acid production. However, these *Lactobacillus* strains are chosen for their high levels of proteinase and peptidolytic activities, which contribute significantly to the development of flavor during ripening. In particular, such strains can compensate for what has been described as a certain degree of blandness in cheeses manufactured using the highly specialized DSS systems. The numbers and range of adjunct starters available from the culture supply companies are expanding, but they are relatively expensive, adding significantly to the overall starter costs incurred by cheese companies.

Another relatively new development is the use of *S. thermophilus* adjunct starters along with the primary cultures in the manufacture of Cheddar and Cheddartype cheeses as added protection should the *Lactococcus* DSS become infected with phage. The underlying motivation is that *S. thermophilus*, being entirely phageunrelated to the *Lactococcus* strains, can take over acid production in the event that the activity of the latter is compromised by phage.

Many of the adjunct starters used to intensify flavor or accelerate the maturation process are actually isolated from a group of microorganisms called nonstarter lactic acid bacteria (NSLAB). NSLAB were originally regarded as adventitious contaminants which can grow to relatively significant levels of 10^6 to 10^7/g of cheese during ripening and are considered to influence flavor development. Specific strains which are deemed to confer positive attributes to the cheese have been isolated and developed into adjunct strains and are now added to some starter blends to harness

these traits in a controlled manner. Screening to select such strains needs to be carried out with care, as some NSLAB may possess undesirable traits, such as the production of biogenic amines (16), or harbor antibiotic resistance genes. Desirable and undesirable traits were traditionally screened for using phenotype-based culture techniques, but the advent of the "-omics" era has largely replaced these methods with genome-based assessments to detect the presence of the genetic determinants of such attributes, with phenotypic confirmations being performed only where such genetic determinants are identified. This significantly reduces the labor-intensive screening processes that were traditionally employed and increases the throughput of screening that may be performed by starter culture manufacturers.

PRODUCTION OF STARTER CULTURES BY CULTURE MANUFACTURERS

When a strain of LAB is considered for commercial application, starter culture producers assess each newly acquired isolate for production and performance attributes. For example, the culture should not display resistance to antibiotics that are used in human therapies. Furthermore, it should be capable of acidifying milk to produce the desired fermentate within a technologically appropriate time frame and at specific temperatures depending on the desired application. For certain applications or fermentations, additional specific properties are desirable, including the production of volatile compounds which are associated with aroma development, the production and potential extracellular release of flavor-associated enzymes, and resistance to infection by bacteriophages. These attributes are assessed using a range of techniques, including culture identification using microbiological tests (growth temperature profiling, sodium chloride tolerance, ability to metabolize compounds such as citrate, and sensitivity to antibiotics) and genetic techniques, which usually include DNA sequencing of either the whole genome or so-called housekeeping genes, such as

the 16S rRNA gene. The production of volatile organic compounds is also assessed using gas chromatography-based tests, and enzymatic capabilities may be identified through PCR-based assays, while microbiological assays can also be used to confirm these activities. Figure 30.4 outlines some of the attributes and methods employed in culture assessment. If the culture is deemed technologically appropriate, it is included in the culture collection and either is produced as necessary as a single culture or is applied as part of a culture blend. Starter culture production for application in the dairy fermentation industry follows these basic steps: determination of the optimum composition and preparation of medium, optimization of growth parameters, harvesting, and preservation. These steps are covered in more detail below.

Medium Preparation and Growth

The majority of commercially produced dairy starter cultures are grown in sterile milk-based media whose composition may be modified based on the specific requirements of a given species or even strain. These media are generally subjected to a thermal sterilization treatment. The starting material needs to be of premium quality in order to ensure the highest level of starter activity; thus, medium selection and preparation are critical steps in the starter culture production process. For example, milk derived from mastitis-infected animals, in most instances, will not support optimal growth of lactic starter cultures, as it often contains lower concentrations of lactose and exhibits a higher pH than normal milk. Heat treatment of the milk generally renders it more suitable for fermentation due to the generation of lowmolecular-weight nitrogen compounds, highlighting the importance of thermal processing of the starting material.

As discussed above, the LAB, including *L. lactis* and *S. thermophilus*, which are the most widely exploited LAB, produce lactic acid as the primary by-product of hexose fermentation. In the absence of buffering, the hydrogen ion concentration becomes limiting near pH 5.0, which restricts further biomass production.

pH control of the starter culture fermentation during its preparation is usually applied to ensure that values remain between 5.4 and 6.3, usually by the addition of gaseous ammonia or concentrated alkali (sodium or more usually ammonium hydroxide). This serves to reduce production times, increase biomass, and increase the reliability of cultures and is employed in the vast majority of commercial operations.

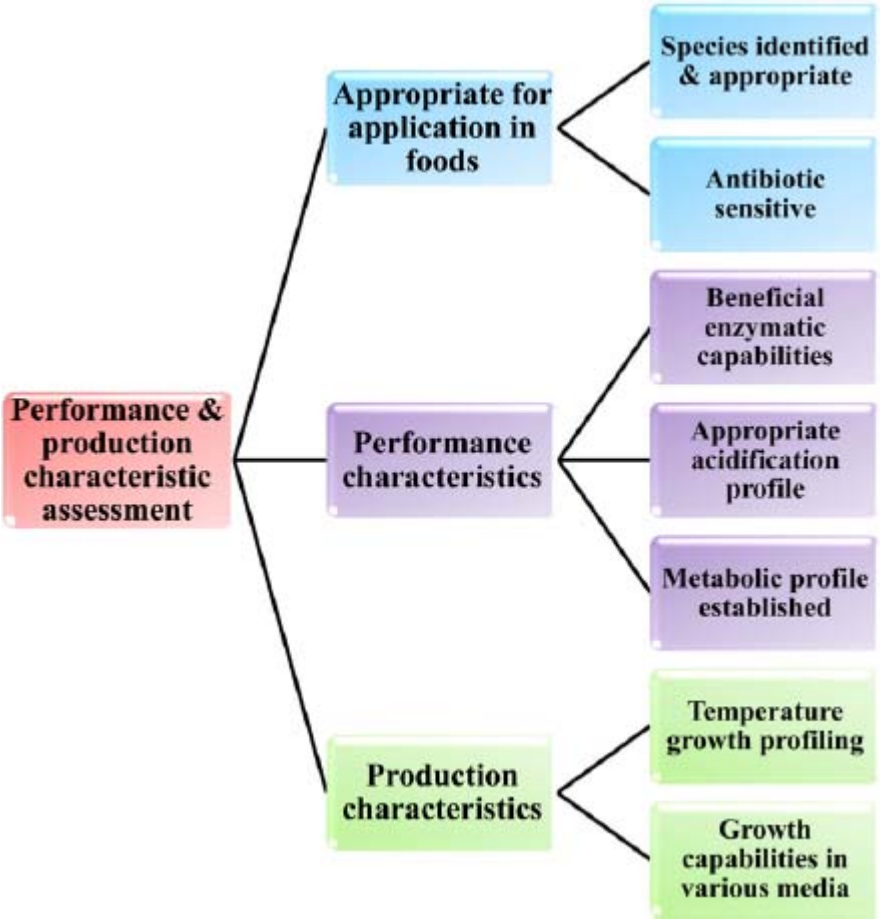


Figure 30.4. Key attributes assessed to determine the technological appropriateness of starter or adjunct cultures by starter culture manufacturers. These attributes include safety for use in foods and performance and production characteristics.

Harvesting and Preparation for Storage

Upon reaching the desired pH and biomass, cells may be harvested using a range of methods. These may include direct aliquoting of

the fermentate into appropriate sterile containers, but more frequently, it is necessary to separate the bacterial cells from the growth medium by either centrifugation or (ultra) filtration. The cells are then standardized to a specified activity and mixed with a cryoprotectant. The concentrated cells may then be packaged for subsequent use or storage. Frozen concentrated cultures typically contain 10^{10} to 10^{11} CFU/g.

The preservation of starter cultures is achieved through freezing or freeze-drying. Frozen concentrated cultures are prepared by rapidly freezing the cell suspension either in liquid nitrogen (–196°C) or in a dry ice-ethanol mixture and are subsequently stored in a freezer. These cultures are immediately active upon thawing.

Alternatively, the concentrated cultures may be freezedried by first suspending the cells in a suitable medium such as milk or a solution of sugar (such as lactose, trehalose, glycerol, or sucrose) and aliquoting in the final storage containers prior to rapid freezing using a cooling mixture or evaporation by cooling under vacuum. The package may contain a single strain or a blend of strains that have been individually propagated, concentrated, and freeze-dried. When the desired moisture content has been achieved (<3%), the cells are blended to achieve a specified activity. These are commercially available in aluminum pouches under nitrogen. The freezedried cultures can be stored at refrigeration temperatures for a period of up to a year without loss of activity. The disadvantage of freeze-dried cultures is that they undergo a longer lag time in the cheese vat than frozen concentrated cultures, with an additional 30 to 60 minutes required to ferment the milk. In addition, while the majority of LAB may be freeze-dried without significant loss of viability or activity, some LAB, such as *Lb. helveticus* and *Lb. delbrueckii* subsp. *bulgaricus*, are not amenable to this form of preservation.

STARTER CULTURE FORMATS EMPLOYED IN COMMERCIAL PRACTICE

The selection of starter culture type by a dairy company is a multifactorial decision which balances economic factors with the production system in use (traditional versus modern practices). In addition, the dairy company must consider the possibility of a specific need for a particular culture, the cost-effectiveness of employing different culture formats, the convenience level required, and the reliability of a culture. This is performed on a case-by-case basis by each dairy company depending on their specific processes and needs.

In-House Preparation and Backslopping

In dairy fermentations, the traditional practice of propagating the starter culture by serial subculturing in the factory (Fig. 30.5) is rarely practiced nowadays. This is highly labor-intensive, and there is considerable risk of exposure to contaminants in the factory environment, particularly to bacteriophages.

The production of some traditional and regionally protected fermented dairy products involves the application of a process known as backslopping (described above). Other traditional methods incorporate the use of wooden vats, for example, and rely on the autochthonous microbiota to initiate the fermentation process. While these fermentations are dependent on either material from previous successful batches or the native microbiota of the vats and equipment to drive the process, there may be inconsistencies in the organoleptic properties of the final product, as the starter mix is undefined and may vary from batch to batch. However, failure of such processes is quite rare, since the diversity of bacteria in any given fermentation will generally be sufficient to successfully acidify the milk. Additionally, traditional fermentations often employ raw cow, goat, or ewe milk, each bringing its own innate microbiota, which may further contribute to the fermentation process and the highly valued flavors associated with traditional rawmilk cheeses.

Bulk Set Cultures

The majority of modern large-scale fermentation facilities employ ready-made starter strain blends that are prepared by starter culture providers based on the specific needs of the processor. These cultures are designed to produce acid at a rate that is reliable and consistent with the requirements of the process and also to generate products with certain desired flavor and texture characteristics. Such cultures are generally provided in the form of deep-frozen or freeze-dried concentrated or highly concentrated (superconcentrated) formats (17). The level of concentration of these starter culture preparations as provided by the starter supplier allows the cheese manufacturer to determine the extent of in-factory propagation that they wish to perform.

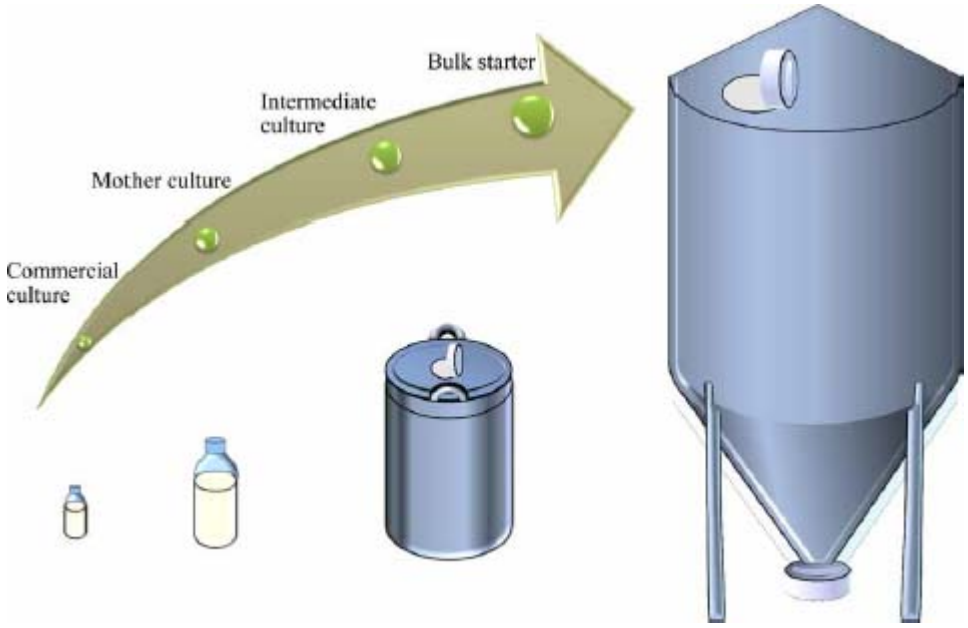


Figure 30.5. Stages in the production of a bulk starter, where intermediate propagation steps are incorporated to scale up the production of the desired starter culture.

Concentrated cultures are usually appropriate for processors using a bulk starter system in the factory. In this case, the frozen or freeze-dried preparations, at concentrations of approximately 10^{10}/g, are slowly thawed or reconstituted and then inoculated into a bulk starter tank. In most cases, the growth medium for bulk cultures is antibiotic-free, skimmed-milk powder reconstituted in water to a 10

to 12% solids content, which is sometimes supplemented with growth stimulants such as tryptone. The process starts by the inoculation of milk that has been heated to 90 to 95°C for 30 to 60 minutes with a commercial culture to produce a bulk culture (Fig. 30.5). The progress of the bulk culture fermentation is assessed by measuring the titratable acidity or pH at regular intervals, and in some cases, neutralization is used to achieve a higher cell number by minimizing acid damage. When a titratable acidity of approximately 0.85 to 0.90% is reached, the fermentation is halted by agitating the cultures and chilling the fermentation vessel to 4 to 5°C.

The bulk culture approach to starter production reduces the cost associated with the in-factory, stepwise propagation of the starter from stock; however, it also incorporates a level of risk. For example, a dairy facility that regularly employs a particular starter may house bacteriophages capable of infecting the culture. The presence of such bacteriophages may slow the acidification process and cause downstream problems in the subsequent fermentation, as the bacteriophages will continue to propagate in the vats. The higher risk of bacteriophage infection associated with bulk starters necessitates increased microbiological testing of the culture, which requires suitably trained personnel, adding further to the costs. The preparation of bulk cultures also necessitates the use of highly engineered and expensive bulk tanks (essentially bioreactors), and the number of bulk tanks that the dairy company possesses will limit the number of different production batches that may proceed on a given day. Furthermore, the bulk starter rooms which house these tanks must be carefully and expensively designed so as to minimize the risk of phage infection and typically incorporate measures such as filtered air, positive air pressure, gowning areas, and sanitizer footbaths. They must also be designed and constructed to facilitate thorough cleaning and sanitization. Therefore, while the preparation of bulk cultures provides dairy companies with reduced direct starter costs and greater flexibility and control in terms of the long-term application of desired starter cultures, it must be accepted

that there is a greater risk of phage infection due to the need to undergo a subculturing step, despite all of the measures that are taken to mitigate this risk.

A routine quality assurance step to ensure that the bulk starter will be fully active when inoculated into the cheese milk is the so-called activity test, in which milk is inoculated with 2% of the bulk culture and incubated at the relevant temperature for mesophilic or thermophilic systems for 4 to 6 hours. The titratable acidity is then determined through titration with N/9 sodium hydroxide, and if the values achieved (measured as percent developed lactic acid) are not at the expected levels, it indicates that the starter is “slow” and may not produce acid in the fermentation at the appropriate rate. This is a simple and inexpensive test that provides same-day results, which is beneficial in the industrial context.

Superconcentrated Cultures: DVS Systems

An alternative approach for the cheese maker is to use superconcentrated cultures (up to 10^{12}/g), which are suitable for direct inoculation into the cheese milk. These are often referred to as direct vat set (DVS) or direct vat inoculation (DVI) cultures. Their use avoids the need for any in-factory propagation, thereby significantly reducing the risk of exposure to phages. They are also very convenient, and there are considerable labor savings compared with bulk starter preparations. Furthermore, in recent years, the number and types of starters that are available in DVS/DVI formats have increased significantly; these benefits have been sufficiently attractive to the cheese manufacturer to outweigh the disadvantages of higher purchase costs and some loss of activity immediately following inoculation into the cheese milk (due to the lag as the cells acclimatize from the frozen or dried state to the milk environment). The use of DVS/DVI starters may be a particularly attractive option in situations where new cheese plants are being commissioned; the capital costs of constructing bulk starter

facilities are considerable, and thus there is a significant savings to be made if this step can be avoided.

Long-term application of DVS/DVI cultures can be expensive for dairy companies, and a commitment to using such systems means that they are continually reliant on starter culture manufacturers to provide these cultures on demand. Furthermore, the supply of particular DVS/DVI cultures preferred by individual dairy companies may be discontinued, with obvious negative consequences for those relying on their continued availability. Dairy companies also need to have adequate storage (freezer) space and good temperature-monitoring systems to maintain the large volume of DVS/DVI cultures if they are purchased in the frozen state (which is usually preferred in the case of cheese starters).

While the culture systems described above are generally very effective in limiting production issues and product inconsistencies, allfermentations are additionally prone to external factors that may influence the acidification activity of starter cultures, as discussed below.

FACTORS AFFECTING STARTER CULTURE PERFORMANCE IN DAIRY FERMENTATIONS

The major components of milk are water, fat, protein, lactose, and minerals. Its exact composition is dependent on a number of factors, including the animal's diet, environmental factors, the breed of the animal, the stage of lactation, seasonal considerations, and the health status of the animal. The most variable component of milk is the fat, while lactose is the least variable constituent. The animal's diet may be designed or altered to manipulate the fat composition. The composition of milk may significantly impact the production regimens and the acidification rates by starter cultures. Therefore, the milk protein levels (and, indeed, the fat-to-protein ratio) are often standardized in cheese milk, as high levels of

protein are associated with a higher buffering capacity, thereby reducing the acidification rate of the culture.

Agglutinins are antibodies that may be present in the protein fraction of raw milk, but their concentration is significantly higher in early-lactation milk (colostrum) or in response to an infection, such as mastitis. Their presence can cause the agglutination of some dairy starters, including lactococci and *S. thermophilus*. Agglutination results in precipitation of the starter cells to the bottom of the fermentation vessel, with negative impacts on their acidification capabilities, which can be a particular problem in the production of cottage cheese and other soft cheeses (18). However, agglutinin-associated issues can be restricted by using agglutinin-insensitive starter cultures or by inactivating the agglutinins through homogenization or heat treatment of the milk or by simply avoiding the use of milk in which agglutinin levels would be expected to be high.

Antibiotics are used routinely in veterinary medicine to treat a range of infections, including mastitis, which is an inflammation of the udder caused by bacterial infections by *Streptococcus agalactiae, Streptococcus dysgalactiae*, and *Staphylococcus aureus*, among others, in the lactating animal (18). Six major groups of antibiotics are currently employed in veterinary medicine. These include the penicillins and cephalosporins, the aminoglycosides, the macrolides, the quinolones and fluoroquinfluoroquinolones, the tetracyclines, and the sulfonamides. Clearly, it is highly undesirable for antibiotic residues to be present in milk, as, apart from inhibiting starter cultures, they may also result in serious allergic reactions in sensitive individuals.

The milk of infected and antibiotic-treated cows is withheld for a period of time, typically six milkings (usually 3 days), to eliminate the possibility of contaminating the bulk milk. Many of the tests for the presence of antibiotics that are currently used were originally designed to detect penicillin, implying that other antibiotics may not be detected by such methods. Concentrations of 0.005 to 0.05 IU of

penicillin are sufficient to cause at least partial inhibition of starter cultures, with *S. thermophilus* being particularly sensitive to this family of antibiotics.

Sanitary practices on farms and in dairy fermentation facilities are continuously improving; often, economic sanctions and/or incentives are applied to farmers to promote the production of high-quality milk, while there is also increasing pressure on processors to conform to stringent modern quality control standards. Although scalding and rinsing of the equipment are part of most sanitization regimens, residual traces of disinfectant may remain in the absence of adequate draining or rinsing. Concentrations of 1 to 5 ppm of the majority of sanitizers employed in dairies are effective in inactivating lactic starter cultures.

In contrast to the short-lived or batch-specific issues associated with sanitizers and antibiotics, bacteriophages present a threat that is much more insidious and more difficult to control. Next, we review the developments in the rapidly advancing areas of research on phages and phage-host interactions and examine how this knowledge can be exploited to better manage cultures and to reduce the risk posed by phage infection.

BACTERIOPHAGES

Phages are bacterial viruses that specifically recognize and infect strains of a given bacterial genus or species. While disruption of fermentations by phages is rarely a significant problem in other food fermentations, it represents the most problematic and consistent issue facing the dairy fermentation sector today. The interplay between bacteriophages and their hosts has been the subject of intensive research over the past several decades. This is not surprising considering the devastating impact that phages can have on fermentation processes that are worth many billions of dollars on an annual basis. This research has recently made significant progress, aided considerably by advances in genomics

and structural analytical technologies. This section focuses on the sources of phages, their structure and classification, and the means by which they replicate. We discuss how phages interact with their hosts and how the host bacteria attempt to counter phage infections by incorporating antiphage strategies.

Sources of Phages in Dairy Production Facilities

There are a number of potential sources of phages in a dairy factory, ranging from the incoming raw materials, including the milk and other ingredients, to the air and the factory environment, where phages are known to survive for long periods of time. Equipment surfaces, personnel, and, more contentiously, the starter culture itself may also be sources; in the latter case, phages may be carried as “pseudolysogens” (incompletely replicated phages which are internalized in the cells and which may become active upon reintroduction to suitable growth conditions) or as low-level contaminants. To limit the proliferation of phages, the majority of modern dairy factories incorporate design features that curb their development and survival at each of the critical points of the fermentation process. For example (with the obvious exception of raw-milk cheeses), the incoming raw milk is pasteurized and homogenized in most dairy factories, which reduces the phage and microbial load; however, it is also known that many phages are not inactivated by pasteurization treatments of 72°C for 15 seconds. Second, positive air pressure is used to ensure that the air flow is unidirectional, and personnel are trained to move in a similar unidirectional manner to prevent contamination of culture preparation rooms with phages from the cheese production areas. Furthermore, strict sanitation regimens are seen as being essential in controlling phage numbers in the plant to manageable levels. This includes sanitization of the vats, piping, and valves to ensure that phages are inactivated and flushed out of the system to the degree possible. Such regimens typically involve routine application of chemical sanitizers such as peracetic acid, sodium hypochlorite, iodophors, and nitric acid, among others. Since phages, if present in

the plant environment, will be concentrated in the whey (in the case of cheese manufacture), it is essential that the management and removal of whey be done in such a manner as to reduce, as far as possible, the likelihood of contamination through dispersal of aerosols.

Many dairy fermentation plants also engage in the process of monitoring the levels of phage present in factory wheys. These tests measure the extent of acidification of the milk by the starter culture in the presence of aliquots of factory whey using time and temperature profiles that are typically applied in the actual fermentations and results are compared with acidification in the absence of whey. Reduction in the starter acidification activity may be attributed to phage inhibition. If the presence of phage is suspected, the actual levels can be determined by plaque assay on the sensitive host.

Phage Structure and Classification

All phages of LAB characterized thus far possess doublestranded DNA packaged in either an isometric or elongated (prolate) head which is connected to a tail structure (Fig. 30.6). The tails of LAB phages may be long and contractile (family *Myoviridae*), long and noncontractile (family *Siphoviridae*), or short (family *Podoviridae*). Lactococcal phages are classified into 10 taxonomic groups based on their morphology and genetic composition (19). Among these 10 groups, eight are classified as *Siphoviridae* phages and thus possess long noncontractile tails, while the remaining two groups are classified as *Podoviridae* and exhibit short tails. While members of all10 groups have been encountered in the dairy environment, three groups predominate irrespective of geographical location. These are named the 936, c2, and P335 phages (allpossess long noncontractile tails), and members of the former two follow a strictly virulent lifestyle, while the P335 phages include both virulent and temperate members (phage lifestyles are covered in more detail below). The 936 phages are the most abundant and frequently isolated lactococcal phages (20). They are highly conserved at the genomic

level, and this has facilitated the development of model systems to unravel the interactions between this lactococcal phage group and their hosts (21, 22).

In contrast to the genetic and morphological diversity of lactococcal phages, those infecting *S. thermophiles* are apparently less diverse. Until recently, only two groups were known to exist, but an additional two were identified more recently (23, 24). The latter groups are infrequently encountered and may in fact represent newly emerging types. All four groups of *S. thermophilus* phages exhibit long noncontractile tails and thus are members of the *Siphoviridae*.

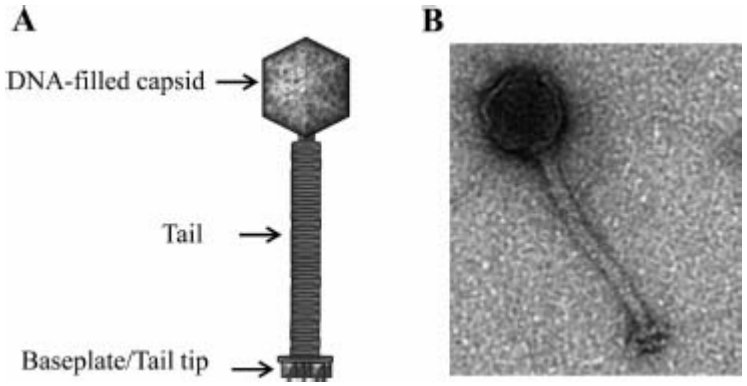


Figure 30.6. (A) Structure and general characteristics of a tailed phage highlighting the DNA-filled capsid, the tail, and tail tip appendages. Some phages possess large multicomponent structures at the distal ends of their tails, termed baseplates. (B) Representative electron micrograph of the lactococcal P335 phage Tuc2009, which exhibits the structures highlighted in the schematic in panel A.

Phage Replication: Lytic and Temperate Cycles

Phages may be largely classified into two groups depending on their lifestyle, i.e., whether they are virulent or temperate. Virulent phages are those that infect a specific bacterial host, replicate intracellularly, and thus cause the death and lysis of the host cell. This is the situation which causes failure of starters in commercial practice. In contrast, when temperate phages infect their bacterial

host, the injected phage genome becomes integrated within that of the host, where it replicates silently *in situ*. When conditions become favorable for lytic development of the phage, the genome is excised and enters the lytic cycle (Fig. 30.7). While at one time, it was thought to be a rare event, genomics studies have now made it clear that lysogeny is a common, and even usual, feature of many genera of LAB. For example, it is notable that the ever-increasing bank of genomic data relating to lactococcal strains demonstrates a very high incidence of integrated prophages (25), while lysogeny has been shown to be much less frequently observed among *S. thermophilus* genomes (26).

Both the lytic and lysogenic cycles involve an initial binding to the cognate receptor on the host cell surface, which may be a (poly) saccharide, protein, or teichoic acid component (this aspect is discussed in more detail below and is directly related to determination of host specificity and host range) and the injection of its DNA into the cytoplasm. A clear picture of the structure of phage genomes now exists, and we know that they are organized in functional modules, with specific genes or clusters of genes controlling functions such as lysogeny (in temperate phages only), replication, morphogenesis, DNA packaging, and lysis (Fig. 30.8).

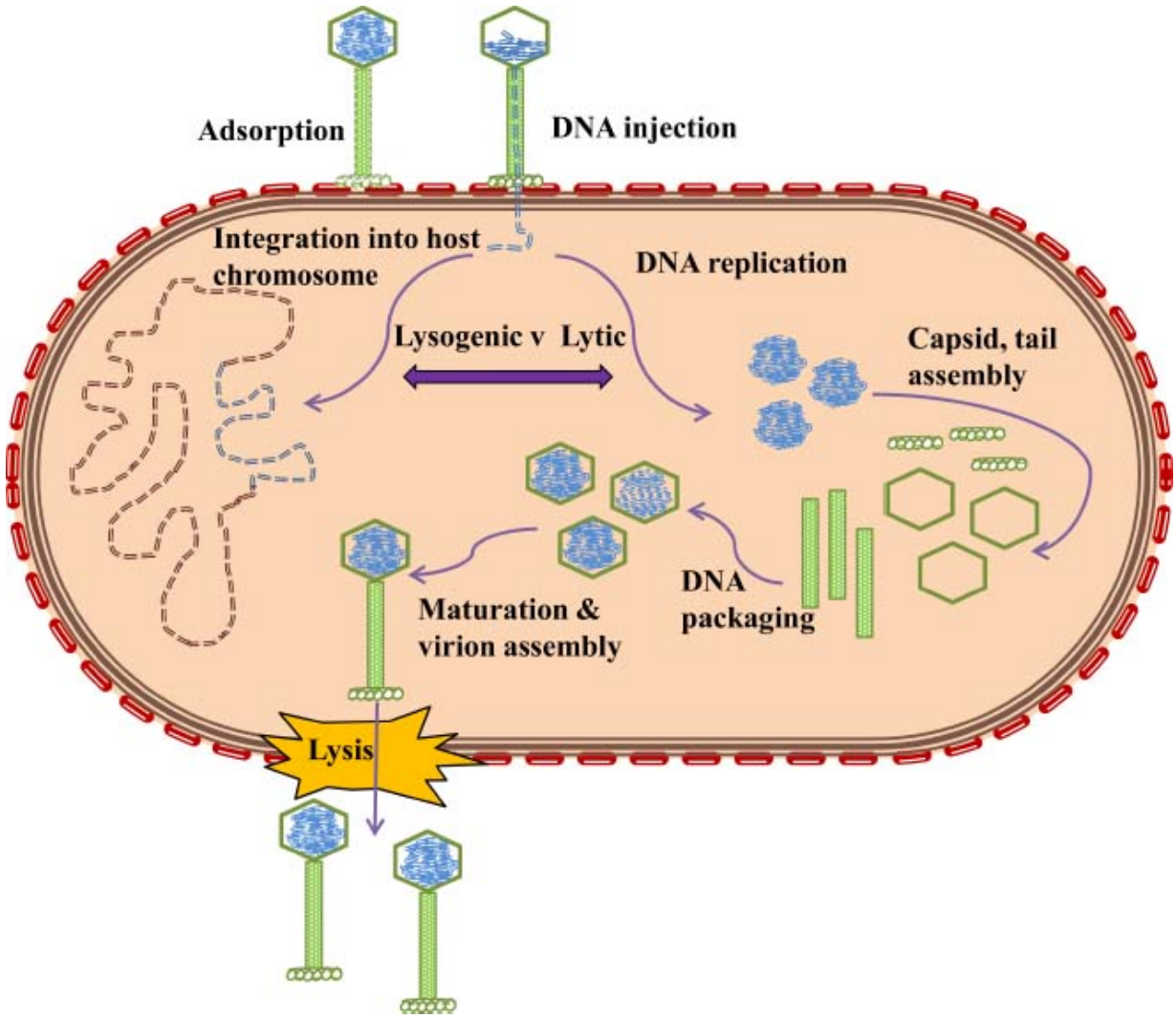


Figure 30.7. Schematic representing the lytic and lysogenic cycles that may be followed by phages.



Figure 30.8. Schematic representation of the genome organization of a typical lactococcal P335 phage highlighting the functional modules of gene clusters, including those associated with lysogeny, replication, and DNA packaging and head and tail morphogenesis functions.

The structural components comprising the head and tail (where relevant) of the phage are produced and are assembled separately.

Following assembly of the immature head (or procapsid), DNA is packaged inside this structure, allowing maturation of the head and attachment of the tail to form the final assembled and packaged phage particle (Fig. 30.7). The final step involves the activity of the components encoded within the lysis cassette, i.e., the holin and lysin. Holins are small proteins that localize at the cytoplasmic membrane, creating a pore through which the lysin gains access to the cell wall, which it then degrades, thereby facilitating the release of the progeny phage particles. A replication cycle can occur in as little as 40 minutes, and typically, each lysed cell can release 80 to 200 new phage particles, although these values can vary considerably and be strain and phage dependent.

While lytic phages are an obvious threat to fermentation practices, temperate phages also constitute a silent risk factor, as they may be induced into the lytic cycle (as mentioned above). Starter culture companies are becoming increasingly aware of this threat, and the identification of potentially inducible temperate phages in starter strains has become part of the standard practice used by many companies when screening new strains. However, it should also be noted that the presence of inducible temperate phages may sometimes be beneficial in fermentations. For example, if it is the case that the acidification rate is not impaired, the lysis of starter cultures through the phage induction process facilitates the release of intracellular flavor-associated enzymes, which can play a key role in the development of the characteristic organoleptic traits of certain fermented dairy products.

Since the first discovery of lactococcal phages by Whitehead and his group in New Zealand in the mid-1930s (2), much of the research linked to LAB has focused on phage-host interactions, with an ultimate expectation of being able to develop and employ starters with a higher degree of confidence in the factory setting. While this earlier research was very valuable, it was limited by the analytical and investigative technologies of the day. However, since the advent of the molecular biology and genomics era, the situation has changed dramatically, making possible findings that are of wider

biological significance, such as the initial discovery of CRISPR (clustered regularly interspaced short palindromic repeat)-Cas systems in *S. thermophilus* (27).

The section below focuses on the tremendous advances that have been achieved in the past decade in terms of understanding phage-host interactions in LAB, but especially the lactococci, where the most dramatic progress has been made, and highlights how this improved understanding has resulted in much more informed and knowledge-based approaches being taken to deal with the issue of bacteriophage infection in industrial settings.

Bacteriophage-Host Interactions

In order to establish effective control and/or preventive measures against phages, it is first essential to understand the intricate interactions and relationships between these predatory entities and their host bacteria. Of all the starter bacteria used in food fermentations, the lactococci are the most extensively studied and much of our understanding of the activities of the LAB in general has been founded on initial observations obtained from research on members of the genus *Lactococcus*. So it is also with the study of bacteriophage-host interactions, a topic that was further stimulated by the fact that Cheddar and related cheese fermentations were among the first to be developed into intensive, more or less continuous, industrialized processes. Under such circumstances (with the continuous presence of very high numbers of microbial cells in the factory environment), phage infection became a more likely possibility and also had more devastating consequences. Thus, it is still the case that we know more about lactococcal phages and their interactions with their lactococcal hosts than we do about other phage-host systems.

The initial step in the infection process involves adsorption of phage in a tail-first orientation to specific receptors on the cell surface. This is one of the best-characterized steps in the phage cycle and involves a phage-encoded adhesion device located at the tail tip that

"docks" the phage on the host cell and a host-encoded receptor moiety, which may be a surface-exposed protein, (poly) saccharide, or (lipo)teichoic acid structure. This critical step has been the subject of considerable research, particularly with respect to *L. lactis* (28), but also to a lesser extent with *S. thermophilus* and *Lactobacillus* spp. (24, 29). The three-dimensional structure of the adhesion device (located at the tail tip) of 936 and P335 group phages is defined as a multicomponent complex that is known to recognize a cell wall polysaccharide structure on the host cell surface.

The majority of phages infecting LAB, including *L. lactis* and *S. thermophilus*, typically display a very narrow host range; in other words, they infect a very small number of bacterial strains. For decades, the biological reason for this was unclear, but the biochemical diversity of particular regions of the polysaccharide structures on the lactococcal cell wall is now proposed to account for the remarkably high degree of specificity of phage-host interactions.

It is now believed that many *S. thermophilus* and *Lactobacillus* spp. phage receptors are likely to be carbohydrate or carbohydrate linked. Among the lactobacilli, carbohydrate moieties of teichoic acid and lipoteichoic acids as well as cell envelope carbohydrates are known to be involved in the initial interactions of tailed phages of *Lb. delbrueckii* subsp. *lactis*, *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. plantarum*, and *Lb. casei*. As there is also now a growing availability of genome sequences for these LAB species (Table 30.2), it is possible to identify the host receptor-encoding genetic elements using comparative genomic analysis of phageresistant derivatives and the parent strain. While in this chapter we focus largely on lactococci, as they are the best-studied phage-host systems, similar principles and approaches could be applied to most members of the LAB which are employed as starter or adjunct cultures.

Table 30.2. Number of completely sequenced genomes of lactic acid bacterial species

LAB	No. of complete genomes published[a]
L. lactis subsp. *Lactis*	19
L. lactis subsp. *lactis* biovar Diacetylactis	1
L. lactis subsp. *Cremoris*	12
S. thermophiles	25
Lactobacillus spp.	193
Leuconostoc mesenteroides	9
Leuconostoc lactis	1
Weissella cibaria	5
Weissella ceti	3
Weissella koreensis	1
Oenococcus spp.	2

[a] A much higher number of incomplete/draft genome sequences are also available.

While the majority of lactococcal phages employ a carbohydrate receptor on the host cell surface, the c2 group of lactococcal phages recognize a membraneanchored protein, termed the phage infection protein (PIP) (30). This protein is highly conserved among lactococci and contains a number of transmembrane domains and a large ectodomain, which is exposed at the cell surface; it is to this that the c2 phages bind. In contrast to the highly host-specific 936 and P335 phages, which bind to carbohydrate receptors, the c2 phages are typified by a wide host range; they can infect multiple strains of *L. lactis*. The presence of PIP-encoding genes in allsequenced lactococcal genomes explains this observed broad host range.

In recent years, the number of reported incidences of c2 phages has declined significantly. It would appear that starter culture companies have selected spontaneous c2-resistant derivatives of lactococci by incubating the strain(s) with its infecting c2 phage(s) and selecting the survivors, which presumably have a mutated *pip* gene, and that these are now prevalent in starters employed successfully throughout the world.

The lactococcal c2 phages are the best-described group of protein-recognizing LAB phages, although others are known to exist. For example, a phage of *Lb. helveticus* recognizes surface S-layer proteins. While the majority of phages infecting Gram-positive bacteria are now presumed to employ a saccharidic component in the primary adsorption step, it is likely that many of these also interact with other cellular components, including proteins, as part of their infection pathway.

Starter Cultures Encode Antiphage Systems

Phages and their host bacteria have coevolved over millennia and continuously adapt to ensure their survival and continued existence. Phage-host coevolution is a well-described phenomenon among the majority of bacterial species; however, it is particularly well studied among LAB due to their commercial importance (31). Each stage of the phage infection process is matched by the presence and activity of host-encoded phage resistance systems (Fig. 30.9), as follows.

1. Adsorption inhibition, where the bacteria have evolved a range of barriers to prevent phage adsorption, including the blocking of phage receptors, the production of an extracellular matrix, or the production of competitive inhibitors (31)
2. Superinfection exclusion systems, where specific proteins block the entry of phage DNA into host cells (32–34)
3. Restriction/modification (R/M) systems, where incoming phage DNA is degraded by a restriction endonuclease (35)

4. Abortive-infection systems, where a range of heterologous proteins target a crucial step of phage multiplication, such as replication, transcription, or translation (36)
5. CRISPR-Cas systems (37), where an immunity system targets foreign nucleic acids, including phage genomes and plasmids. Short segments of DNA of the infecting phage are incorporated into the CRISPR spacer array, thereby preventing subsequent infection by the same phage

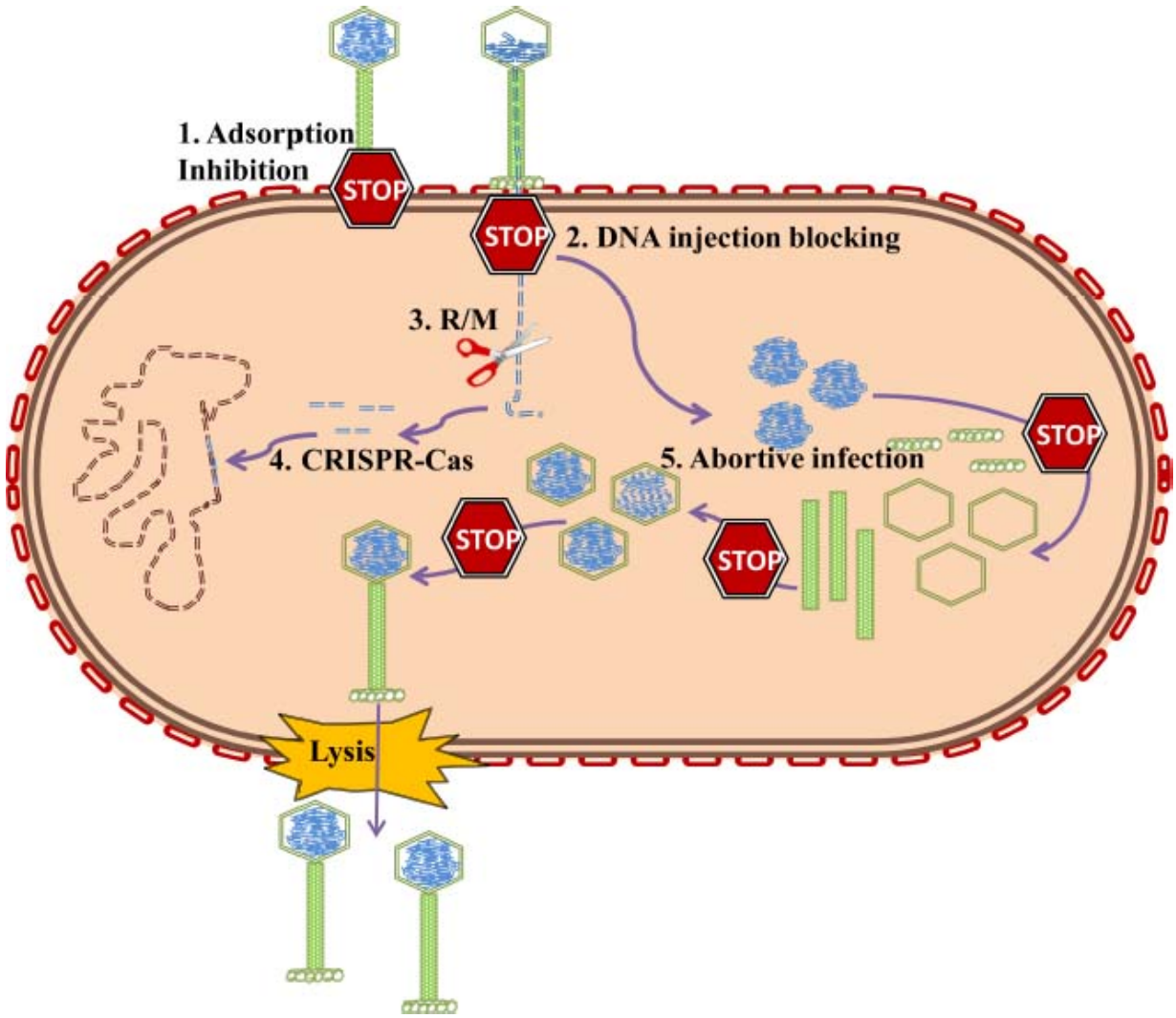


Figure 30.9. Schematic depicting the five known phage resistance systems that are active at the various stages of the phage cycle, from adsorption inhibition at the initial binding step to DNA injection blocking, R/M systems that cleave and modify the incoming DNA, CRISPR-Cas systems that provide immunity to "foreign" DNA, and abortive infection systems that may be active at the stage of DNA replication, assembly, or maturation of the phage particle.

S. thermophilus strains appear to rely mostly on CRISPR-Cas systems for phage protection, and their presence limits their ability to acquire plasmids that may encode phage resistance systems (among other traits), since this is recognized as "invading DNA" in the same way as phage DNA. A few R/M systems have been

reported among some *S. thermophilus* strains, but these are rare. Lactococci, on the other hand, do not appear to encode active CRISPR-Cas systems and are reliant on the other phage resistance mechanisms listed above for protection against phage infection or development. In fact, the genomes and plasmids of lactococci are observed to stack multiple phage resistance systems to provide the widest possible array of antiphage activities, as individual systems may have a limited effect.

It is important to note that the evolutionary process is a continuous one, with phages responding to the presence and activities of specific resistance mechanisms. For example, by mutating the phage-encoded target of the abortive-infection, R/M, or CRISPR system, the phage can overcome these particular barriers to infection. In turn, this requires that the host evolve to prevent the next generation of phages from infecting, and so the process repeats itself to ensure the survival of both antagonists. Knowledge of their presence and of the modes of activity of phage resistance systems in LAB is beneficial for the development of starter cultures with increased technological and phage robustness, and, as outlined below, this knowledge has been successfully harnessed to generate improved strains for the dairy fermentation industry.

Development of BIMs

The identification of new cultures that produce cheese with characteristics similar to the ones that they are replacing when they have succumbed to phage infection is a time-consuming and labor-intensive process involving the identification of phage-unrelated strains, screening to ensure that they exhibit the appropriate technological characteristics, and performing small-scale production trials. An alternative to the identification of new strains is to develop bacteriophage-insensitive mutants (BIMs) of the originals through simple exposure of the relevant phagesensitive component to its infecting phage and selecting for the presumed phage-resistant survivors. BIMs of thermophilic starter cultures such as *S. thermophilus* are readily produced, while many mesophilic cultures

are not amenable to spontaneous BIM generation. The genetic basis of this phenomenon was recently discovered to be due to the presence of active CRISPR-Cas systems in *S. thermophilus* and *Lactobacillus* spp., where the incorporation of a spacer sequence can occur at relatively high frequency, resulting in resistance. In contrast, lactococcal strains, which generally do not contain CRISPRs, instead rely on incorporating mutations in the receptor-encoding genes in many cases, a process which occurs at a much lower frequency than CRISPR-mediated spacer incorporation.

BIM development is a critical but short-term solution to phage infection; mutations and spacer incorporations can readily be overcome by single point mutations in the phage genome. Therefore, longer-term strategies must be considered which will inevitably be based on the knowledge that is being generated through the genomics and detailed structure-function studies of different phage-host systems of LAB. These are discussed in more detail below.

IDENTIFYING NEW STRAINS FOR APPLICATION IN STARTER CULTURES

To ensure the continued success of dairy fermentations and also to meet demand for cultures with novel organoleptic or technological characteristics, starter culture providers and producers alike continually seek to expand their catalogue of cultures and component strains. This traditionally involved the application of empirical methods based on screening for novel strains or, alternatively, adapting existing cultures using traditional or modern knowledge-based systems. The section below deals with each of these aspects.

Evolution of Strain Characterization Processes

Since the starter culture industry began, the standard method for identifying new strains and adding them to the catalogue on offer to

the fermentation industry has typically involved isolating strains from sources known to be rich in LAB and subjecting these to a range of tests to ensure that they met the criteria for whichever process they were intended for. These included rate of acid production, range of sugars metabolized, proteolytic and autolytic properties, the ability to produce compounds such as bacteriocins and EPS, antibiotic resistance profiles, lysogenic status, and phage sensitivity patterns (Fig. 30.4). More subtle tests, such as measuring production of volatile compounds using gas-liquid chromatography, later became a part of the screening process. While assessing the characteristics of such strains in this manner is still valid, the approach is time-consuming and laborintensive. In addition, screening for particular traits is often hampered by the lack of good screening methods, while upon scaling up, some of the strains do not perform as well as they did in the small-scale laboratory testing, leading to loss of time and profit. Therefore, it was clear that new approaches needed to be adopted.

In the 1980s, the application of molecular biological methods provided, for the first time, new information regarding the technologically important traits of the LAB, with the most notable early work being focused on members of the genus *Lactococcus*. Thus, the plasmid location of genes for many key traits of lactococci (lactose metabolism, proteinase production, citrate uptake, and phage resistance) explained the frequent phenotypic instability of these properties. Furthermore, it was often possible to demonstrate that such plasmids possessed genes that allowed them to be transferred to other hosts by conjugation. This raised the possibility of transferring desirable traits (particularly those associated with phage resistance) to industrial strains in a nonrecombinant manner. Thus, it was by this method that commercially desirable cultures were generated following conjugal transfer of (often multiple) phage resistance plasmids from donor strains. These did not require regulatory approval before deployment in the field and they have been used quite successfully for the past 30 years. Such constructed

strains represent the first generation of “knowledge-based” improved starter cultures.

However, this approach too was limited largely to the development of phage-resistant derivatives of existing strains and was confined to the specific antiphage systems that were encoded on the particular plasmid(s) that were transferred, which in many cases exhibit a limited range of activity. This approach was also limited by the relatively small number of phage resistance plasmids with conjugative functions, thereby restricting the adaptability of the cultures.

Most recently, the advent of high-throughput genome sequencing and the ensuing explosion in the number of complete and draft genome sequences of LAB that are now available in public databases (Table 30.2), combined with ground-breaking biochemical and biophysical studies, have resulted in a significant leap forward in our knowledge of dairy strains (and their phages). Indeed, such technologies have been very significant contributors to our wider understanding of other technologically important traits, such as proteolysis and ripening, in addition to other metabolic activities. Thus, it is now almost a universal practice for starter culture companies to routinely sequence newly isolated strains that exhibit potential for application or novelty and subject them to comparative genome analysis with previously sequenced isolates, which may lead to the identification of conserved, differentiating, or even unique features that could enhance their commercial relevance. An example of this approach is the identification of strains possessing different *cwps* phage receptor genotypes (and/or subgenotypes) and, based on this information, development of strain blends that can be scientifically certified as phage unrelated. Such approaches can be expected to eliminate the need for labor-intensive, timeconsuming, and high-cost starter selection and rotation systems designed to limit the risk of phage infection while simultaneously achieving the desired flavors, textures, and aromas in the final product.

Starter cultures encode myriad metabolic functions that render them technologically robust and/or desirable. Genomic data can be used to identify the extent of genetic diversity, the level of adaptation to the dairy environment, and the presence of specific metabolic pathways or factors that may influence their application in starter culture blends or rotations. For example, the two subspecies of *L. lactis* that are widely employed in the dairy industry, *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*, show significant differences in their genetic detail. *L. lactis* subsp. *cremoris* strains exhibit extensive genome decay, which is reflective of their adaptation to the dairy environment and the associated heavy reliance on milk as a rich source of direct nutrients. As a further adaptive measure, *L. lactis* subsp. *cremoris* strains are observed to harbor a more extensive plasmid complement than *L. lactis* subsp. *lactis* strains, which encode functions such as lactose and casein utilization, among other advantageous functions for the dairy fermentation environment. *L. lactis* subsp. *cremoris* strains are used preferentially in the production of Cheddar-style cheeses due to their general ability to generate more desirable flavor profiles, while *L. lactis* subsp. *lactis* strains are associated with faster growth and higher acidification rates and are preferred in European continental cheeses such as Brie.

Detailed genome analysis provides the means to generate a database of genetic characteristics of starter cultures. Knowledge of the predicted genetic functions of a culture is useful in selecting strains for targeted cheese trials to assess the functional capabilities and technological appropriateness of such cultures. Therefore, genomic approaches afford starter culture providers with the information to guide preemptive assessments of particular strains and strain blends. However, while this approach is very powerful and facilitates the identification of novel strains, there remains the challenge of adapting strains (where required) in a manner that is acceptable to regulatory bodies, i.e., in a non-GMO (genetically modified organism) manner.

The above-mentioned genomic approaches to analyzing starter cultures and their functional properties have revealed that there is limited genetic diversity among lactococcal and *S. thermophilus* strains. This implies that although commercial starter culture providers may have large collections of strains, many of these may be closely related, thus causing a certain level of redundancy in terms of functional and/or genetic diversity. Furthermore, strains in the collection may be phage related, which would pose major problems in terms of building good strain partnerships for the widely used DSS culture systems. Therefore, it is imperative that starter culture providers continue to adopt the parallel approach of sourcing novel strains as well as modifying and improving existing strains which already exhibit desirable traits and meet the required performance criteria in commercial practice.

CONCLUSIONS

The business of producing fermented foods has a very long history and has undergone significant evolution, especially in the past century. However, even though most consumers' experience of fermented foods relates to those that are produced on an industrial scale, there still remains a positive association with the artisanal, traditional, and healthful characteristics of the original, preindustrial products. The successful transition from small-scale, local, artisanal production to a large-scale, factory-based activity that retains the qualities that are attractive to the consumer can, to a major degree, be attributed to the development of high-performance starter cultures. These have been continuously and successfully adapted and refined to meet the ever-changing challenges posed by changing consumer preferences and increasingly stringent manufacturing and technological demands. This transition could not have occurred without the application of scientific studies which were essential in unravelling the underlying basis for the key functional and technologically important properties that allow starter bacteria to successfully perform in

highly intensive industrial settings. As the demands on starter cultures have increased in the modern era, so too have the advances in our knowledge of these microorganisms, thanks to the effective application of the modern -omics and other analytical technologies. Indeed, the era of constructing and engineering specific starter strains for particular industry needs is rapidly approaching, notwithstanding the restrictive regulatory environment in many jurisdictions. However, it is also important that the food fermentation industry retains its values of providing products that maintain their link with the "natural goodness" and the highly diverse organoleptic characteristics long associated with traditional fermented foods. This tension between the demands of modern manufacturing processes and the need to maintain the wholesomeness and traditionality of fermented products will remain an interesting challenge for those who provide and use starter cultures in the future.

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31
Diet, Health, and the Gut Microbiota

INTRODUCTION

The traditional view of the human body is that eukaryotic cells form the structures and superstructures that make up the body. More recently, this view has been updated to include the microbes that live in and on us. These communities of microbes, known as the microbiota, have previously been a neglected area of research. They consist of bacteria, fungi, and viruses and provide a range of potential functionality due to the large size of the metagenome. This functionally diverse population is known as the microbiome, and it is now known to be essential for our health and well-being. A number of factors contributed to a lack of awareness in the wider scientific community of the importance of our microbiota. Most important of these was the lack of research tools that would allow us to characterize these communities. This resulted in a naïveté within the wider scientific community regarding the symbiotic relationship between eukaryotic and prokaryotic organisms, which led to a eukaryote-centric view of our health. Now, the microbiota is increasingly regarded as an auxiliary organ of the human body and is considered an important factor for an individual's health and response to illness. In this chapter, we discuss the importance of the microbiota diet relationship and how this relationship influences our health.

History of the Microbiome

The bacteria in our guts have evolved within with us over the millennia. This closely knit evolution has allowed these organisms to form mutually beneficial relationships with their human hosts. The human microbiome covers every surface in and on our bodies and is one of the most complex and diverse biological systems ever observed. If we consider the number of cells and the genetic information and functionality that these organisms contain, it is no surprise that these organisms are key regulators of our development and health. Diet-health associations are well established in the scientific literature, with well-balanced diets that contain a mixture of proteins, fats, and carbohydrates from plant-based foods being regarded as optimal in the maintenance of the health and well-being of an individual. What is perhaps less appreciated is the impact that our microbiome has on how this diet influences our overall nutrition and health.

The vast majority of the research to date on the associations between microbiome and diet has focused on the fecal microbiota, which is used as a proxy for the proximal colon. This is primarily because fecal material is readily available, in contrast to colonic biopsy samples, which tend to be difficult and expensive to harvest. The gut microbiome is the most heavily studied human microbiome due to the direct association between fiber in a diet and the utilization of this fiber by bacterial species. Therefore, this chapter focuses on the gut microbiota.

The Gut Microbiome

Traditional research on the gut microbiome was carried outusing culture-dependent techniques, such as cell counts, to characterize a few easily cultured organisms. This method provided a distorted view of the composition of our microbiome, with organisms such as *Escherichia coli* and *Bifidobacterium*, which are present at low levels in adults, being extensively studied due to their ease of cultivation, and failed to provide information on the full gut microbe composition.

The 16S rRNA gene is evolutionarily conserved across bacterial species and can be used to identify the taxonomy of a bacterium by comparing it to a reference database. With the advent of high-throughput sequencing, it became possible to characterize the microbiota through the sequencing of this 16S rRNA gene amplicon sequence. This is one of the two most common ways to characterize the microbiota, with the second being the use of whole-metagenome sequencing, which characterizes the microbial genes and thus reveals the functional potential of the microbiota.

Because of the ability to characterize both the microbiota (taxonomy) and the microbiome (genes, genomes, and potential functionality), the study of communities of microbes has become an exciting and rapidly expanding field of research, as seen by the rapid increase in the number of microbiota papers. These studies have allowed us to identify the huge impact of the microbiome on our health, nutrition, and even well-being, with almost 90% of all disease and disorders being associated with microbiota or microbiome alterations. The microbiota is now seen as an auxiliary organ of the human body and is thus considered an important factor for an individual's health and response to illness.

When we discuss the microbiota, it is important to understand the different levels of taxonomy that a bacterium can belong to. At the highest level is the domain, *Bacteria*. This domain is subdivided into phylum, class, order, family, genus, and species. Each bacterial species can be further subdivided into strains, which are genetic variants or subtypes of the species. The gut microbiome is dominated by two main phyla, *Firmicutes* and *Bacteroidetes*, which account for 90% of the abundance of taxa in our gut, with *Proteobacteria* and *Actinobacteria* accounting for most of the rest (1). The genera *Prevotella* and *Bacteroides* of the phylum *Bacteroidetes* are two of the most common genera in our gut. They are functionally similar and tend to be negatively associated with each other, so that an individual can be said to have *Bacteroides* predominance or *Prevotella* predominance. As will be discussed, this strong negative association forms the basis for two of three

subject groups in the enterotype model of the human gut microbiome (2), with the third group, “other,” tending to be the genus *Ruminococcus* of the phylum *Firmicutes*. There are a large number of genera within the phylum *Firmicutes*, with some of the most common genera in the gut being *Lactobacillus*, *Roseburia*, *Clostridium*, *Ruminococcus*, *Blautia*, *Dorea*, *Lachnospira*, and *Faecalibacterium*. Other abundant taxa in the human gut include the genus *Akkermansia* in the phylum *Verrucomicrobia* and the genus *Bifidobacterium* in the phylum *Actinobacteria*.

The microbiota is viewed as being essential to health throughout life. The microbiome forms over our lifetime through the acquisition of microbes from our environment. Therefore, the majority of differences in the microbiome between individuals can be described as stochastic. However, it is notable that the initial acquisition of bacteria occurs at birth. In the days and weeks after birth, the evolving neonatal gut acquires a nascent microbiota of low diversity and high instability. It has been suggested that if the establishment of the stable adult microbiota is programmed in infancy, this early microbiota profile leaves a lifelong signature with a potentially significant effect on lifetime health.

MICROBIOME AND DIET IN EARLY LIFE

As with adults, the most extensively studied microbiota niche in infants is the gut. There has been an explosion of interest in the infant microbiota in the past 10 years, as researchers have begun to recognize the potential importance of early-life programming of the microbiota. In this overview, the gut microbiota is the primary focus, with a brief mention of other populations where appropriate, such as the skin and oral microbiota. The most important determinants of the gut microbiota at 1 month of age have been found to be the mode of delivery, type of infant feeding, gestational age, infant hospitalization, and antibiotic treatment of the infant. The main factors responsible for the development of the infant gut microbiota are illustrated in Fig. 31.1, which also illustrates how the

microbiota may influence the health of the infant. There is likely maternal influence on the establishment and subsequent maturation of the infant microbiota via vertical transmission.

The microbiota establishment is now thought to be initiated *in utero*, so the evidence for this hypothesis is discussed first, and then how birth mode affects the initial founder bacterial populations and how these are subsequently succeeded by increasingly diverse bacterial populations as the neonatal gut matures are reviewed. A crucial factor after mode of birth is the feeding regimen of the infant, which may be exclusive breast-feeding, bottle feeding, or a mixture. The concurrent development of the infant immune system with the microbiota is currently not well understood from a mechanistic viewpoint, but it is acknowledged that there is likely to be close interaction between the two. This section on the early-life microbiome describes current knowledge on the development of the infant microbiota, from an early period of instability and low diversity to a more adult-like microbiota with increased stability and diversity. Despite recent advances in identifying the sources of the neonatal microbiota, many questions remain regarding the true origin of the bacteria which colonize the newborn and the factors which drive the high level of interindividual variation observed in the gut microbiota.

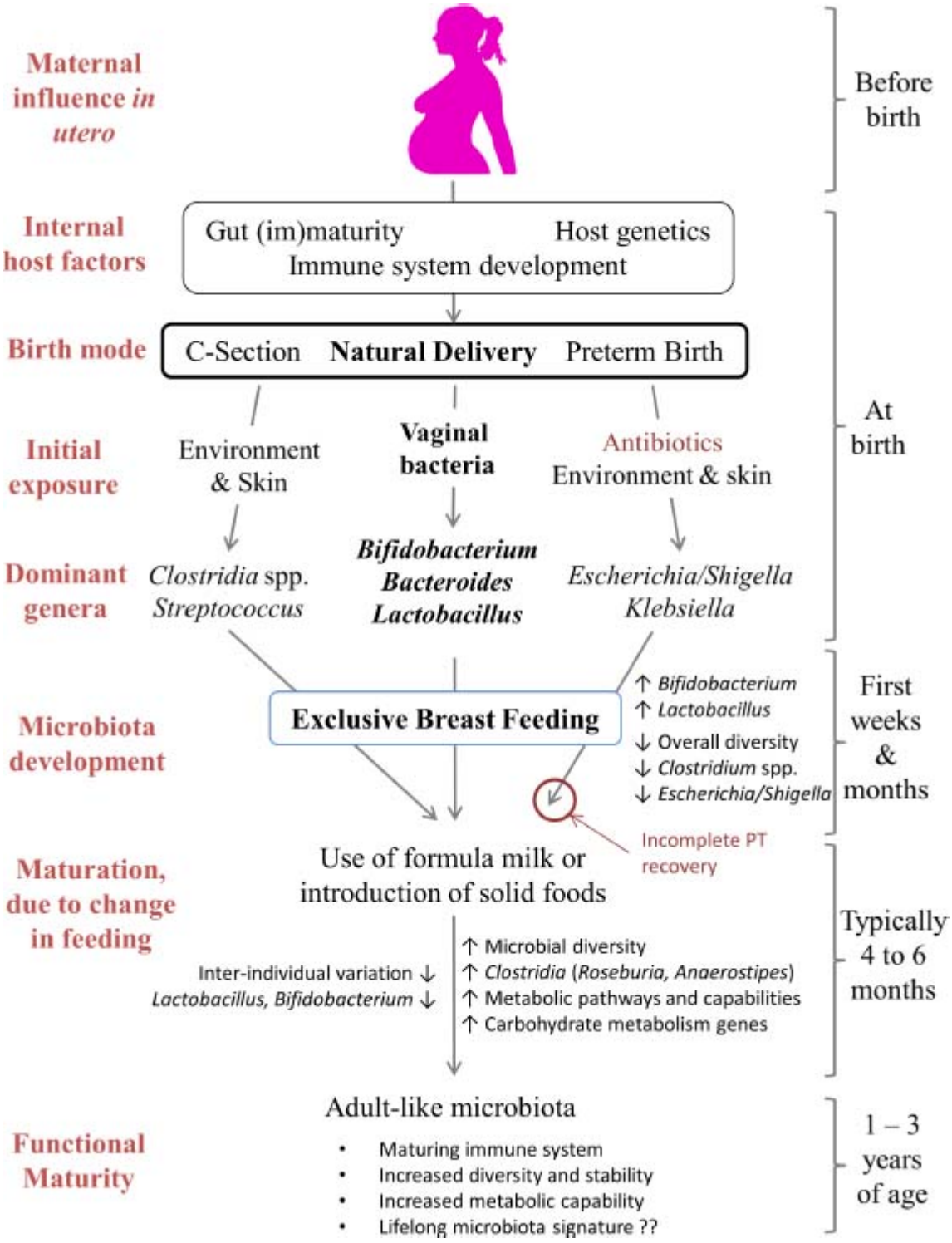

Maternal influence *in utero*
Before birth
Internal host factors
Gut (im)maturity
Host genetics
Immune system development
Birth mode
C-Section
Natural Delivery
Preterm Birth
At birth
Initial exposure
Environment & Skin
Vaginal bacteria
Antibiotics
Environment & skin
Dominant genera
Clostridia spp.
Streptococcus
Bifidobacterium
Bacteroides
Lactobacillus
Escherichia/Shigella
Klebsiella
Microbiota development
Exclusive Breast Feeding
↑ *Bifidobacterium*
↑ *Lactobacillus*
↓ Overall diversity
↓ *Clostridium* spp.
↓ *Escherichia/Shigella*
First weeks & months
Incomplete PT recovery
Maturation, due to change in feeding
Use of formula milk or introduction of solid foods
↑ Microbial diversity
↑ *Clostridia* (*Roseburia, Anaerostipes*)
↑ Metabolic pathways and capabilities
↑ Carbohydrate metabolism genes
Inter-individual variation ↓
Lactobacillus, Bifidobacterium ↓
Typically 4 to 6 months
Adult-like microbiota
Functional Maturity
• Maturing immune system
• Increased diversity and stability
• Increased metabolic capability
• Lifelong microbiota signature ??
1 – 3 years of age

Figure 31.1. Factors influencing the establishment of the infant microbiota. The development of the microbiota from the womb to adult-like functional maturity as a toddler is influenced by a myriad of factors. An overview of some of the current knowledge surrounding this developmental process is outlined conceptually.

MATERNAL INFLUENCE AND PREBIRTH FACTORS

Transmission of Bacteria from Mother to Neonate

An extensive review details the universality of vertical transmission in the animal kingdom (3), arguing that it is so important that it "should be viewed as an additional and important mechanism of genetic and functional change in human evolution." With this in mind, maternal health parameters, particularly microbiota populations from various body sites, should be investigated in infant microbiota studies as a potential source of influence on the establishment of the microbiota. Maternal prepregnancy body mass index (BMI) has been found to influence the microbiota of the offspring, and it has yet to be demonstrated if this effect is maintained as the infants age. A study of lifestyle factors found no difference in the overall microbiotas of mothers or their infants despite large differences in lifestyle between the two groups studied but did find a change in the abundance of specific genera, such as an increase in *Bifidobacterium* and a decrease in *Bacteroides* and *Veillonella*. *Bifidobacterium* in particular is a keystone bacterium in the developing microbiota, with *Bacteroides* also being important in the infant gut. Previous studies have found a significant effect of environment on the infant microbiota: infants in Malawi and rural Burkina Faso were found to have microbiota compositions very different from those of infants in metropolitan areas of the United States (4, 5). The authors hypothesize that the different diets that the infants receive may drive this significant effect, in addition to the potential environmental influence.

Maternal Microbiota Alterations during Pregnancy

Specific maternal microbiota change occurs during pregnancy, with alterations in both the vaginal and gut microbiota, which is significant, as they are the two main sources of vertical bacterial transfer from a mother to her naturally delivered infant. These changes are likely part of an adaptive response to protect and promote the health of the newborn infant by providing a specific bacterial inoculum at birth. Aagaard et al. (6) found that microbiota diversity was lower in the vaginas of pregnant women and there was a dominance of *Lactobacillus* species, especially during later gestational stages. The changes in the maternal gut are suggestive of a greater energy harvest to support both the fetus and the mother.

ESTABLISHMENT OF THE MICROBIOTA BEFORE AND AFTER BIRTH

The founding bacterial populations of the infant microbiota at birth are significantly dependent on birth mode (7–15). Twenty-four hours after birth, infants born by spontaneous vaginal delivery (SVD) are found to have acquired bacterial communities resembling the vaginal microbiota of the mother, while communities in infants delivered by cesarean section (CS) resemble the skin microbiota of the mother (16). This is due to SVD infants receiving an inoculum of bacteria from the birth canal during delivery, which is absent in a CS birth. This study also showed that there was no significant difference between the microbiotas of different body niches (oral, skin, and gut) at this early time in the infant life.

The differentiation of microbiota across different body niches occurs within 1 week, and birth mode has been shown to have a significant effect on the infant oral microbiota at 3 months of age (17). The microbiota of an SVD infant is more closely related to its own mother's vaginal microbiota than that of a random mother, while microbiotas of CS infants are no more closely related to their own mother's than to that of a random mother (16). This suggested

a strong environmental influence on CS infants, which was confirmed in a separate study showing that the microbiotas of infants in neonatal intensive care units (NICUs) had bacteria that resembled those found in swabs of the NICU ward environment, including from equipment, general surfaces, and the hands of healthcare providers and parents (18); worryingly, these included bacteria harboring antibiotic resistance genes. The developing neonatal microbiota is characterized by low diversity and high variability (4, 19). A high proportion of *Bifidobacterium* species has been consistently associated with SVD and shown to have a lower relative abundance in CS infants (8, 20–22). There is also a greater proportion of *Bacteroides* and *Lactobacillus* in SVD infants than in CS and preterm (PT) cohorts (23).

Infants born by CS continue to have an altered microbiota profile in the first weeks of life, regularly characterized by underrepresentation, or delayed acquisition, of *Bifidobacterium, Escherichia-Shigella*, and *Bacteroides* species. In addition to specific changes, the initial overall diversity of the microbiota is often found to be lower in CS infants than their SVD counterparts.

EFFECT OF FEEDING TYPE ON INFANTS' GUT MICROBIOTAS

Nutritional Composition and Importance of Breast Milk

Breast milk is the only food source that has coevolved with humanity and, as such, is the gold standard food source for neonates. Jain and Walker's review (24) gives an excellent overview of how, in addition to providing pronutrient effects, the microbiota can be stimulated by breast milk to limit nutrient resources available to potential pathogens, by physically occupying niches within the gut and out competing them for metabolic substrates. Mother's milk contains a number of bioactive ingredients to aid the development of the growing infant, including lipids, secretory IgA, live bacteria, and antimicrobial proteins, which can have a large

influence on the development of the gut microbiota. Live bacteria are present in breast milk, including *Streptococcus*, *Lactobacillus*, and *Bifidobacterium*. Breast milk changes over time to reflect the changing nutritional needs of the infant, from colostrum to less diverse milk as lactation progresses, while milk composition has even been found to be altered depending on the gender of the offspring among rhesus macaques (25).

Exclusive Breast-Feeding versus Partial Breast-Feeding

It is generally accepted that exclusive breast-feeding increases the proportional amount of *Bifidobacterium* in the infant gut. CS infants who are breast-fed may rapidly increase the proportion of *Bifidobacterium* in their gut microbiota. Maternal milk also contains a wide range of human milk oligosaccharides (HMOs) which cannot be digested by the host and require specific bacterial strains to break down (26). Certain bifidobacterial species, such as *Bifidobacterium infantis*, are of great importance, as they are capable of digesting these HMOs into metabolically useful substrates. Similarly, *Bacteroides* species prevalent in breast-fed infants are also capable of consuming HMOs as a sole carbon source. It is clear that the species (or indeed strains) of bacteria present in the infant gut are an important factor in the infant's efficacy in metabolizing milk and extracting the maximum nutritional benefit from it. A recent study demonstrated that bovine milk oligosaccharides promoted the growth of *Bifidobacterium* in formula-fed infants (27).

The use of formula milk has been shown to impact metabolism (28) and development of the neonatal immune system (29). Breast-feeding has been associated with a higher abundance of bacterial cells but less diversity than formula feeding, as the introduction of formula milk or solid food perturbs bacterial colonization and may lessen the benefits of breast-feeding. It has been shown that when breast-fed infants receive even a small proportion of their nutrition

from formula milk, the microbiota closely resembles that of infants fed formula only and that this alteration is similar to the one observed when the switch is made to solid food (30).

Interestingly, shotgun data have revealed that the cessation of exclusive breast-feeding appears to be the main driver of the metabolic shift in the gut, rather than the introduction of solid food (15). In that study, exclusive breast-feeding for a prolonged duration was associated with a younger functional "microbiota age," with lower diversity and reduced levels of central carbohydrate metabolism genes.

GUT DEVELOPMENT IN PT INFANTS

PT neonates have several unique challenges to the establishment of their microbiota. They are typically delivered by CS, have maternal and/or neonatal exposure to antibiotics, are incubated in the sterile environment of the NICU, and have an immature immune system and a compromised gut mucosa, all of which can significantly alter the natural pattern of acquisition of gut microbiota. The microbes in the NICU resemble those found in the PT gut (18), indicating a clear environmental influence in this crucial early period of bacterial colonization. Worryingly, antibiotic-resistant microbes from the hospital environment were among those found colonizing these infants' guts in the NICU.

Early-life perturbations of the PT infant's gut microbiota result in an atypical microbial profile, with the proportion of *Bifidobacterium* in guts of PT infants being commonly found to be very low until at least 1 month of age (31, 32). Nutrition may also be affected in PT infants, as they may be fed cow's-milk-based formula, pasteurized donor breast milk, or human milk fortifiers, rather than breast milk from their own mothers. This can create a different metabolic environment in the gut and thus favor a different microbial community. The strong influence of breast-feeding is discussed above, and even when PT infants are breast-fed, mothers of PT

infants produce significantly altered milk with a novel mix of HMOs that could influence microbial growth. Despite these challenges, the PT gut can progress to a gut microbiota resembling that of an SVD infant if it is not repeatedly exposed to antibiotics and if breast-feeding is continued.

The significant perturbations to PT infants' gut microbiotas strongly indicate that they may be a suitable target for intervention or modulation of their microbiotas at an early age to restore the microbiota to a more beneficial population.

GUT MATURATION AND IMMUNE SYSTEM DEVELOPMENT

The gut microbiota plays a vital role in the maturation of the infant gut, including barrier function, integrity, and immune response, which has been extensively reviewed elsewhere (24, 33), and a detailed discussion of specific associations is largely beyond the scope of this overview. In brief, a symbiotic relationship exists between the gut microbiota and the developing infant gut. CS infants demonstrated reduced responses from a type of T helper cell called Th1 during the first 2 years of life, enforcing this theory. The nutrient-rich environment provides an ideal habitat which the microbes can rapidly colonize to form a stable ecosystem. This, in turn, provides a new range of metabolic substrates for the gut, which increases the digestive capacity and the energy harvest of the infant. Inflammatory responses to bacterial exposures can activate both the innate and adaptive immune responses by a variety of methods, including interaction with the mucus layer; activation or deactivation of Tolllike receptors, interleukins, and cytokines; activation of B cells in the mesenteric lymph node system; and many more.

Understanding how the adaptive immune system identifies antigens from commensal bacteria, and how it responds in conjunction with the innate immune system, holds immense potential for identifying

the initial drivers of immune disorders, in particular autoimmune disorders, which occur at a higher rate in CS infants. Elucidating the exact mechanisms underpinning this response may identify the roles of the initial founder populations of gut bacteria, if any, in the progression of such disorders. It is likely that the combination of relatively low (breast-fed infants), high (formula-fed infants), or delayed (CS and PT infants) exposure to specific antigens and the resulting inflammatory responses in this crucial window of dynamic host-microbiota interaction profoundly influence the long-lasting gut and systemic health of the individual throughout life.

PROGRESSION TO AN ADULT-LIKE STABLE MICROBIOTA

As the infant ages and begins weaning, significant development occurs in the microbiota composition. The cessation of breast-feeding and/or introduction of complementary feeding induces the replacement of the breast-feeding-associated microbiota, as characterized by lactobacilli and bifidobacteria, with a more adult-like microbiota often dominated by *Clostridiales* spp. and *Bacteroides* spp. The overall diversity increases, with increased functional similarity to the maternal microbiota occurring by 1 year of age (15). The 1-year-old gut environment has been found to be enriched in genes involving the degradation of complex sugars and starch and genes involved in central carbon metabolism. This suggests that the microbiota adapts to availability of nutrient substrates as the infant ages. In developed countries, the infant gut microbiota appears to mature to an adult-like status more quickly than that of infants in developing countries, but differences in functionality remain as development is ongoing. As development progresses to an adult microbiota type, it is clear that environmental, diet, and lifestyle factors combine to greatly influence the microbiota, as evidenced by the distinct difference between gut microbiota compositions observed in individuals in

urban areas of the United States and those of individuals in developing countries (4).

THE MICROBIOME AND LONG-TERM DIET IN ADULTHOOD

This section describes the composition and functionality of the gut microbiome of healthy adults. The gut microbiota of healthy adults is commonly dominated by two bacterial phyla, *Firmicutes* and *Bacteroidetes*, with interindividual variability in their proportions. Members of the *Proteobacteria*, *Actinobacteria*, and *Verrucomicrobia* are present at lower abundance (1). Although there is great inter- and intrasubject variability, a well-established categorization of microbiome profiles of individuals based on the most abundant genus in their gut microbiomes is widely used to simplify the research area. The classification is based on the idea that all the subjects may be classified into three groups, called enterotypes, based on the prevalence of *Prevotella*, *Bacteroides*, or *Ruminococcus* in their gut microbiota (2). This classification, although appealing for understanding microbial change in health and disease, has been accused of leading to an oversimplified vision of the gut microbiome (34, 35). Indeed, it is probable that associations of dominant taxa within the gut may vary smoothly in the human population, going from one enterotype to another with only a weak association. Nevertheless, it is a useful tool when presenting the microbiome literature and allows a generalized discussion of the most common microbiota compositions. With these limitations in mind, the general associations in the literature are as follows: studies have associated the *Bacteroides* enterotype with a high-fat, low-fiber diet, while the abundance of *Prevotella* was linked to a diet rich in fiber and low in animal products (36).

While both *Prevotella* and *Bacteroides* possess a large catalogue of genes for carbohydrate-utilizing proteins, the *Prevotella* enterotype tends to be associated with high-fiber diets while the *Bacteroidetes*

enterotype tends to be associated with low-fiber diets, an apparent paradox due to the similarity of the potential functionality encoded by their genomes. This can be partially explained by a mouse study (37) which examined the mucin-degrading abilities of *Bacteroides* and provided evidence that *Bacteroides* species preferentially utilize dietary fiber when it is available; however, when the mouse was provided with a low-fiber diet, some *Bacteroides* organisms had the ability to utilize mucin as an alternative energy source, which may deplete the protective layer of the epithelium. Differences such as these may explain the health associations between the microbiota and the number of diseases and disorders.

These studies were pioneers in highlighting the power of long-term diet in shaping gut microbiome and how a short-term dietary switch from a high-fat, low-fiber diet to a high-fiber, low-fat diet can rapidly stimulate changes in a number of taxa within the gut microbiome. However, these short-term changes are not durable, and therefore, the long-term habitual diet seems to be more important in shaping the gut microbiome (36). Basically, what is food for humans is also feeding our commensal microbes.

DIET, THE MICROBIOTA, AND BMI

Obesity is associated with a range of serious diseases and health conditions. The percentage of the world population that is overweight has been steadily increasing. An area of much controversy in the literature on the microbiome and further afield is the factors that contribute to obesity. Dietary type, consumption of excess calories, and genetics all have roles to play in the worldwide obesity epidemic. Recently, the microbiome has been implicated in the obesity epidemic, with Turnbaugh et al. (38) showing that obesity is associated with a reduced bacterial diversity and an altered functional potential encoded by the bacterial genes. This area of research is complicated due to confounding effects of associations between diet and BMI, microbiota and BMI, and diet and microbiota. Indeed, Davis et al. showed that for increased BMI,

the influence of diet was greater than the influence of the microbiota (39). However, even if the effect of the microbiota is modest, it cannot be ignored as a confounding factor, as even a modest effect could represent large changes at the population level.

The link between the microbiota and BMI has been firmly established in animal models, with Ley et al. (40) showing that genetically obese mice had an altered microbiome profile, with increased proportions of *Bacteroidetes* and decreased proportions of *Firmicutes*, as well as a reduced diversity compared to their lean siblings. They formulated the first theory of how the microbiota influences body weight by postulating that the obese mice were more efficient at extracting and/or storing energy, which pushed the individual animals into a high-BMI state. However, the microbiota taxonomic alterations observed to be associated with high BMI are not consistent between studies (41).

Sze and Schloss showed that there were few if any consistent taxonomic alterations seen in studies looking at high-BMI versus normal-BMI individuals (41). Low diversity and the associated metric, low gene richness, have been identified as being associated with high BMI (42). Sze and Schloss confirmed that there is indeed a small but significant association between low diversity and high BMI.

WESTERNIZED VERSUS TRADITIONAL DIETS

In Western countries, dietary and lifestyle habits have changed extensively in recent decades, with an increase in the consumption of red meat, high-fat foods, and refined sugars. The "Westernization" of the diet is surely involved in the increase in the incidence of inflammatory disorders, such as obesity, diabetes, cardiovascular diseases, and allergies. However, a strictly food-based view of these conditions, while useful, may miss some important details. The French paradox, for instance, is the fact that

on average, the French eat a high-fat diet but do not have the same high rates of coronary heart disease as other Western populations. To investigate the influence of the microbiota on these diseases, several studies have looked at rural populations living in South America or Africa as a model of traditional, healthful diets, to understand how the Westernization of diet and lifestyle influenced our gut symbionts. All these studies consistently found that the composition of the gut microbiota dramatically differs between urbanized, Western people eating a high-fat and high-protein diet and rural populations, usually hunter-gatherers or agriculturalists, still consuming a subsistence, agrarian diet, with low intake of animal-origin products and high consumption of fibrous foods, such as fruit, vegetables, tubers, and roots. Western populations often show a loss of microbial diversity (5, 43, 44), with a decrease or disappearance of fiber-degrading bacteria, such as *Prevotella*, *Treponema*, *Succinivibrio*, and *Lachnospira*. In particular, *Treponema* was found only in gut microbiotas of nonhuman primates (45–47) and human populations with more traditional lifestyles from Africa and South America (5, 43–45), suggesting that this member of the gut microbiome was recently lost in urbanized people. Moreover, genomic analysis showed a selective adaptation of this genus to the gut environment and to the use of complex polysaccharides (43). In contrast, Western gut microbiotas are often enriched in bile-tolerant microorganisms, such as *Bacteroides*, *Alistipes*, and *Bilophila*, associated with the consumption of a diet rich in fat and protein and poor in indigestible carbohydrates (44, 48).

Gomez et al. (49) compared the gut microbiomes of two traditional African populations (BaAka and Bantu) with those of American controls. The two rural populations had different lifestyles and dietary habits: the BaAka are hunter-gatherers living in the rainforest and consuming a diet rich in fibrous tubers, such as manioc roots and wild yams, while the Bantu recently switched to a subsistence agriculture. Interestingly, the authors observed a progressive decline in the abundance of fiber-degrading bacteria

from the BaAka to the American controls, with the Bantu showing intermediate features, reflecting the consequences of a recent change in their lifestyle (49).

A high diversity of microbiota organisms will be reflected in an increased functional potential and increased functional redundancy. The microbiomes of Hadza hunter-gatherers were found to contain plenty of genes that encode enzymes able to break down a broad set of polysaccharides, in contrast to the microbiomes of Italians (50). This highlights the enrichment in metabolic pathways related to the degradation of several xenobiotics of human origin in the latter (50). Moreover, a Westernized diet selects a gut microbiome with a higher capacity to degrade amino acids and bile salts, as well as simple sugars (4).

THE GUT MICROBIOME AND THE PRODUCTION OF METABOLITES

The gut microbiome is responsible for the production of several metabolites that may positively or negatively affect host health and are collectively defined as the gut metabolome. The gut metabolome reflects both the chemical diversity of the substrates available and the different metabolic potential of the gut microbiota. Therefore, it is influenced both by the gut microbiota composition and by the type of nutrients we feed it. Indeed, traditional and Westernized populations show distinctive fecal metabolomes (Fig. 31.2). Most studies focused on fecal levels of short-chain fatty acids (SCFAs), in particular, acetate, propionate, and butyrate, that are produced by bacterial fermentation of indigestible polysaccharides in the colon and are associated with several health-promoting effects, such as anti-inflammatory and anticarcinogenic effects (51, 52). Indeed, fecal SCFA levels were significantly higher in the agrarian and rural populations studied around the world (5, 44). Undoubtedly, this is explained by the substantial enrichment in fiber of their diet, which selects for a fiber-degrading and SCFA-producing gut microbiota.

Besides SCFAs, gut microbiota can produce other metabolites, with a potentially beneficial effect on the host health (Fig. 31.2).

Phytochemicals and micronutrients entrapped in plant cell walls are released by microbial fermentation in the colon, where bacteria can metabolize them and convert them to a wide range of bioactive molecules (53). As an example, daidzein, a soy isoflavone, can be converted to equol, which may be associated with anticarcinogenic properties (53). However, due to the wide diversity of chemical molecules commonly included in the phytochemical group, it can be expected that different gut microbiota members may be involved in their degradation, with the resulting production of a comprehensive assortment of bioactive metabolites. Gut microbiota can also produce detrimental metabolites (Fig. 31.2). Fermentation of aromatic amino acids produces phenylacetic acid, phenols, indoles, and *p*-cresol, associated with a proinflammatory and carcinogenic effect (51), while the catabolism of sulfur amino acids and taurine by sulfate-reducing bacteria leads to proinflammatory and toxic sulfides (52). Moreover, amines derived from microbial fermentation of proteins in the colon can be nitrosated to produce *N*-nitroso compounds, which were previously correlated with the incidence of colorectal cancer (54).

In addition, a high-fat diet leads to an increase in bile secretion and consequently to a higher quantity of bile acids in the colon, where they are converted into secondary bile acids. The gut microbiomes of African Americans were found to be enriched in microbial genes encoding secondary bile acid production compared to those of rural Africans, which showed lower levels of fecal bile acids and a lower risk of colorectal cancer (55).

Another important microbial metabolite associated with a detrimental effect on host health is trimethyl amine-*N*-oxide (TMAO) (Fig. 31.2). Intestinal microbiota metabolism of phosphatidylcholine, choline, and l-carnitine produces trimethylamine, which is oxidized in the liver; the synthesis of TMAO was recently implicated as a risk factor in the development of

cardiovascular diseases (CVDs) and atherosclerosis (56, 57). One commonly observed contradiction to this observation is that saltwater fish are rich in choline and carnitine and are therefore a potential source of TMAO, but the consumption of fish is associated with lower rates of CVDs and atherosclerosis.

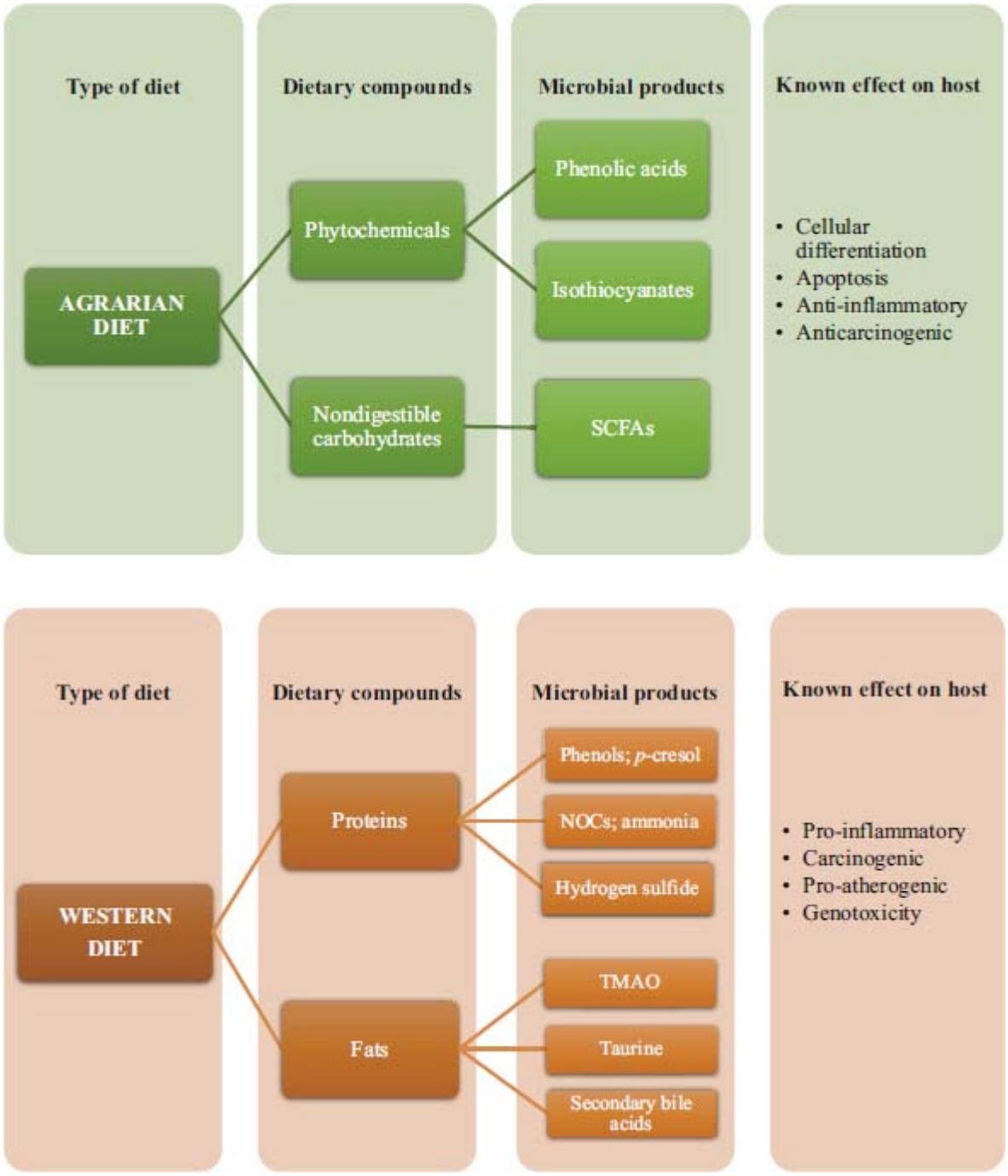


Figure 31.2. Microbiome-diet interaction. The type of diet consumed affects the quantity and nature of the microbial metabolites produced. These metabolites can have beneficial or deleterious effects on the host. NOCs, *N*-nitroso compounds.

A potentially important but less well-known risk factor for atherosclerosis may be the production of bacterially derived lipids

that are quickly absorbed by the digestive tract. We assume that the lipids in our circulatory system are derived from our diet; however, recent evidence points to the presence of lipids produced by *Bacteroidetes* in our blood, and these may be causative of CVDs and atherosclerosis due to their inflammatory nature. Immune cells that are present on the blood vessel walls recognize these lipids as foreign and bind them. Furthermore, the *Bacteroidetes* lipids may be broken down by enzymes in the body, leading to the production of inflammation-enhancing molecules. Together, these processes may lead to an inflammatory response with negative health outcomes (58).

THE MICROBIOME AND DIETARY TYPES

Western diet-microbiota interactions are not always detrimental. Indeed, the number of people choosing a vegetarian or vegan diet has surprisingly increased in the last few years. According to an American poll in 2016, approximately 3.3% of American adults are vegetarian or vegan (59). Although these dietary patterns are becoming more prevalent, few studies have tried to characterize the microbiota of the vegetarian's or vegan's gut. De Filippis et al. (60) recently explored the gut microbiomes and metabolomes of 153 Italian vegetarians, vegans, and omnivores. Consistent with the higher dietary intake of fiber, vegans and vegetarians showed higher abundances of plant-degrading bacteria, such as *Lachnospira* and members of the family *Prevotellaceae*. Vegans had significantly higher abundances of fecal SCFAs than omnivores, while vegetarians showed intermediate levels. In addition, the urinary abundance of proatherogenic TMAO was higher in omnivores (60). Indeed, Wu et al. (36) found striking differences in the plasma metabolome of a smaller cohort of vegans and omnivores, with higher levels of metabolites likely derived from microbial catabolism of plant polyphenolic compounds, as well as of equol. Differences in equol concentration were not associated with a significant variation in the consumption of food rich in its

precursors (such as soy-based products). Indeed, only about 30 to 40% of Western adults are able to convert isoflavones to equol, regardless of their dietary intake (61), emphasizing that both the intake of the right substrates and the presence of specific microbial players are necessary for the development of a health-promoting metabolome.

Without reaching the extreme of a vegetarian or vegan diet, the habitual consumption of a healthful dietary pattern, such as that based on the Mediterraneanstyle diet, can help to develop a health-associated microbiome and metabolome, without completely renouncing meat and animal-origin products. The Mediterranean diet is characterized by high intake of fruit, vegetables, legumes, nuts, and whole grains, moderate consumption of fish, and low intake of saturated fat, meat, and dairy products (62), and it has been demonstrated to be beneficial for the treatment of obesity, type 2 diabetes, and inflammatory and cardiovascular diseases (63–65). Indeed, in the study mentioned above (60), the adherence level to the Mediterranean dietary pattern was evaluated. The authors observed a progressive increase in fecal SCFAs going from low-adherence to high-adherence subjects and an opposite trend for urinary TMAO. Interestingly, even when omnivore subjects alone were considered, a higher adherence to the Mediterranean diet correlated with a higher abundance of SCFAs. This study highlighted the idea that a healthful dietary pattern can deliver the proper substrates and shape the gut microbiome to boost the production of health-promoting metabolites even in an overall omnivore-type diet.

AGE-RELATED CHANGES IN THE GUT MICROBIOME

As people transition from middle to late adulthood, the microbiota composition, which has been relatively stable over the previous few decades, starts to undergo changes. These changes are particularly

evident in centenarians, where decreased levels of *Faecalibacterium* and *Bifidobacteria* and an increase in the mucin-degrading bacterium *Akkermansia* have been reported (66).

The ELDERMET project investigated the microbiome changes that occur after the age of 60 and found that both the microbiota and the metabolic activities of the microbiota change in the elderly in a diet-dependent manner (67). The diet in an elderly population can change for a number of reasons, including institutionalization, loss of taste sensation, frailty, tooth loss, and difficulties with chewing or swallowing. For this reason, the diet of elderly persons tends to be less diverse, with a reduction in the proportion of calories coming from unprocessed plant-based sources and an increased consumption of processed, high-sugar, high-fat foods.

As mentioned above, changes in the long-term diet have a significant impact on the microbiota, and this is also true for the elderly population, in whom a dramatic observable reduction in a number of key taxa, such as *Faecalibacterium*, *Ruminococcus*, and *Blautia*, is observed. Between healthy young adults and the general aged population, there is a reduced abundance of *Blautia* and *Ruminococcus* species and an associated increase in *Escherichia*, of which *E. coli* is a member (67).

However, these changes do not occur in a uniform manner across the elderly population. It is observed that the decline is strongly correlated to the decline in the diversity of plant-based foods that have been consumed, and in a subsequent study, it was shown that there are only subtle differences between the microbiomes of healthy elderly individuals and those of healthy adults (67, 69). Therefore, within the elderly population, there are a large number of microbiome states, ranging from one that is indistinguishable from that of young healthy adults to distinct microbiome profiles that can be related to varying health phenotypes.

The changes in the microbiota that can occur can be summarized into three trends: the loss of commensals, the loss of fiber-responsive taxa, and the gain of pathobionts. The loss of the fiber-

responsive taxa and, to a lesser extent, that of the commensals are associated with a reduction in the diversity of the microbiota. The loss of these taxa and the subsequent low diversity are associated with a prefrail state (70). We can speculate that it is at this stage where a dietary intervention to prevent this loss would be most effective. As people age further, a proportion of the population continue to lose taxa, becoming very low-diversity individuals. However, these individuals are in the minority within the frail aging population, with the majority of frail individuals experiencing a small increase in diversity compared to the average microbiome. This increase in diversity is the gain of rare taxa that are present at low levels in healthy young adults but whose increased presence in the elderly is associated with an altered immunological and metabolic state and a large increase in frailty. These increases in rare taxa, though not directly associated with diet, are associated with the risk of malnutrition. Also, the gain of these potentially pathobiont rare taxa seldom occurs before the loss of the more abundant fiber-responsive proportion of the microbiota.

These changes in diversity are associated with changes in the predominant taxa within the microbiota profile. As mentioned above, the two main phyla within the gut microbiota are the *Bacteroidetes* and *Firmicutes*. The genus-level alterations in the *Bacteroidetes* go from a predominantly *Prevotella*-associated microbiome to a predominantly *Bacteroides*-associated microbiome as people move from a high-diversity microbiome to one that is low diversity. The *Bacteroidetes*-associated taxa that are associated with the increase in frailty are *Parabacteroides* and *Alistipes*. The *Firmicutes* that are generally lost as elderly individuals become frailer are *Roseburia*, *Lachnospiraceae*, *Ruminococcus*, and *Faecalibacterium*.

These alterations in the taxonomic profiles are associated with an altered functional potential. The utilization of nitrogen sources is increased, with a detectable increase in the proteolytic functions (71). We also see a decrease in the production potential and the amount of SCFAs produced due to the reduced consumption of fiber

and the associated reduction in key genes necessary for SCFA production (67). We can associate these changes with the reduction in abundance of several key butyrate producers belonging to *Clostridium* clusters XIVa and cluster IV (67, 72, 73). In the study of elderly individuals, the authors also demonstrated that there were a reduced number of gene types in the elderly gut that were related to the production of butyrate, acetate, and propionate production in the fecal metagenome of the oldest, frailest individuals (67). Previous reports also indicate that an increase in the abundance of facultative anaerobes, of which *Escherichia* is a member, is associated with increased immune activity associated with aging, sometimes called “inflammaging” (72).

The loss of taxa, and potential functionality, associated with frailty is strongly associated with the quality of the diet of the individual (68). However, this does not exclude the possibility that the composition of the gut microbiota can vary due to a number of physiological and immunological factors, such as a reduction in immune system functionality, loss of epithelial barrier integrity, gastrointestinal motor function, altered food transit, and altered functionality of the intestinal wall (74).

GUT MICROBIOME MODULATION AND DIETARY INTERVENTIONS

Although long-term dietary habits seem to be the main force shaping the gut microbiome, several studies suggest that the appropriate dietary intervention can boost the development of beneficial microbes in the gut. These studies focused on the addition of indigestible fiber to the diet and highlighted the possibility of boosting the development of fiber-degrading bacteria in the gut (75). These changes in the gut microbiota were often associated with the increase of beneficial metabolites, such as SCFA, and with the reduction of inflammatory markers (76, 77). The presence of diet-responsive gut microbiome members highlighted

the possibility of modulating the gut microbiome through the appropriate diet for therapeutic purposes. However, it is still not clear how and to what extent these changes may occur in a predictable way. Indeed, recent findings suggested that the response to a dietary intervention is person specific and strongly connected to the gut microbiome configuration. During a dietary intervention with barley kernel fiber, Kovatcheva-Datchary and coworkers (78) observed that only a fraction of the population studied showed improved glucose metabolism after the intervention and that only the responders showed increased levels of *Prevotella copri*. Therefore, a personalized intervention considering differences in the gut microbiome across subjects is often necessary. Accordingly, people eating identical meals showed different metabolic responses, consistently associated with gut microbiota features (79). Integration of gut microbiota signatures in a complex algorithm that included anthropometrics, blood parameters, and dietary habits allowed a precise prediction of postprandial glycemic response, associated with type 2 diabetes development (79). Moreover, this algorithm may be successfully used for defining personalized dietary intervention to lower postprandial blood glucose. These results emphasized the possibility of personalized medicine based on a patient-tailored modulation of the gut microbiome, which may help in the treatment of a wide range of disorders in the near future.

CONCLUSION

A wealth of knowledge clearly exists surrounding diet and the microbiota. Diet is a major determinant of the microbiota populations in our gut. In early life, diet is a driver of the microbiota as initial populations are replaced. As we progress into adulthood, a healthful plantbased diet is associated with a high-diversity microbiota which encodes a range of beneficial functionalities. As we approach the end of our life span, the beneficial microbiota may become depleted, and this is strongly associated with dietary changes.

It is clear from the literature that there are a wide number of factors outside diet that also affect the dynamic process of bacterial acquisition and retention. A broad, holistic approach is necessary to gain insight into the specific mechanisms for the sequential replacement and change in bacterial populations over time as individuals progress from a fetus through birth to adulthood and old age. Nevertheless, the diet is a key determinant of the microbiome and may therefore be utilized in interventions to maintain health, life span, and overall quality of life.

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32
Probiotics and Prebiotics

For decades, probiotics and prebiotics have been studied for their beneficial health effects. More recently, research on the composition and activities of the microbes colonizing the human body has established the important role the microbiota plays in health. A convergence of these fields has increased awareness of the value of probiotics and prebiotics as tools to improve the function of the microbiota and thereby improve host health. Clinical evaluations have accumulated, with positive results leading to probiotics being included in some medical guidelines (1, 2). Probiotic and prebiotic targets have expanded considerably beyond the original focus on the gut to the oral cavity, vaginal tract, brain function, metabolic endpoints (diabetes, obesity, and cardiac risk factors), nutritional factors (mineral absorption, vitamin production, and lactose malabsorption), and the immune system. The range of target hosts for probiotic and prebiotic interventions has expanded beyond humans to include numerous agricultural species (mammalian, aquatic, and avian) and companion animals. Mechanistic understanding of how probiotics and prebiotics function has also advanced considerably. Knowledge of how prebiotic structure relates to function has increased (3), providing insights into the ability of these substances to stimulate beneficial commensal (autochthonous) microbes. As externally introduced (allochthonous) microbes, probiotics have been shown to both directly (through interactions with epithelial and immune cells) and indirectly (through interactions with autochthonous microbes and metabolic end products) impact the host (4). Many model probiotic

strains amenable to focused genetic manipulation have been developed and investigated via "-omics" technologies, leading the way for developments in strain improvement, mechanistic studies, and construction of genetically modified strains capable of functioning as vaccines or delivering other biologically important molecules (5). Finally, global markets have increased product options for consumers and health care professionals. Notably, although most commonly found in food and supplement products, probiotics and prebiotics are being developed and investigated to address therapeutic needs related to microbiome manipulation. This chapter reviews the science behind these dynamic substances.

PROBIOTICS

Definition of "Probiotics"

Probiotics, first popularized at the beginning of the 20th century by the Russian Nobel laureate Elie Metchnikoff (Fig. 32.1), have undergone numerous definition revisions. The term "probiotic" does not entail a legal definition in most countries, but a definition of "probiotics" was adopted by a joint United Nations Food and Agricultural Organization/World Health Organization working group in 2001, and it is currently one of the most widely recognized definitions of these microorganisms (6). This definition was refined (and grammatically corrected) by a consensus panel of experts convened by the International Scientific Association for Probiotics and Prebiotics (ISAPP) to "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (7). Figure 32.2 shows substances that fall under the umbrella of this definition and distinguishes between substances that are probiotics and those that are not. The wide acceptance of this definition has not deterred some from proposing many related terms, which may serve specific purposes (Table 32.1). Important implications of this definition are that a probiotic must be administered alive and must have been shown in a properly

controlled study to confer a health benefit. In order to be the subject of research, the probiotic must be taxonomically identified and suitably defined, which precludes inclusion of undefined microbial mixtures such as those found in some fermented foods and in fecal microbial transplants. The definition of a probiotic applies to any type of health benefit for any type of host and spans regulatory categories, which, as long as the definition is met, could include foods (those consumed by humans, animals, marine animals, birds, etc.), dietary supplements, medical foods, therapeutics, medical devices (including nonoral applications), and cosmetics. Importantly, a probiotic must be safe for the intended use.

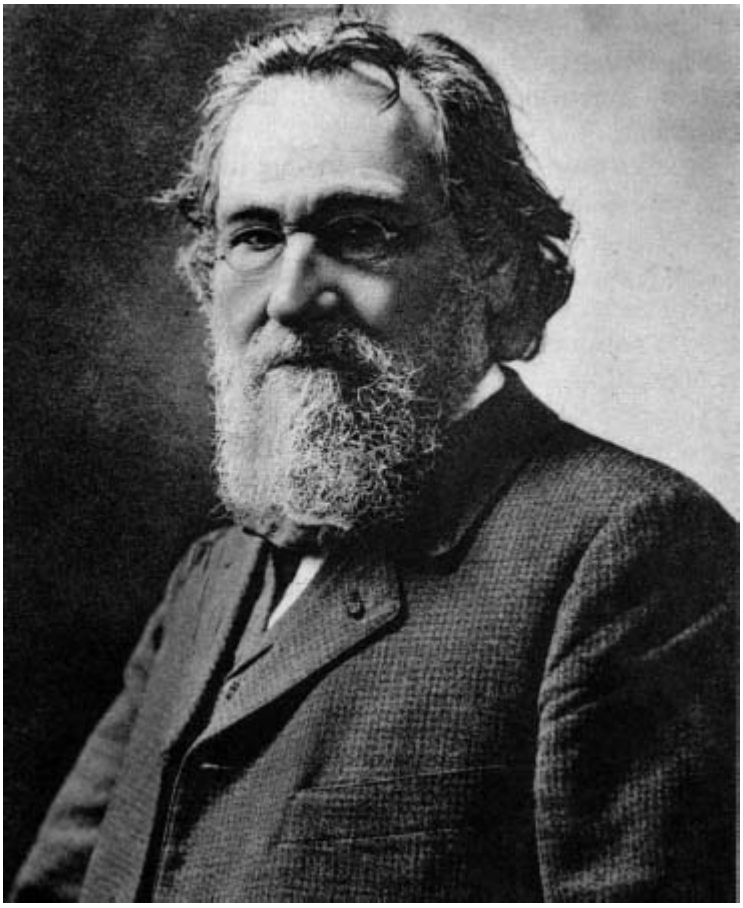


Figure 32.1. Elie Metchnikoff (1845–1916) (125).

There is also interest in the potential health effects of consuming killed bacteria or cellular components. Since the definition of probiotic stipulates use of a live microorganism, these products do

not fit the probiotic definition. Several terms have been coined to include nonviable microbial cells or products (Table 32.1). The preponderance of research, however, has been conducted using live microbes.

Mechanisms of Action of Probiotics

While the mechanisms through which probiotics exert their beneficial effects are not entirely understood on a clinical level, several major avenues exist through which probiotic cultures are likely to carry out beneficial activities (8, 9) (Table 32.2). Some key mechanisms are described here.

1. Interaction with the immune system. The intestinal tract is a prominent part of the immune system, and it constantly interacts with "non-self" components as they transit through the gut. Consumed foods, live microbes, microbial components, and commensal microbiota are in regular contact with gut-associated immune cells. Through *in vitro* assays, *in vivo* animal models, and in human studies, probiotic strains have been shown to interact in a strainspecific manner with immune cells. Some probiotics alter cytokine production by exposed macrophages and dendritic cells and can shift the production of cytokines from the inflammation-inducing to antiinflammatory pathways (10–12). Some probiotics can upregulate adaptive and innate immune function, to better respond to pathogen or cancer cell challenge, as demonstrated by improved seroconversion after vaccination (13). Research into effector molecules has identified ways that probiotics specifically interact with immune cells (9), such as the surface layer proteins of *Lactobacillus acidophilus* (14) and bacterial glycoproteins on the pili of *Lactobacillus rhamnosus* GG, which are important in the immunomodulation of the host (15). The net effect is that probiotic strains and their display of cell surface components can modulate immune function.

2. Strengthening the mucosal barrier. The barrier between the lumen of the intestine and the inside of the body is essential to maintaining health. This mucosal barrier is selectively permeable, allowing transit of nutrients while keeping outmost other luminal components. Dysfunctional barriers are associated with a number of disease states (16). Probiotics have been shown to reinforce and repair this barrier, in part by stimulating the production by intestinal epithelial cells of protective proteins, such as mucins (17).
3. Exclusion and inhibition of pathogens. Decadesold literature speaks of a concept known as colonization resistance (18). This phenomenon, likely the aggregate result of multiple functions of colonizing microbes, grew from observations from many different lines of research showing that an intact, normal microbiota enables the host to resist pathogens. Some of these mechanisms demonstrated by colonizing microbes are found in probiotic bacteria as well, including production of antimicrobials (bacteriocins, organic acids, and others), preemptive binding of probiotics to receptor sites to block pathogen adhesion, and stimulation of host cells to produce mucin, thereby tightening the mucosal barrier and others (19).
4. Production of bioactive substances. Probiotic growth in the intestinal tract can yield bioactive substances with a range of physiological activities (20). For example, certain probiotics can produce enzymes such as lactase, which can improve lactose digestion, or bile salt hydrolases, which can favorably impact circulating blood lipids. Probiotics may also produce, or enhance host or commensal microbiota production of, opioid substances that may contribute to gut pain alleviation (21, 22) and neuroactive substances such as gamma-aminobutyric acid, serotonin, catecholamine, and acetylcholine, which may contribute to antidepressive, antianxiety, and satiety effects (23).

5. Impact on colonizing microbiota. Although a common understanding is that probiotics "promote a healthy microbiota composition," research has shown that probiotics administered to healthy adults have minimal impact on fecal microbiota composition (24, 25), although -omics techniques to reveal taxonomic changes at the species and strain level have not been employed. Typically, transient increases in the species of the fed probiotic are detected, but no changes in overall bacterial community structure are observed. Recent evidence suggests that probiotic intervention in subjects with unstable or dysbiotic microbiota may lead to notable changes. Administration of *Bifidobacterium lactis* Bl-04 and *L. acidophilus* NCFM to colon cancer patients resulted in increased abundance of butyrate-producing bacteria in the tumor, nontumor mucosa, and fecal microbiota (26). Healthy newborn infants fed *Bifidobacterium infantis* EVC001 harbored increased levels of this species for 1 month postfeeding (27). *Bifidobacterium longum* AH1206 was stably maintained by about 30% of healthy subjects after cessation of oral administration (28). The microbiota of successfully colonized subjects was subsequently shown to be characterized by a low abundance of *B. longum*, suggesting the availability of an ecological niche that was exploited by this strain. Research into the impact of probiotics on microbiota functions has suggested that even though bacterial populations are not significantly altered by probiotic administration to healthy individuals, microbiota functions may be. Probiotic yogurt containing *B. lactis* fed to healthy subjects did not alter fecal microbiota composition but did alter microbiota transcriptomes (29). Eloe-Fadrosh et al. (30), using a transcriptomic approach, found that oral administration of *L. rhamnosus* GG to healthy elderly subjects resulted in enrichment of anti-inflammatory pathways expressed by resident microbes. This research area is ripe for exploration. It is possible that nontraditional probiotic species may more effectively change microbiota structure. Further, how the characteristics of an individual consumer's microbiota,

diet, and genes may influence microbiota responses to probiotics needs to be better understood.

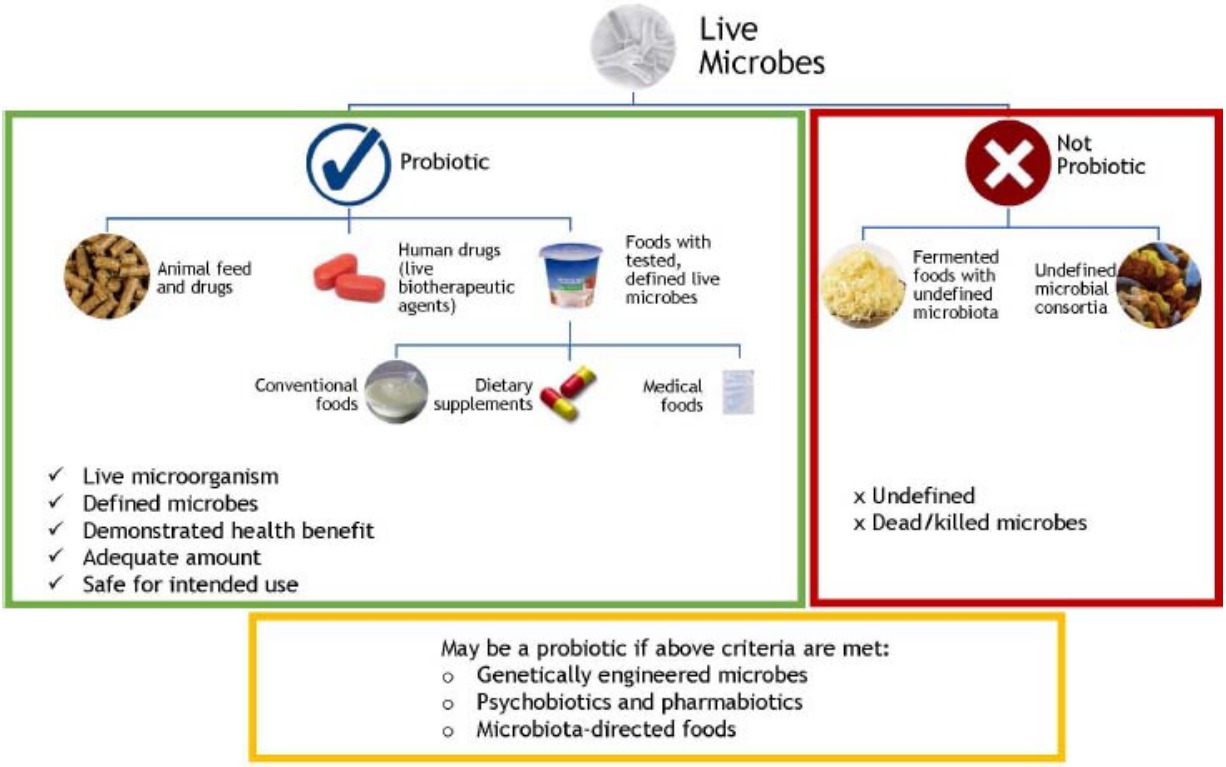


Figure 32.2. Probiotics: what is encompassed under this term. By definition, a health benefit must be demonstrated for a probiotic, either at a strain-specific level or at a taxonomic level where mechanisms are shared. Probiotics can be administered via different routes (oral, intravaginal, and topical routes and via mouth sprays) and are not limited to human use (i.e., they may also be given to companion animals, livestock, and fish). The probiotic definition is not restricted by regulatory category for probiotics and encompasses the spectrum from foods to drugs (live biotherapeutic agents). Dead microbes, microbial end products, microbial components, and undefined microbial mixes do not come under the probiotic classification. Adapted from reference 34 with permission.

Table 32.1. Terminology related to probiotics[a]

Term	Definition	Reference or comment
Probiotic	Live microorganism that, when delivered in adequate amounts, confers a health benefit on the host	7
Prebiotic	Substrate that is selectively utilized by host/commensal microorganisms conferring a health benefit	3
Synbiotic	Combination of prebiotics and probiotics	Prebiotic may (synergistic synbiotic) or may not (complementary synbiotic) be utilized by the probiotic
Antibiotic	Medicine that inhibits the growth of or destroys microorganisms	
Abiotic, postbiotic	Nonviable probiotic organism or cellular component thereof that exerts beneficial effects on health or well-being	128, 129
Pharmabiotic	Any form of therapeutic exploitation of the commensal flora, including the use of live probiotic bacteria, probiotic-derived biologically active metabolites, prebiotics, synbiotics, or genetically modified commensal bacteria	19

Immunobiotic	Bacterium that promotes health through activation of the mucosal immune apparatus	130
Psychobiotic	Live organism that, when ingested in adequate amounts, produces a health benefit in patients suffering from psychiatric illness	131
Probioceutical	Probiotic-derived factor	132
Live biotherapeutic agents	Biological product that contains live organisms, such as bacteria; applicable to the prevention, treatment, or cure of a disease or condition of human beings (not a vaccine)	133

[a] These terms differ with regard to the presence of live microbes, the intended effect, the mechanism of action, and the regulatory category.

Table 32.2. Mechanisms of action that may mediate health benefits conferred by specific probiotic strains

Stimulate intestinal mucin production
Pili expressed on bacterial cell surface enhance colonization/gut retention
Produce antimicrobial substances such as bacteriocins, targeting pathogens or shaping microbial ecosystem
Produce short-chain fatty acids
Produce enzymes, such as providing lactase to humans deficient in β-galactosidase
Enhance adaptive and innate immune function, to better respond to pathogen or cancer cell challenge
Downregulate inflammation, a risk factor for negative outcomes of many chronic disease states
Digest prebiotic fibers, to improve metabolic/growth opportunities in the gut
Improve gut barrier integrity
Modulate gene expression or activity of host microbiota, including cross-feeding and altered enzymatic activities such as bile salt hydrolase
Signal the central nervous system through production of neuroactive compounds
Produce vitamins

The final expression of a health benefit mediated by a probiotic or prebiotic may be due to the action of several different relevant mechanisms, leading to the overall observed effect.

Strain Specificity

Strain specificity of probiotic effects is a foundational principle underlying the science of probiotics. There are many examples of microbial traits, genetic and metabolic characteristics, and cell

physiology features that vary among different probiotic strains. This was investigated in human-derived strains of *Lactobacillus reuteri* using a comparative genomic approach (31). Although reuterin may not be made *in vivo* due to the lack of an essential precursor, glycerol, in the human gut, this example is still illustrative. Gene content was found to be highly conserved among clades within this species, yet only 70 to 90% of total gene content was shared. Differences in sequences occurred in genes related to bacteriophages, vitamin biosynthesis (B_{12} and folate), antimicrobial production (reuterin), arginine catabolism, and immunomodulation. Such observations have led to the widely held notion that each probiotic strain must be regarded as unique and any efficacy studies conducted on one probiotic strain cannot be extrapolated to another strain (although this notion is being refined; see below). What is less clear, however, is the extent to which measurable differences in expression of characteristics, even those presumed to be important mechanistically, impact an overall clinical benefit. Most clinical trials compare one probiotic strain or strain blend to a placebo, and a few compare different probiotics. Studies comparing different strains of the same species with regard to a clinical endpoint are rare. One example is a pilot study that looked at immune responses in subjects treated with the same dose of different strains of two species, *L. acidophilus* La-14 versus *L. acidophilus* NCFM, and *Bifidobacterium animalis* subsp. *lactis* Bl-04 versus *B. animalis* subsp. *lactis* Bi-07 (32). This study showed some differences in host immunoglobulin response to different strains compared to placebo, but unfortunately, the statistical comparisons between the different strains of the same species were not reported. Therefore, it is not possible to state that the strains are different from each other in the case of this clinical endpoint. A research need is for studies comparing different strains of the same species on clinical endpoints to determine the extent to which strain differences detected in preclinical tests translate into clinical differences.

Numerous systematic reviews evaluating the totality of evidence for a specific clinical benefit of different probiotics show that similar clinical benefits are achieved by many different strains (33). This of course does not mean that all strains can be expected to perform similarly, but it does suggest that certain determinative functions are likely shared among many different strains and that differences observed in some preclinical assessments may not predict differences in clinical outcomes. A 2014 consensus panel recommended that strains of certain well-studied species could reasonably be expected to provide some digestive benefits and therefore could be considered probiotics even at the species level. However, it is not possible to reach 100% assurance that any given strain will yield a given clinical benefit, since physiological fitness may be lacking or traits that override adequate expression of needed functions may exist.

This concept was developed further by Sanders et al. (34), who presented evidence for several mechanisms that are shared among probiotic taxonomic groups at higher levels than the strain level. The distribution of such mechanisms among all members of a taxon provides a rationale that some general probiotic benefits can be expected in a non-strain-specific manner. Indeed, the absence of certain mechanistic functions (in mutants or similar strains devoid of the function) has been shown to result in loss of physiological benefit. For example, compared to wild-type strains, loss of bacteriocin production by *Lactobacillus salivarius* UCC118 led to loss of protection against *Listeria monocytogenes* infection in mice (35), and a histamine decarboxylase-deficient *L. reuteri* mutant unable to generate histamine no longer suppressed inflammation-associated colorectal cancer in mice (36). Such results suggest that specific mechanisms might be essential for clinical benefit, and the expression of such mechanisms may be able to predict clinical benefit.

A cornerstone mechanism that likely contributes to many of the benefits attributed to probiotics is their ability to produce short-chain fatty acids (SCFAs). Metchnikoff, the founder of the probiotic

concept, proposed that a normal gastrointestinal microbiota dominantly engaged in metabolism of complex carbohydrates yielding SCFAs was preferred over a microbiota engaged in degradation of proteins, which results in putrefactive metabolites (37). Today, we better appreciate the important role that SCFAs—especially butyrate and propionate—can play in health and homeostasis. SCFAs are important to gut health through their action as signaling molecules and by inhibiting pathogens, but they are also important to the function of peripheral tissues, such as adipose tissue, skeletal muscle, and liver tissue (38). Although lactobacilli and bifidobacteria do not produce butyrate and propionate, their metabolic products (acetate and lactate) can increase levels of these important SCFAs through cross-feeding of other gut microbes (39). The metabolic pathway for production of lactate and acetate is conserved among all *Bifidobacterium* species and homofermentative (lactate) and heterofermentative (lactate and acetate) lactobacilli. These pathways are the most important metabolic pathways conserved among lactic acid bacteria and other probiotic genera.

Microbes Used as Probiotics

The most common microbes used as probiotics today are members of the genera *Lactobacillus* and *Bifidobacterium*. However, some nonpathogenic microorganisms that occur within niches in the host gut or tissues, such as *Bacillus* species, *Enterococcus*, *Escherichia coli*, and some yeast species, are also used as human and animal probiotics (Table 32.3). As studies have emerged correlating certain members of the normal gut microbiota with the absence of certain disease states, researchers have proposed an expanded list of microbes that may make suitable candidates for the next-generation probiotics (40). For example, *Akkermansia muciniphila*, which is inversely associated with metabolic disorders including obesity, diabetes, and cardiometabolic risk factors, has been proposed for inclusion in novel foods or drugs (41). *Eubacterium hallii*, a

butyrate-producing species which improves insulin sensitivity in mice (42), is being tested in humans.

Table 32.3. Examples of probiotic strains with research documentation in human subjects[a]

Species	Strain(s)
Lactobacillus acidophilus	NCFM, DDS1
Lactobacillus rhamnosus	GG, GR1
Lactobacillus casei-L. paracasei	Shirota, CRL431, DN 014 001, F19
Lactobacillus plantarum	299V
Lactobacillus reuteri	DSM17938, ATCC PTA 6475
Lactobacillus johnsonii	La1, Lj1
Lactobacillus salivarius	UCC118
Bifidobacterium breve	Yakult
Bifidobacterium animalis subsp. *lactis*	BB12, HN019, DN173 010
Bifidobacterium longum	SBT-2928, BB536
Streptococcus salivarius	K12
Saccharomyces boulardii	lyo CNCM I-745
Escherichia coli	Nissle 1917
Bacillus coagulans	BC30, IS2, MTCC 5856

[a] Strains researched as part of a multistrain blend are not listed.

Criteria for Selection of Probiotic Cultures

Different probiotic species and strains within a species exhibit distinctive properties that can markedly affect their survival in foods, survival through the digestive tract, and important probiotic properties. Therefore, strain selection becomes a critical parameter to ensure both the culture's stability and its probiotic performance.

Potentially useful traits for selection of functional probiotics are listed in Table 32.4, including minimum criteria needed to meet the probiotic definition, as well as criteria important to technological utility and *in vivo* functionality. Impacts on health targets are complex, because the underlying mechanisms by which probiotics exert these functional roles *in vivo* are multifaceted and not completely understood. An important research need is to better understand how results from *in vitro* assessments of these traits relate to *in vivo* functionality.

Our understanding of probiotic properties has been revolutionized by whole-genome sequencing and functional genomics. Genome sequencing projects examining the collective attributes of strains with probiotic properties have identified a number of similarities that suggest critical traits for these strains, including an extensive network of glycosyl hydrolases in bifidobacteria and oligosaccharide transport and metabolism enzymes in lactobacilli that allow metabolism of complex, indigestible carbohydrates available in human milk and the gut (43). Complete genome annotation and functional analysis support the identification of key genes that are expected to direct functions important in probiotic activity. For example, within the *L. acidophilus* genome (Fig. 32.3), a series of surface-associated proteins (e.g., mucin-binding and fibronectin-binding proteins) have been identified and correlated with the bacterium's ability to adhere to intestinal epithelial cells *in vitro*. Across several *Lactobacillus* species, a number of gene regions are implicated in probiotic activity, including bile salt hydrolase activity, acid tolerance, bacteriocin activity, prebiotic/oligosaccharide utilization, oxalate degradation, exopolysaccharide production, mucus and fibronectin binding, and communication with immunomodulatory cells of the host (5).

Table 32.4. Characteristics important for probiotic strains

Category	Criteria
Appropriateness	Ability to exert one or more clinically documented health benefits Taxonomic identification as determined by current genomic and phylogenetic methods Origin—although efficacious probiotics have been developed that are not normal inhabitants of the target species, there may be an ecological benefit to a strain that was isolated from a healthy individual of the target species Safety—nontoxic, nonpathogenic, and safe for the intended use
Technological suitability	Amenable to mass production and storage: adequate growth, recovery, concentration, freezing, dehydration, storage, and distribution Suitable viability retained during culture preparation, storage, and delivery Provides desirable organoleptic qualities (or no undesirable qualities) when included in foods or fermentation processes Genetically stable and consistent phenotypic properties Genetically accessible for research and potential genetic modification
Performance and functional properties often considered in strain selection[a]	Functionally capable of survival, proliferation, and metabolic activity at the target site, *in vivo* (e.g., gastrointestinal tract, mouth, vagina, skin, etc.), if required for the specific mechanism of function Resistant to bile Resistant to acid Compete with the normal microbiota, including the same or closely related species

	Resistant to bacteriocins, acid, and other antimicrobials produced by residing microbiota Adheres or interacts with the mucosal epithelium Adequate retention to achieve beneficial effects Ability to metabolize wide range of carbohydrates in the host environments (e.g., gastrointestinal tract and vaginal tract) as an energy source to maintain survival and probiotic functionality Antagonistic toward pathogenic/cariogenic bacteria Produces antimicrobial substances (bacteriocins, hydrogen peroxide, organic acids, or other inhibitory compounds) Immunomodulatory (stimulation or downregulation of immune responses, as needed for particular use) Antimutagenic Production of bioactive compounds (enzymes, bacteriocins, vaccines, peptides)

[a] Listed properties *may* be useful, depending on desired probiotic functions, expected site of action, and target host. Methods used to assess these properties typically are not standardized or validated, so results must be interpreted with caution.

Probiotics for Human Health

Substantial research has been conducted to examine the clinical effects of probiotic microorganisms. A thorough review of this research is beyond the scope of this chapter and has been presented in other publications (see reference 2 for a summary of gut-related clinical evidence; Sanders and coworkers [134] have prepared a summary of clinical evidence). A list of studied health outcomes related to probiotic consumption is compiled in Fig. 32.4. Compelling evidence has accumulated for several clinical outcomes, including treatment of infantile colic (44), prevention of antibiotic-associated diarrhea (1), prevention of necrotizing enterocolitis (45), prevention of *Clostridioides* (formerly *Clostridium*) *difficile*-

associated diarrhea (46), reduction of incidence and duration of common infectious diseases (47), adjunct treatment of *Helicobacter pylori* (48), adjunct treatment of bacterial vaginosis (49), and treatment of acute pediatric diarrhea (50). However, the body of efficacy research on probiotics comprises a heterogeneous compilation, with studies using different strains, doses of viable cells delivered, product formulation, clinical endpoints, study subject characteristics, and study design. The study quality has also varied over the years, with earlier studies more often being underpowered and not conducted or reported according to CONSORT guidelines (51), resulting in outcomes marred by a high risk of bias. Further, there are few studies that compare dose, different probiotic strains or strain blends, carrier matrix material for the probiotic microbes, or timing of administration, leaving a gap of knowledge regarding how important these parameters are to clinical success.

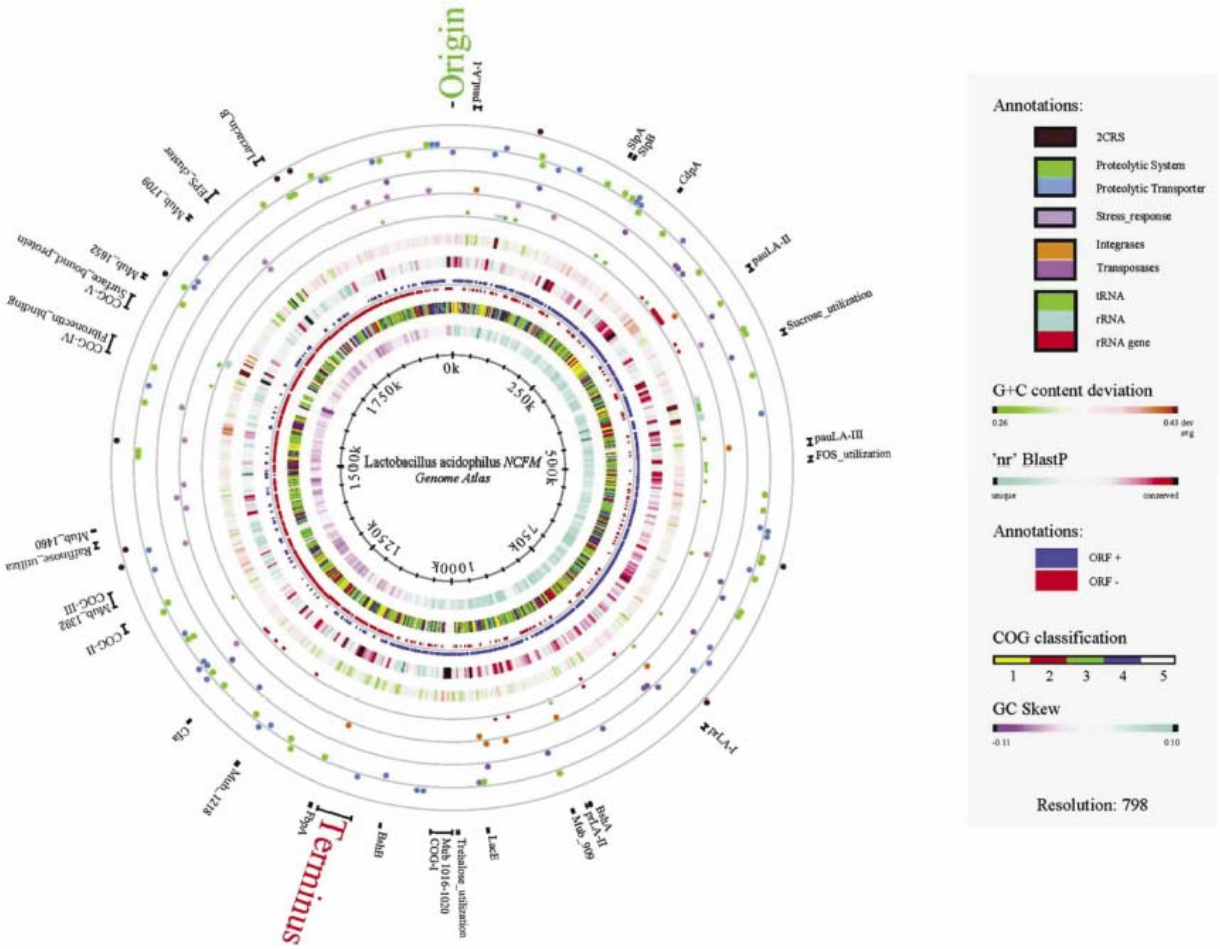


Figure 32.3. Genome atlas of the probiotic *L. acidophilus* strain NCFM, presenting a circular view of the complete genome. The key describes the single circles in the top-down outermost-innermost direction (126).

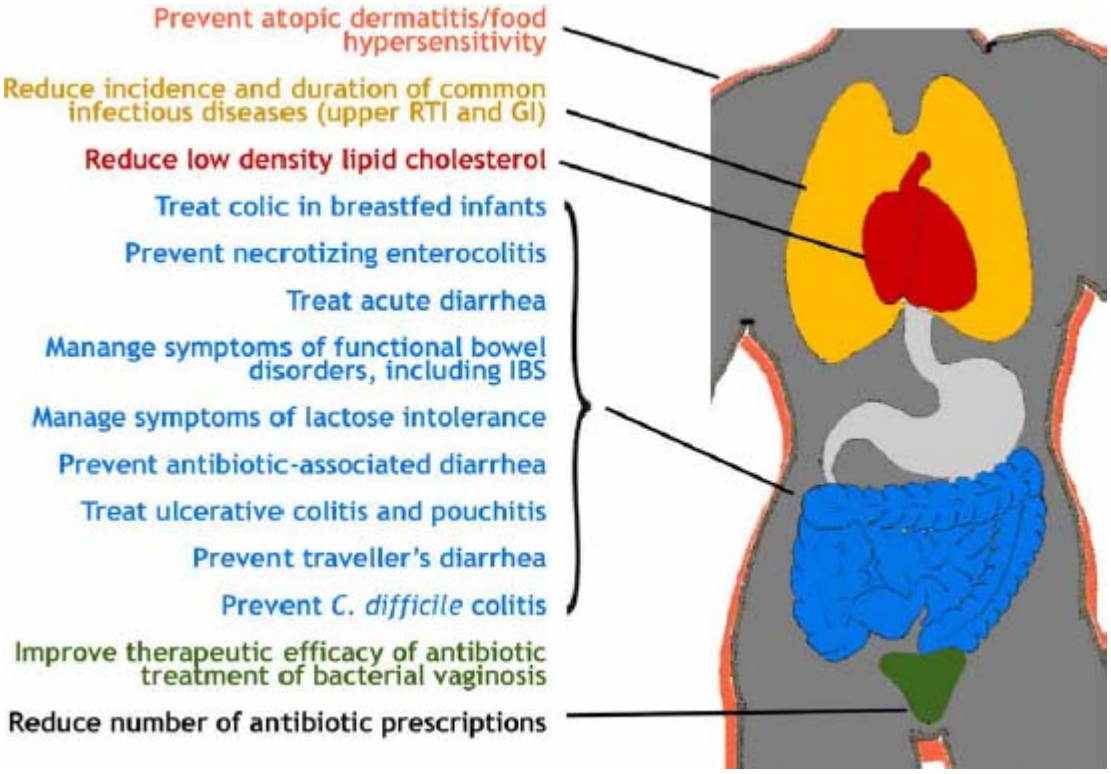


Figure 32.4. Overview of indications where probiotics have been researched with varying but solid evidence for clinical benefit. RTI, respiratory tract infection; GI, gastrointestinal.

A useful tool to gain insight into the totality of evidence for specific clinical endpoints is the systematic review and meta-analysis process. It is noteworthy that the number of human studies assessing health effects of probiotics has increased to the point that it is possible to use these techniques for an unbiased approach to reviewing the totality of evidence. Hundreds of such studies have been published, addressing a range of clinical effects. Some considerations should be brought to bear, however, when considering meta-analyses of probiotics (52). Most importantly, there must be a scientifically sound basis for combining evidence on different strains, species, or genera. Such a rationale is possible, especially when effects are expected to be based on common, underlying mechanisms.

A novel application of probiotics for health is their use to deliver vaccines and other biotherapeutics to the mucosal immune system

of the intestine. An optimized probiotic carrier could increase antigen potency and reduce the potential side effects incurred through other, more traditional delivery routes. Oral ingestion of proteins (vaccines or enzymes) results in denaturation, degradation, and loss of biological activity. In contrast, these bioactive molecules can be protected by viable microbial cells during passage through the stomach and then released into the intestine. Bioactive molecules targeted for delivery by probiotics include vaccine antigens against viral and bacterial pathogens, digestive enzymes for humans, and growth-promoting enzymes in animals. Probiotics are an attractive choice for mucosal delivery vehicles because of their ability to survive intestinal passage, record of safety, and ease of genetic manipulation (53). Wells et al. (54) pioneered the development and use of lactic acid bacteria as vaccine delivery vehicles, and developed recombinant strains designed to treat a number of conditions, including HIV infections. Klaenhammer and colleagues successfully developed effective oral vaccine delivery platforms in the probiotic species *L. acidophilus* and *Lactobacillus gasseri* for protection from anthrax and botulism challenges in mice (55–57).

Earlier it was demonstrated in a mouse model that a genetically modified *Lactococcus lactis* designed to produce and secrete the cytokine interleukin 10 in the gastrointestinal tract could decrease inflammation associated with colitis (58). This group also addressed the important issue of biocontainment of the recombinant microorganism by devising an elegant solution of replacing the *L. lactis thyA* gene with the interleukin 10 gene. ThyA is responsible for the synthesis of the nucleic acid thymidine. Once the gene is removed, *L. lactis* requires an exogenous source of thymidine that must be provided in the culture medium. Thymidine is scarce in the environment, thereby preventing growth and spread of the microorganism outside laboratorycontrolled culture conditions (58). This recombinant microorganism was tested in a phase I clinical trial for Crohn's disease, as well as the effectiveness of the engineered biocontainment system (59). The clinical study

demonstrated the feasibility of the approach but was unsuccessful, and no further human trials have been published.

There are many exciting opportunities to employ probiotic bacteria as vehicles to deliver bioactive molecules to targeted locations in the gut. This is likely to be one of the most important areas for practical application of probiotic cultures that are derived through recombinant-DNA technologies.

Probiotics for Animal Health

Considerable effort has been directed toward the development of probiotic cultures for both agricultural and companion animals. Important health targets for agricultural animals are enhancing animal growth, promoting weight gain, and reducing carriage of human enteric pathogens. Reduced pathogen carriage is of significant interest because control of enteric pathogens at the farm level can greatly reduce the risk of foodborne illness. While antibiotics have been used extensively as prophylactics in animal feeds since the 1950s to improve growth and feed conversion, concerns over the development, transmission, and spread of antibiotic resistance determinants through this practice have led to its ban by some regulatory agencies [regulation (EC) no. 1831/2003 of the European Parliament and of the Council on additives for use in animal nutrition; https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32003R1831] and has revived interest in developing probiotic cultures for livestock and poultry. Decades ago, Nurmi and Rantala (60) discovered that newly hatched chicks could be protected from *Salmonella* infection by exposure to a suspension of gut contents from the adult chicken. This concept was later revived in a probiotic mixture composed of 29 species of nonpathogenic bacteria isolated from the chicken gut. The probiotic mixture is sprayed over the chicks so that as they preen, their intestinal tract is seeded with microbes which occupy this ecological niche to provide colonization resistance against *Salmonella*, *Campylobacter*, and *Listeria* (61). This seeding mimics the colonization that occurs naturally in small flocks, where mothers

are in close contact with the hatchlings. Generally, it has been shown that undefined competitive exclusion mixtures (e.g., mixtures derived from gut contents) perform better than simpler defined mixtures (e.g., a single strain or defined mix of strains) (62). Similarly, Zhao et al. (63) specifically targeted colonization of *E. coli* O157:H7 in cattle by creating a probiotic cocktail of 17 isolates of *E. coli* and one *Proteus mirabilis* strain. These were isolated from cattle on the basis of their ability to inhibit *E. coli* O157:H7 *in vitro*. In challenge studies with *E. coli* O157:H7, the probiotic mixture reduced the level of pathogen carriage in most of the probiotic-treated animals. Probiotics have been studied for their role in reducing *Campylobacter jejuni* colonization in poultry (64). In the animal probiotic field, host specificity, animal age, and targeted benefit are critical in selecting cultures for specific probiotic applications. There are currently a number of commercially available competitive exclusion products available for agricultural animals (65).

Probiotic use in companion animals has largely focused on cats and dogs (66, 67). Study endpoints include reduction of diarrhea, treatment of gastrointestinal disorders, prevention or treatment of allergies, treatment urinary tract infections, and treatment of bad breath.

Safety

Live microorganisms of many species have been consumed safely by humans in various forms and in high numbers for centuries. Safety assessments of the commonly used and studied probiotic genera *Lactobacillus* and *Bifidobacterium* document a low potential for adverse health effects in these species (68). However, proposed probiotic strains are not limited to these genera and can include members of the genera *Bacillus*, *Enterococcus*, and *Escherichia*, which contain species that are foodborne pathogens. Novel probiotic strains must be assessed for safety before addition to foods and supplement products. Safety review of potential probiotics generally

involves, but may not be limited to, the strainlevel assessment of four criteria:

- identity of the microorganism
- history of safe use of the microorganism
- fermentation and processing conditions for the microorganism
- the microorganism's potential for pathogenicity, toxigenicity, and resistance to clinically relevant antibiotics

Although reviews conclude that the infective and toxigenic potential of lactobacilli and bifidobacteria is low (68), case studies have described opportunistic infections caused by lactobacilli in the immunocompromised or elderly. With extended survival of chronically immunocompromised individuals and an aging Western population, increased scrutiny must be given to strains that may be administered to these individuals. In particular, some intestinal isolates, such as *L. rhamnosus*, have warranted increased surveillance due to a higher correlation with secondary infections. Ongoing genome sequencing projects have not revealed any identifiable pathogenic determinants for probiotic cultures. However, genome sequencing projects have identified genes in probiotic species that are annotated as antibiotic resistance genes or multidrug resistance transporters (69). This is not surprising, given the widespread use of antibiotics in agriculture and medicine and the apparent widespread distribution of antibiotic resistance genes among commensal and food bacteria (70). When these are identified in probiotic cultures, it will be important to determine whether any antibiotic resistance determinant is functional, is clinically relevant, and has any reasonable probability for genetic transfer to other microbes.

Taxonomy and Molecular Identification of Probiotic Strains

The correct identification of probiotic strains is critical to a number of activities associated with these microorganisms, including product labeling, safety assessment, scientific communication, and attribution of beneficial effects. Historical problems with phenotypic classification of probiotic microbes led to misidentification of many commercial strains prior to 1995, and therefore the genus and species names found in older probiotic literature should be viewed carefully. Further, some product manufacturers persist in using old nomenclature. The advent of molecular techniques and efficient and costeffective whole-genome commercial sequencing facilities has made bacterial species identification by genome sequencing and phylogenetic analysis commonplace. The availability of molecular-analytic tools has enabled proper identification of probiotic species and individual strains over the last decade and has helped dispel confusion over species identity and ancestry. Phylogenetic analysis can be conducted in varying degrees of depth and combined with information on other phenotypic characteristics (e.g., microscopic morphology and carbohydrate fermentation profile) as needed to gain a complete view of taxonomic classifications.

A number of DNA sequence-based typing systems have been used to analyze conserved regions of the ribosomal DNA operon or other conserved genes in probiotic strains. These include the following:

- PCR amplification and sequencing of the 16S rRNA gene (1,500 bp) or the □450 bp of the internal transcribed spacer region between the 16S and 23S rRNA genes were used to identify members of the *L. acidophilus* complex (71).
- Modern phylogenetic trees are being constructed based on whole-genome sequencing, as is the case for the genus *Lactobacillus*, which is undergoing a major taxonomic upheaval (72).
- PCR amplification and sequencing of alternative genes that are universally present and highly conserved, e.g., the *recA* gene of

bifidobacteria (73)

- Whole-genome sequencing for comparative genomic analysis (74, 75)

The ability to properly identify probiotic species provides the first giant step toward eliminating confusion over strain identity and ancestry. The practice is expected to also uncover new probiotic species that have been hidden below the surface of traditional taxonomic descriptions. DNA sequence analysis of the rRNA operons of strains loosely classified as *L. acidophilus* revealed six closely related species that comprise the *L. acidophilus* complex: *L. acidophilus*, *Lactobacillus crispatus*, *Lactobacillus amylovorus*, *Lactobacillus gallinarum*, *L. gasseri*, and *Lactobacillus johnsonii*. All of these are considered to have probiotic potential, but the specific roles and benefits of each species remain to be determined.

Lactobacillus is a member of the phylum *Firmicutes*, class *Bacilli*, order *Lactobacillales*, and family *Lactobacillaceae*. This genus was identified by Beijerinck in the early 1900s, and members were first classified by their optimal growth temperatures and sugar fermentation pathways. Later, the species were classified based on physiological assays such as the homo- and heterofermentation profiles of the members (76). However, identification and classification utilizing 16S rRNA gene sequences is now the most widely accepted method for species-level typing of microorganisms and offers a fast, reproducible, and an inexpensive method for discrimination. Beyond phylogenetic analysis, the most detailed recent examination of probiotic and commensal microbes was whole-genome analysis of the lactobacilli. Complete genome sequencing of representative strains across 213 species comprising lactobacilli revealed the complexity of the genus and the precise taxonomic relationships between the numerous species that occupy diverse ecological niches in plants, animals, humans, and fermented foods (75).

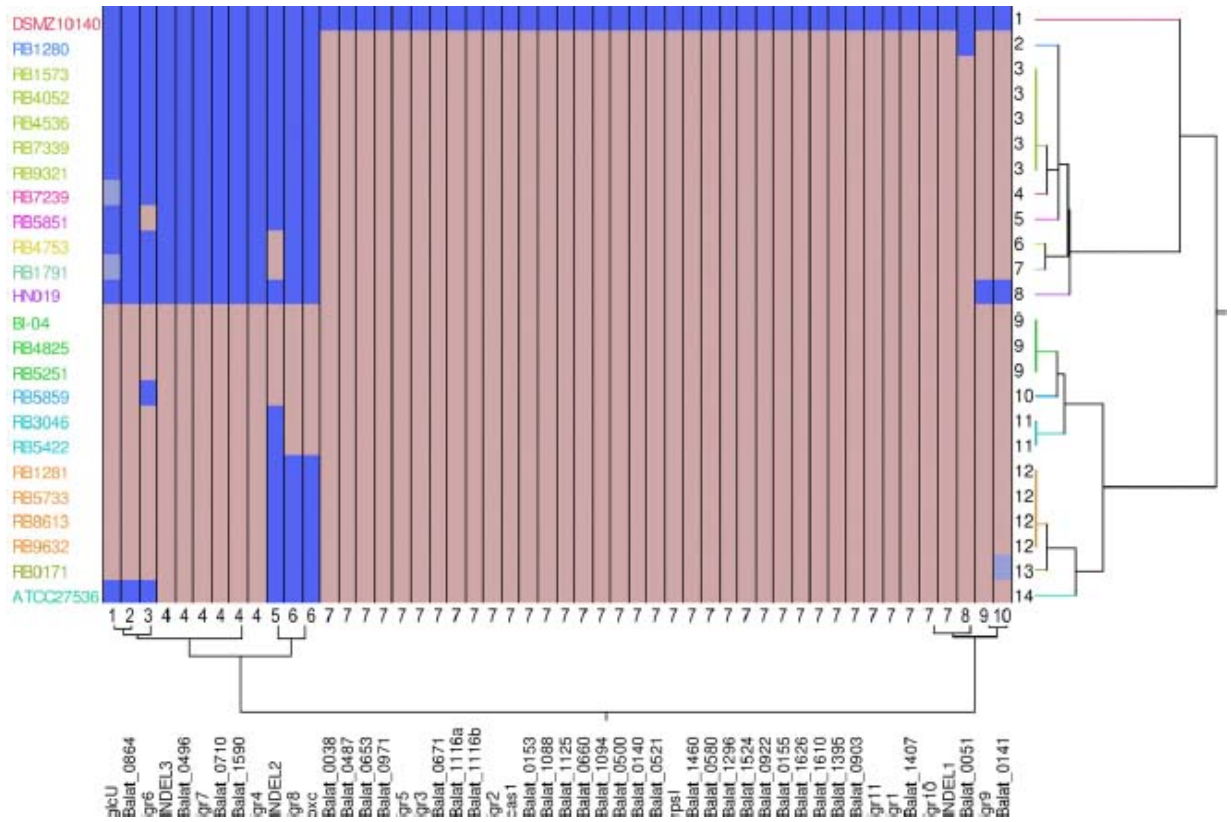
Bifidobacterium, another widely studied genus of probiotic microorganisms, belongs to the phylum *Actinobacteria*, class *Actinobacteria*, order *Bifidobacteriales*, and family *Bifidobacteriaceae*. Similar to the lactobacilli, historic methods of species identification relied on phenotypic differences, including the origin or host from which the species was isolated (e.g., *B. longum* subsp. *infantis*, isolated exclusively from infants, or *B. longum* subsp. *longum*, isolated from adults), as well as their sugar fermentation profiles (77). The taxonomic classification of bifidobacteria was significantly improved through use of 16S rRNA gene sequencing. Whole-genome sequencing shows that multiple, commercial *B. lactis* strains are highly similar (74) (Fig. 32.5), yet specific strains can be distinguished (78). A current list of all recognized bacterial species, including *Bifidobacterium* and *Lactobacillus* species, can be found in the Approved List of Bacterial Names at http://www.bacterio.net.

Comparative Genomics

Comparative genomic analysis is a powerful tool for discerning the relationships between microbes and identifying key similarities and differences. Alignment of whole genomes has illustrated the overall synteny between the closely related members of the *L. acidophilus* complex and substantial differences with *Lactobacillus plantarum* (79). Moreover, genomic content can be compared between strains of the same species, allowing an inventory of genetic content or deficiencies. Molenaar et al. (80) compared the genomic content of 20 *L. plantarum* strains using DNA microarrays based on the *L. plantarum* WCFS1 genome. Remarkably, considerable variation was observed among strains, notably in properties considered important for probiotic activities, such as exopolysaccharide synthesis, bacteriocin production, and carbohydrate utilization.

Genomic analysis has revealed important differences and similarities within these probiotic bacteria (81). First, these bacteria are rich in transporters for uptake of sugars and amino acids, emphasizing their dependence on importing, rather than

synthesizing, nutrients from the gastrointestinal tract. In this regard, members of the *L. acidophilus* complex (*L. acidophilus*, *L. gasseri*, and *L. johnsonii*) are largely deficient in their ability to synthesize amino acids and other essential nutrients. This is consistent with the evolution of these bacteria within the small intestine, where nutrients are abundant and can be acquired via transporters. In contrast, *B. longum* encodes complete pathways for synthesis of all amino acids and nucleotides, reflecting the more nutrientcompetitive environment of the colon, where bifidobacteria naturally reside. In addition, *B. longum* harbors a surprisingly large number of genes predicted to encode proteins required for catabolism of complex carbohydrates and plant polymers (43). Such compounds, like fructooligosaccharides (FOSs), are considered prebiotics which can promote the growth of bifidobacteria and some lactobacilli.


DSMZ10140
RB1280
RB1573
RB4052
RB4536
RB7339
RB9321
RB7239
RB5851
RB4753
RB1791
HN019
Bl-04
RB4825
RB5251
RB5859
RB3046
RB5422
RB1281
RB5733
RB8613
RB9632
RB0171
ATCC27536
1 2 3 4 4 4 4 4 4 5 6 6 7 7 7

pathways which are clustered in a 600-kb "lifestyle adaptation region." This metabolic capacity and flexibility explain why *L. plantarum* is found naturally in the intestinal tract and can also dominate microbial populations in fermenting plant materials.

Functional Genomics

As a result of genomics, correlations between gene function and mechanisms of probiotic activity are now rapidly emerging in this field, and the potential functionality and safety of probiotic cultures can now be rationally considered based on genome content. Genomic information also allows surveys for potentially problematic genes that might be harbored by probiotic strains, including antibiotic resistance genes. The tetracycline resistance determinant (*tetW*) was found by sequencing of the genome of *B. lactis* BB-12, a widespread commercial probiotic (82). This strain was subjected to an in-depth review by FDA and remains classified as "generally recognized as safe" due its long-term, safe use as a probiotic and the widespread distribution of *tetW* throughout the genus *Bifidobacterium*, in both commensal and probiotic strains (83).

Among probiotic lactobacillus strains, genes identified and confirmed to be involved in carbohydrate metabolism (lactose, sucrose, galactooligosaccharides, FOSs), bile tolerance, and host interaction (e.g., cell surface proteins) are common. There are other species of lactobacilli, however, that are primarily involved in the fermentation of milk that have markedly reduced gene content and genome size (84). Further approaches to validate the importance of probiotic genes include the use of transcriptomics and directed mutagenesis for gene knockouts to link gene to function. One landmark example examined the effects of the bacteriocin-producing *L. salivarius* strain UCC118 on infection challenges by *L. monocytogenes* in a mouse model. This study unequivocally demonstrated that wild-type *L. salivarius* prevented infection, while a mutant strain unable to produce the listeria-active bacteriocin did not prevent infection and death of the mice (35) (Fig. 32.6). More

recently, cell surface pili (Fig. 32.7) were discovered via genomic screening of both lactobacilli and bifidobacteria and linked to the ability of these bacteria to bind mucin (85, 86).

Establishing mechanisms that link *in vitro* phenotypic criteria to *in vivo* functionality remains one of the major scientific challenges for probiotics in the future (5). In this regard, this field is poised perfectly to further exploit the recent progress in high-throughput sequencing capacity and functional genomics toward the investigation of these bacteria and their probiotic capabilities. There remains a myriad of possible probiotic strains, representing a diverse set of phenotypes, which are being linked increasingly to a variety of benefits. Defining and screening genetic traits important for functional probiotic activities promise to identify superior strains and allow the construction of strain combinations that elicit unique or multiple effects.

PREBIOTICS

The human diet is generally rich in complex plant carbohydrates, but the human genome encodes fewer than 20 carbohydrate-hydrolyzing enzymes capable of breaking down the disaccharides sucrose and lactose and, to some extent, starch polysaccharides (87). It is now established that most of the indigestible carbohydrates pass through our small intestine and reach the colon intact. There they nourish the microbiota, which possesses specialized carbohydrate-scavenging machinery to harvest nutrients from these dietary glycans. In fact, the “bifidus factor” in human milk, discovered during the 1950s (88) and later identified as human milk oligosaccharides (HMOs), has long been observed to influence the gut microbiota composition in breast-fed infants by stimulating the growth of bifidobacteria. Based on this specialized host-microbiota mutualistic relationship, certain indigestible carbohydrate ingredients that mimic bifidogenic effects have been exploited as dietary interventions to modulate gut community structure and enrich health-promoting microorganisms.

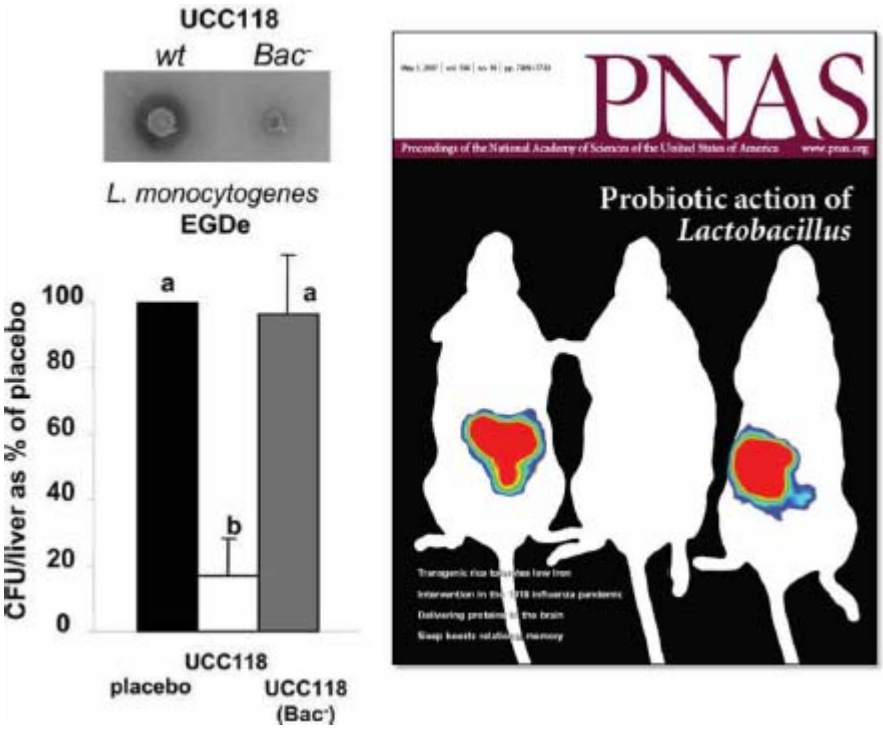


Figure 32.6. Bacteriocin production by *L. salivarius* UCC118 protects against *L. monocytogenes* EGDe infection of mice. (Top left) Bacteriocin production by wild-type (wt) UCC118 and the non-bacteriocin-producing mutant. (Bottom left) Bars represent mice fed placebo (black), UCC118 (white), or a non-bacteriocin-producing mutant of UCC118 (gray). (Right) Image on the cover of *Proceedings of the National Academy of Sciences* shows livers of mice that are colonized with luminescent *L. monocytogenes* EGDe, in red. Adapted from reference 35 with permission.

Prebiotic Definition and Composition

The term “prebiotic” was formally conceptualized in the mid-1990s by Gibson and Roberfroid (89) and was recently updated by a consensus panel of experts convened by ISAPP. A prebiotic is currently defined as a substrate that is selectively utilized by host microorganisms conferring a health benefit (3). Gibson and Roberfroid also proposed the synbiotic approach, where a prebiotic ingredient is combined with a probiotic culture, often the prototypical probiotics bifidobacteria and lactobacilli, to enhance

their retention and efficacy in the host (89). Well-documented prebiotic ingredients are long- and short-chain β-fructans (inulin and FOSs [also called oligofructose]), the HMO-mimetic β-galactooligosaccharides (GOS), and lactulose disaccharide. FOSs are linear fructose oligomers (Fig. 32.8) naturally found in Jerusalem artichoke, chicory roots, and onion and are commercially either produced from hydrolysis of inulin or synthesized using sucrose as the starting substrate by the transfructosylation action of β-fructofuranosidases or β-fructosyltransferases. Originally developed to simulate HMOs, GOSs are typically linear galactose oligomers (Fig. 32.8), most often contain a terminal lactose moiety at the reducing end, and are commercially synthesized by the transgalactosylation activity of β-galactosidases using high concentrations of lactose as starting substrates. Other promising prebiotic candidates include raffinose trisaccharide, isomaltooligosaccharides, xylooligosaccharides, pectin oligosaccharides, resistant starch, and selected dietary fibers. Also included recently as potential prebiotics are noncarbohydrate compounds such as polyphenols, other phytochemicals, and polyunsaturated fatty acids, which have demonstrated positive impacts on the microbiota and health outcomes. The present definition expands prebiotic applications beyond the gut to include administration to extraintestinal sites such as the vaginal tract and skin (3).

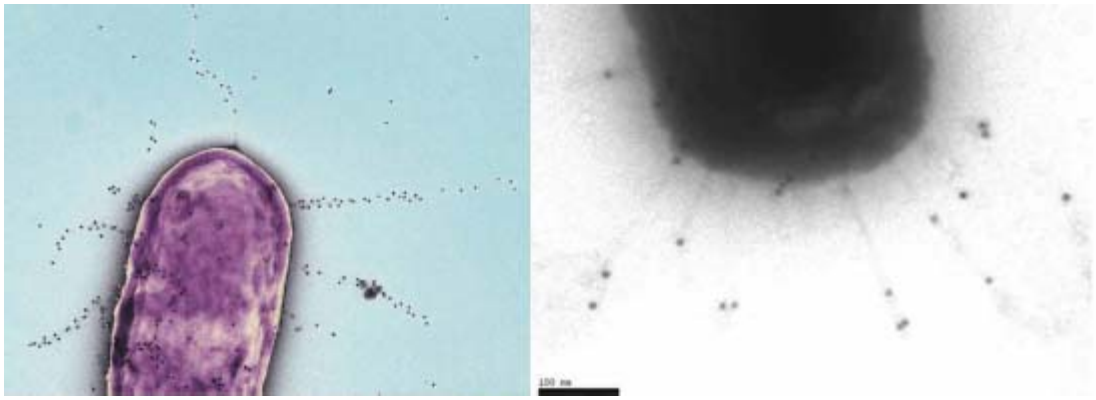


Figure 32.7. Pili expressed by *L. rhamnosus* (left) (85) and *B. breve* (right) (86).

Next-Generation Prebiotics

The prebiotic concept continues to evolve, as gut microbiome and -omics studies have revealed that other indigenous genera in the gut, such as *Faecalibacterium*, *Akkermansia*, *Roseburia*, *Bacteroides*, *Ruminococcus*, and *Eubacterium*, are also stimulated by prebiotics. Metabolism of these nondigestible compounds apparently involves protocooperative efforts and substrate crossfeeding among a broad consortium of the resident microbiota, challenging the initial selectivity criterion for prebiotic ingredients. Studies have also demonstrated metabolic cross-feeding among the beneficial taxa; for example, lactate and the SCFA acetate produced by bifidobacteria during carbohydrate fermentation serve as growth factors for the beneficial members of *Faecalibacterium*, *Roseburia*, *Eubacterium*, and *Anaerostipes* (90–92). Collectively, these recent findings highlight cross-feeding as a key mechanism for which prebiotics exert their effects on the gut microbiota and indicate that the concept should emphasize the overall ecological and functional attributes of the microbiota (93, 94). Most importantly, the core of the prebiotic concept remains conferring health-promoting physiological effects to the host, specifically via prebiotic-stimulated modulation of the beneficial microbiota composition and activities (95).

In one recent example, feeding GOS to lactoseintolerant subjects resulted in a significant modification of the microbiome (96) and concurrent improvement of clinical symptoms and tolerance to lactose (97). The studies documented the ability of a prebiotic to modify the microbiome, leading to a health benefit for lactoseintolerant individuals.

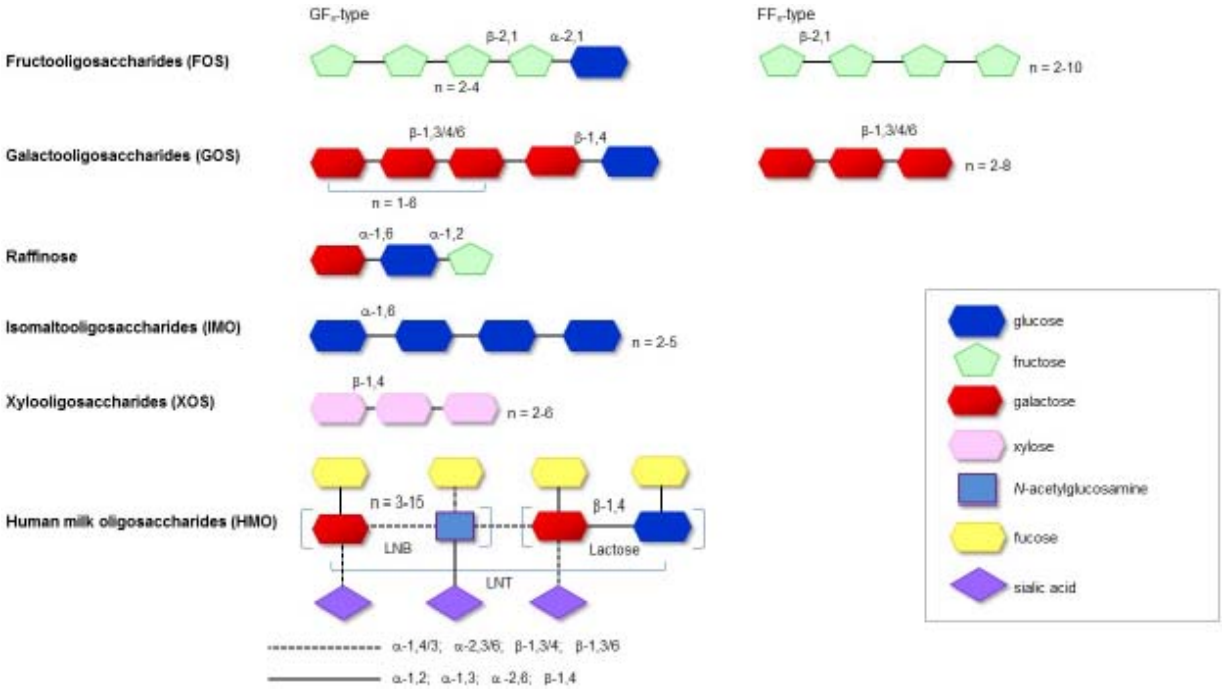


Figure 32.8. Examples of prebiotic oligosaccharides with demonstrated health effects through modulation of beneficial microbiota. FFn, FOS generated from partial hydrolysis of inulin; GFn, FOS synthesized from sucrose; LNB, lacto-*N*-biose I; LNT, lacto-*N*-tetraose. The structure of HMO is modified from reference 121; figure modified from reference 107.

Prebiotic Mechanisms of Action

In an effort to distinguish prebiotics from other dietary components or antimicrobial agents that may also impact the microbiota and host health (e.g., some nonfermentable dietary fibers, antibiotics, etc.), scientists proposed that the classification be restricted to compounds that are utilized (fermented or metabolized) by the microbes as nutrients, leading to modulation of the microbiota and subsequent health effects (3, 94). Accumulating evidence has shown that intestinal and systemic health benefits elicited by a microbiota shift are achieved via some of the following proposed mechanisms (Fig. 32.9):

- decrease in colonic pH resulting from SCFA production during prebiotic fermentation, such as acetate (C2, mainly by bifidobacteria), propionate (C3, by propionibacteria and *Bacteroidetes*), and butyrate (C4, by *Faecalibacterium*, *Roseburia*, *Eubacterium*, and *Anaerostipes*) (98–100)
- improvement in mucosal immunity from production of anti-inflammatory cytokines stimulated by beneficial microbial members (reviewed in reference 101)
- improvement in intestinal barrier function and mucosal epithelial development
- promotion of colonization resistance by commensal microbiota and enhanced protection against enteric infections
- production of secondary metabolites and bioactive compounds, including bacteriocins (reviewed in reference 102), vitamin B (e.g., biotin, cobalamin, folate, nicotinic acid, pantothenic acid, pyridoxine, riboflavin, and thiamine), vitamin K (reviewed in reference 103), and neurochemicals (e.g., gammaaminobutyric acid and serotonin) (104; reviewed in reference 105)
- bioconversion of dietary glycosylated polyphenols/phytochemicals, leading to the potential release of bioactive components (e.g., anti-inflammatory, anticarcinogenic, antimicrobial, and antioxidant substances) from the prebiotic substrates, thus improving their bioavailability in the host (106)

In order to develop effective prebiotic and synbiotic interventions, it is essential to understand how the prebiotic compounds are metabolized by the targeted microbiota and probiotic microbes, especially under *in vivo* conditions. High-throughput genome sequencing and transcriptome analyses coupled with genetic studies in some of these microbes have gradually unraveled the molecular mechanisms involved in the uptake and catabolism of prebiotic substrates. The depolymerization of these substrates often relies on the interplay of multiple glycosyl hydrolases, located intra- and/or

extracellularly, and dedicated transport systems for the uptake of intact or intermediate substrates. For example, the genome sequences of *Bifidobacterium*, *Lactobacillus*, *Bacteroides*, and several other gut commensal genera reveal broad repertoires of transporters and enzyme machinery to metabolize a wide range of complex carbohydrates (reviewed in references 107, 108). These genome features are key determinants for survival in the gastrointestinal tract and reflect the specialized adaptation of these microorganisms to the lower small-intestinal (e.g., lactobacilli) and colonic niches (e.g., bifidobacteria and *Bacteroides*), where complex oligosaccharides and plant polymers, as well as host-derived glycans (e.g., mucin, glycogen, HMOs, etc.), are abundant.

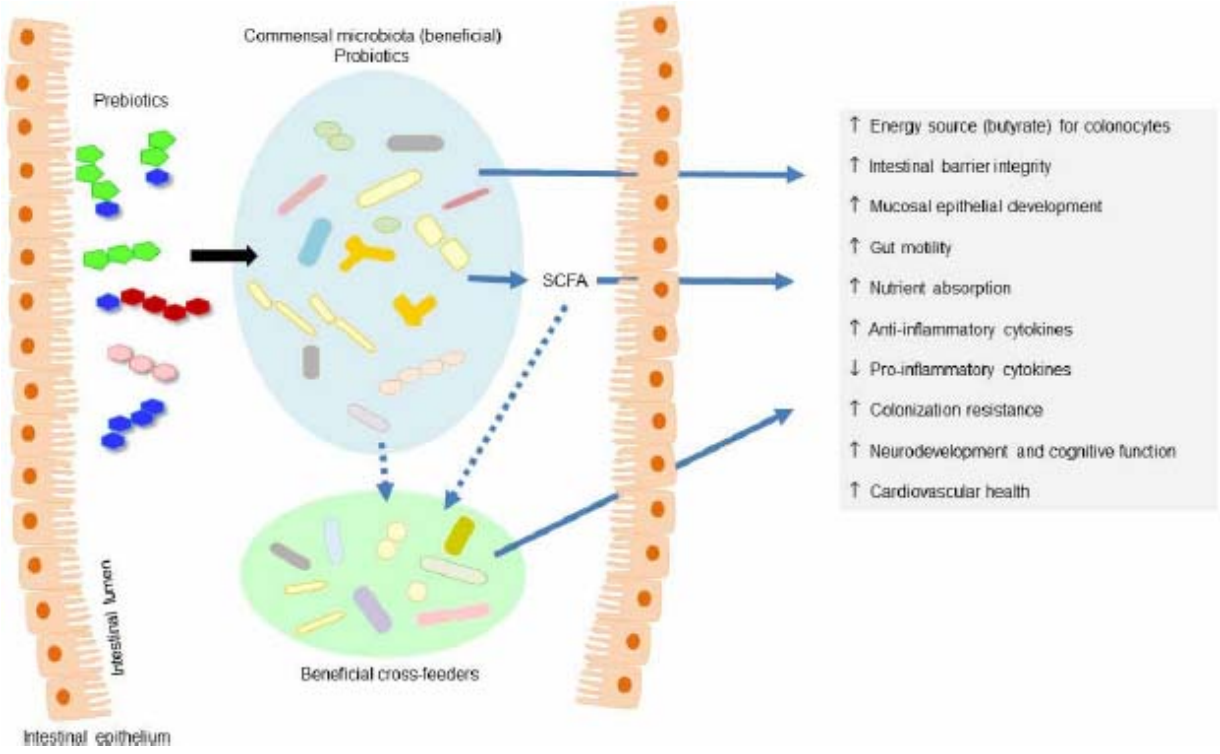


Figure 32.9. Metabolism of prebiotic compounds by beneficial gut microbiota and probiotic microorganisms, by which the resulting population is enhanced directly and indirectly (via substrate cross-feeding and metabolic cross-feeding) and which has health-promoting effects on the host. SCFAs are an essential energy source for colonic epithelial cells and serve as growth factors for some beneficial bacteria. These metabolites have also been associated with maintenance of gut barrier integrity, immunomodulatory functions, and host signaling (39, 100). Dashed arrows indicate cross-feeding of hydrolyzed prebiotic substrate intermediates and SCFA metabolites.

The genetic mechanisms for metabolizing FOSs, GOSs, and HMOs have been studied extensively among lactobacilli and bifidobacteria. The majority of the FOSfermenting species of *Lactobacillus* and *Bifidobacterium* possess specific transporters (e.g., ATP-dependent binding cassette [ABC] transporter, phosphotransferase system [PTS], and sugar permeases) for the uptake of mainly short-chain FOSs, followed by intracellular hydrolysis of the substrates by cytoplasmic β-fructofuranosidases (109–112) (Fig. 32.10). On the

other hand, species in the *Lactobacillus casei-Lactobacillus paracasei* group are capable of utilizing FOS and larger fructan polymers, such as inulin and levan, owing to the presence of a cell wallanchored β-fructosidase (113, 114). This enzyme hydrolyzes the fructan polymers externally, generating substrate intermediates (consisting mainly of fructose moieties) that are subsequently imported by the cells via dedicated fructose PTS transporter.

Overall, the internal sequestering of FOS in its intact form allows inclusive intracellular catabolism and confers a competitive advantage to the microbe in a nutrient-rich environment (109). In contrast, the unique extracellular FOS degradation strategy demonstrated by the *L. casei-L. paracasei* group provides cross-feeding of hydrolytic products from FOS and allows some versatility to catabolize more complex fructan substrates (114). This strategy could be viewed as logical for establishing a mutually beneficial relationship with other resident microbiota sharing the same niche where complex fructoside substrates are readily degraded and made generally available to the entire microbial ecosystem. The molecular mechanisms of GOS utilization were first identified in the commercially ubiquitous probiotic organism *L. acidophilus*, which involves a lactose permease (LacS) and two cytoplasmic β-galactosidases that are induced by GOS (115). Separate studies further reported upregulation of this GOS catabolic system upon exposure to bile *in vitro* (116), revealing intertwined gut-adaptive traits for bile tolerance and oligosaccharide utilization in the small-intestine environment. These observations support the strategy for delivering *L. acidophilus* with GOS as a synbiotic. When the probiotic microbes transit through the intestine, GOS metabolism would be induced by both GOS and an environmental signal, bile.

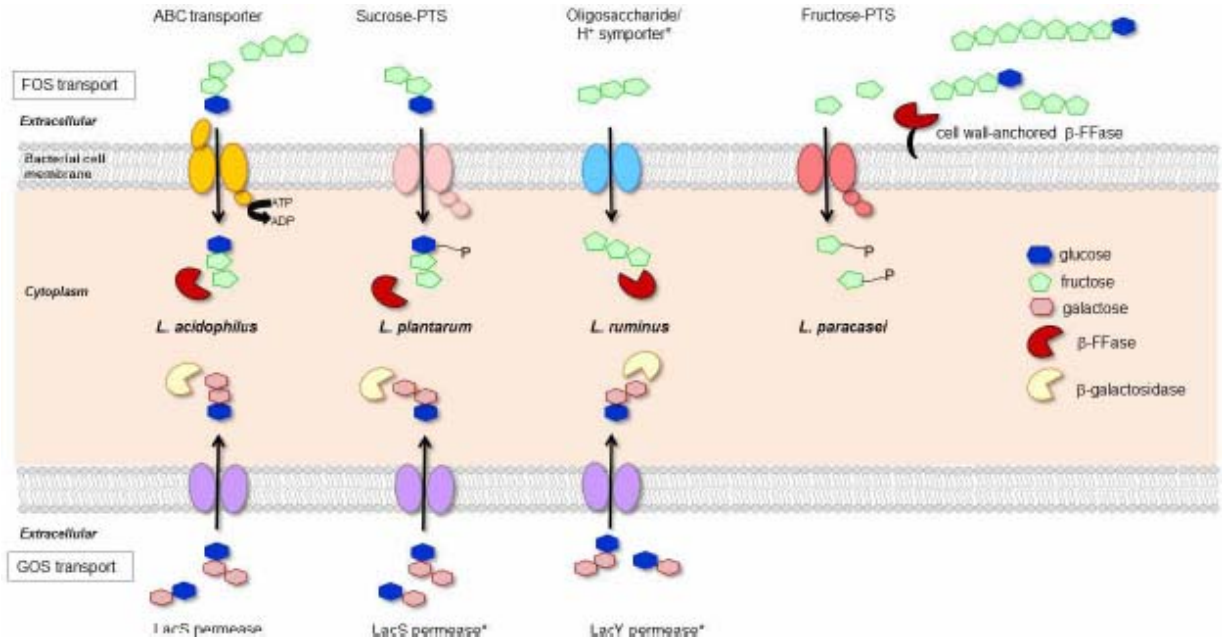


Figure 32.10. Molecular mechanisms of prebiotic FOS and GOS uptake and catabolism identified among intestine-associated lactobacilli. A diverse array of transport systems (ABC transporter, sucrose and fructose PTSs, symporter) and hydrolytic pathways (intracellular versus extracellular) have evolved for FOS utilization in these organisms (top). Uptake of GOS is mainly mediated by lactose permeases and is hydrolyzed by cytoplasmic β-galactosidases (bottom). Note that no LacS permease ortholog was identified in the *L. casei-L. paracasei* group. FFase, β-fructofuranosidase or β-fructosidase; ~P, phosphorylation of the substrate intermediate during the uptake process via PTS; an asterisk indicates that the transporter system was predicted based on *in silico* analysis.

Among bifidobacteria, certain strains of *B. longum* and *Bifidobacterium breve* encode a cell membranebound endogalactanase (GalA) that degrades GOS and plant-derived galactan. The enzyme liberates galactotriose from GOS and galactan polymers in an exo-type fashion (117). The resulting galactotriose is imported via an ABC transporter and hydrolyzed by a cytoplasmic β-galactosidase (118). In *B. longum*, the same catabolic system is also associated with the utilization of lactose and FOS (112). Meanwhile, glycoprofiling of GOS consumption among bifidobacteria revealed

that infant isolates of *B. breve* and *B. longum* subsp. *infantis* metabolized longer-chain pure GOS more efficiently than the adult isolates of *B. adolescentis* and *B. longum* subsp. *longum* (119). This differential GOS consumption phenotype reflects specific GOS catabolic systems that evolved through adaptation to host nutrient environments and provides a framework for targeted GOS enrichment of bifidobacteria in specific host populations.

As the third most abundant solid component in human milk after lactose and lipids, HMOs are a complex mixture of nondigestible oligosaccharides composed of glucose, galactose, *N*-acetylglucosamine (GlcNAc), l-fucose, and sialic acid (*N*-acetylneuraminic acid), the latter two of which confer structural diversity to the HMOs (120). Not surprisingly, the ability to metabolize HMOs or lacto-*N*-biose (LNB; Galβ1-3GlcNAc), the main building block of HMOs, is a common trait among infant-derived species of *B. longum* subsp. *infantis* and *Bifidobacterium bifidum* but is not observed among bifidobacteria found in adults, i.e., *B. adolescentis* and *Bifidobacterium catenulatum*. Regarded as the archetypical HMO phylotype, *B. longum* subsp. *infantis* possesses a unique 43-kb gene cluster that encodes an array of glycosyl hydrolases, ABC transporters and oligosaccharide-binding proteins dedicated to HMO utilization (43). This gene cluster, along with several other HMO utilization loci, is conserved among all HMO-positive *B. longum* subsp. *infantis* strains but is absent in strains of the closely related organism *B. longum* subsp. *longum*, which are specialized degraders of plant glycans derived from their adult host diet. In *B. longum* subsp. *infantis*, short-chain HMOs (degree of polymerization of ≤7) are transported via a dedicated HMO ABC transporter and are subsequently disassembled by a series of cytoplasmic β-galactosidase, fucosidases, sialidase, and β-*N*acetylhexosaminidase (121). By comparison, in *B. bifidum*, another specialized HMO degrader, hydrolysis of HMOs occurs extracellularly prior to uptake and is initiated by membrane-bound fucosidases and sialidases that remove the decorated fucose and sialic acid moieties, followed by liberation of LNB by a membrane-

associated lacto-*N*biosidase and subsequent uptake of LNB intermediates by an LNB ABC transporter (122–124). This extracellular catabolic strategy is thought to circumvent transporter capacity limitation and provide a competitive advantage by enabling the organism to metabolize a wider range of HMO structures.

In general, the majority of gut commensals and probiotic microorganisms of intestinal origin are equipped with a remarkably diverse glycobiome for efficient utilization of complex carbohydrates in the hosts. Compared to lactobacilli and bifidobacteria, *Bacteroides* and *Ruminococcus* possess more elaborate cell membrane-bound enzyme complexes—the starch utilization system (Sus) and the cellulosome/amylosome, respectively—for the degradation of starch and insoluble fibers (108). The carbohydrate gene repertoires among the gut members dictate their substrate preference based on chain length, glycosidic linkages, monosaccharide constituents, and the overall structural complexity of the substrates. These genetic capacities in turn are shaped by adaptation to nutrient availability, and consequently, niche differentiation and resource partitioning within the microbiota, presumably to minimize nutrient competition while promoting metabolic syntrophy (cross-feeding) to sustain a mutualistic community and host symbiosis.

The inherently complex and dynamic nature of the human microbiota is magnified by a variety of host factors (e.g., diet, environmental influences, host genetics, and health status), which undoubtedly pose challenges to designing effective prebiotics for targeted host populations. Increasing research efforts in recent years have focused on understanding the precise molecular mechanisms of how prebiotics and dietary glycans are metabolized and consequently modulate the microbiota and impact host health. For example, *in vitro* mixed-culture models, metagenomics and metatranscriptomic studies, *in vivo* animal models, and comprehensive clinical studies are aimed at comprehending how the metabolic fate of prebiotic substrates can positively impact and shape the microbiota. The mechanistic insights into this causal

effect will provide the molecular framework for the development of next-generation prebiotics and synbiotics with tailored specificity and enhanced effectiveness.

CONCLUSIONS

The probiotic field has offered considerable promise since the early observations by Metchnikoff about the importance of limiting putrefactive metabolism in the gut. The associated prebiotic concept proposed by Gibson and Roberfroid in 1995 launched efforts to understand how to manipulate colonizing and probiotic microbes through rationally designed, selective substrates. Today, microbiological and molecular methods provide new insights into prebiotics, probiotic bacteria, the microbiota, and the interactions that may be responsible for eliciting beneficial outcomes realized through probiotic and prebiotic consumption. Advancing our understanding of the probiotic and prebiotic concepts provides a worthy challenge to scientists due to the complexity of the host, dynamic interactions within microbial ecosystems, and the multifaceted impacts of our associated microorganisms on health and well-being. Key issues and important challenges for food microbiologists working on probiotics and prebiotics in the years ahead will include the following:

- clarifying mechanisms of action of both probiotics and prebiotics
- characterizing responders and nonresponders to probiotic or prebiotic interventions
- clarifying how structure relates to function of prebiotic substances and correlating that function to health outputs
- matching expression of probiotic mechanisms with specific clinical benefits and understanding the strain specificity of clinical benefits

- deepening our understanding of the impact of probiotics and prebiotics on the composition and expression patterns of normal colonizing microbiota
- understanding how manufacturing or harvesting conditions and delivery matrices influence clinical outcomes for probiotics and prebiotics
- stabilization of probiotics for effective delivery in expanded applications
- developing next-generation probiotics (novel microbes) and prebiotics (novel structures)
- developing the use of probiotic cultures for oral delivery and expression of vaccines and biotherapeutics to the gastrointestinal tract and associated mucosal immune system
- translation of scientific discoveries into commercial development • identification of physiologically relevant biomarkers that can assess parameters of probiotics and prebiotic effectiveness
- epidemiological and long-term studies in target hosts to determine the impact of probiotic and prebiotics on long-term health and chronic disease

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33
Fermented Foods

DIVERSITY OF FERMENTED FOODS AND TECHNOLOGICAL AIMS OF FOOD FERMENTATIONS

In fermented foods, microbial and enzymatic conversions determine and maintain food safety and quality. Fermentation is one of the oldest methods of food processing, and a substantial proportion of contemporary foods are processed by fermentation (Fig. 33.1). The intentional use of food fermentations likely predates agriculture. The case was made, although not undisputed, that Neolithic hunter-gatherer societies in the Fertile Crescent generated sufficient surplus to create a demand for festivities and ritual or recreational drugs, which was met by cultivation and fermentation of (yet-undomesticated) cereals (1, 2). Whether or not human desire for mind-altering drugs during ritual or social festivities represents “ground zero” for agriculture and food fermentations, many of the fermented foods are produced to meet that demand (Fig. 33.1, groups 1 to 4 and spirits).

A second motivation for food fermentations is the detoxification of raw agricultural commodities. Owing to their compact structure, cereal grains and legumes are poorly digestible without processing. Cereals and legumes also contain inhibitors of digestive enzymes—proteins as well as phenolic compounds—and lectins (3, 4). These compounds are reduced or removed by soaking, milling, fermentation, and cooking or baking, unit operations that have been used for processing of cereals since the onset of agriculture (5). Fermentation also removes toxic or bitter-tasting phytochemicals, e.g., linamarin in cassava or oleuropein in olives. Fermentation of tubers, cereals, pulses, and some other plant material thus primarily aims to improve palatability and nutritional value of raw agricultural commodities (Fig. 33.1, groups 5 to 10). The

current debate concerning the role of FODMAPs (fermentable oligosaccharides, disaccharides, monosaccharides, and polyols) and amylase/trypsin inhibitors in wheat and their role in wheat (or gluten) intolerance demonstrates that this aspect remains relevant even in societies with an abundant and diverse food supply (6).

Fermentation with lactic acid bacteria preserves perishable raw materials such as vegetables, fish, meat, and milk (Fig. 33.1, groups 12 to 18). In climates with long winters, fermentation is a straightforward way to preserve vegetables and to supply vitamins until the following spring. This was exemplified by the British explorer James Cook (1728 to 1779), who used sauerkraut to prevent scurvy during long voyages by ship, which allowed him to boldly go where no Europeans had gone before (7). Lactic fermentation of milk or vegetables achieves a storage life of several days or weeks. When combined with drying, lactic fermentation converts meat and milk to tradable commodities with a storage life that is comparable to that of dry cereal grains or canned foods.

Last but not least, fermented foods please the palate. Tea, coffee, chocolate, and condiments such as vinegar are fermented for no other purpose. The unique sensory properties of other fermented foods have ensured their popularity, although the initial aim of their production—detoxification or preservation—could be readily achieved by other unit operations in food production. For example, the use of sourdough has rebounded as the default fermentation step in bread production after a century of almost exclusive use of baker's yeast in wheat baking. This recurrence is largely attributable to the superior sensory quality of sourdough bread.

Most fermented foods are based on tradition and artisanal fermentation processes and are deeply rooted in the culture and the economy of specific regions. This strong connection to specific locations is brought about by climatic conditions that determine the availability of specific crops which require fermentation, e.g., rye, sorghum, or cassava; by economic constraints, e.g., preservation of crops that are harvested only once a year or the preservation of milk that is produced on pastures that are remote from the consumers; or by (religious) festivities that are celebrated with specific (fermented) food items. Most fermented foods are, or originate from, "indigenous" or "ethnic" foods, even though a few are available worldwide.

The discovery of yeasts and bacteria as food-fermenting organisms in 1857 by Louis Pasteur enabled the industrialization of food fermentations (8). Baker's yeast and starter cultures for dairy fermentations were commercialized less than 30 years after Pasteur's discovery; lactic starter cultures for fermentation of cereals (1910), meat (1966), and wine (1985) were commercialized in the 20th century. Industrial food production allows global production of fermented foods with a relatively standardized quality. New food product developments also employ fermentation processes that are unrelated to traditional procedures; examples include the use of *Gluconobacter* in lemonade production, the fermentation of yogurt-like oat products with probiotic lactobacilli (9), and fermentation processes for imitation cheese products from plant proteins.

SCOPE AND LIMITATIONS OF THIS CHAPTER

This chapter focuses on major categories of fermented foods and fermentation organisms (Fig. 33.1). For additional information on fermented foods and for information on the fermentation of wine, beer, vinegar, coffee, and cocoa, the reader is referred to more comprehensive textbooks that are dedicated to fermented foods and to the respective chapters of the fourth edition of this book (10–15).

Fermented foods are a major contributor to the dietary intake of viable bacteria: a meal that includes 100 g of cheese, salami, kimchi, or yogurt contains 10^{11} viable lactic acid bacteria. Food-fermenting bacteria interact with human intestinal microbiota and with the gut-associated immune system. Accordingly, the concept of probiotics, "live bacteria that, when administered in adequate amounts, confer a health benefit on the host," was developed by Metchnikoff in the early 20th century with yogurt as the conceptual template. Owing to the conceptual link between fermented foods, viable bacteria, and health, the supplementation of food initially focused on fermented dairy products, particularly yogurt. The list of bacterial species with probiotic activity and that of species that reliably occur at high cell counts in fermented foods overlap significantly. Several authors have made the case that specific fermented foods may have probiotic activity; however, others excluded fermented foods from the probiotic concept because, in

addition to the presence of viable probiotic bacteria, health effects of fermented foods are inextricably linked to other food components. This chapter does not explore the link of fermented foods and the probiotic concept further; the reader is referred to two opinion papers that make the case in favor (16) and against (17).

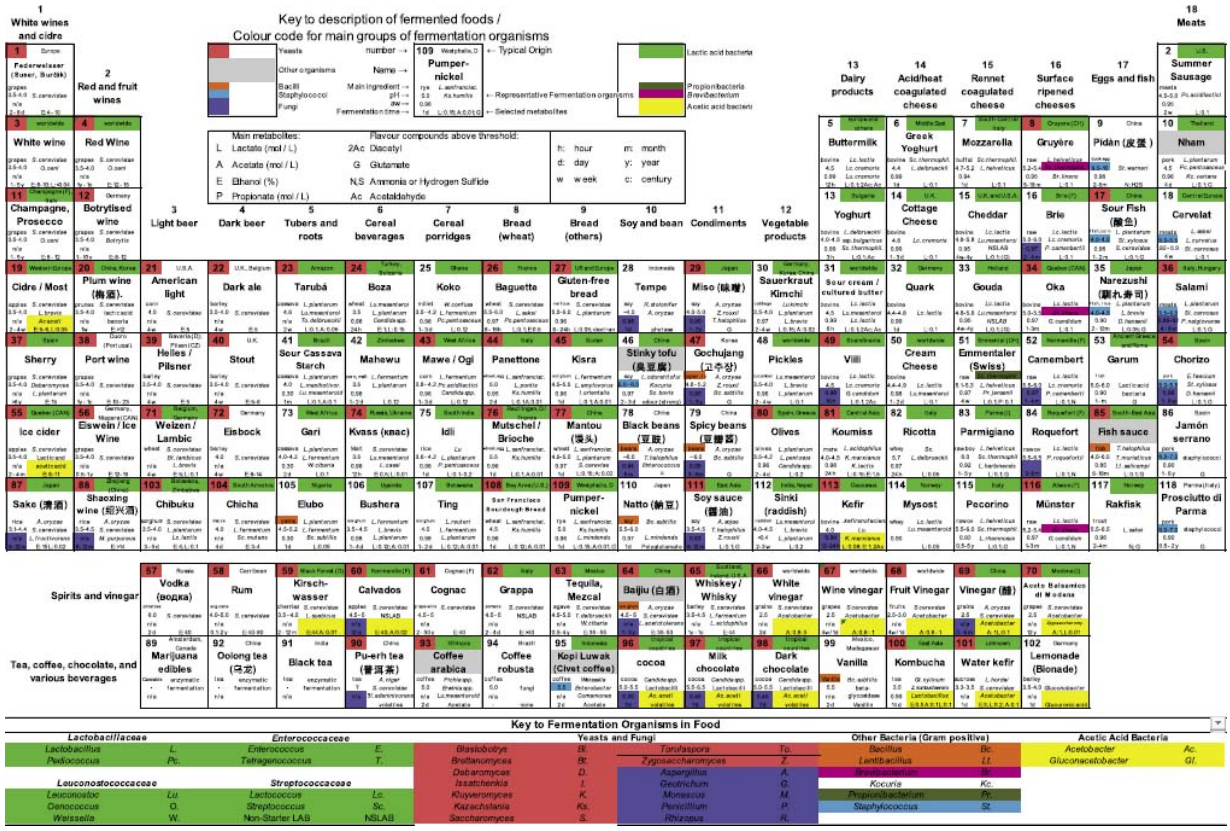


Figure 33.1. Periodic table of fermented foods, indicating the diversity of products, fermentation organisms, and raw materials. Fermented foods are grouped by product category and ranked within a group by flavor intensity or ripening time, if applicable. Fermentation organisms that contribute to fermentation are indicated by color coding of specific fields (see the key at the top of the table). Typical organisms, the concentration of relevant metabolites, and the ripening or fermentation times are also indicated. Genus abbreviations used in this chapter are introduced in the figure. Food products are generally listed in the language of origin. The figure is formatted for large-scale (A0) printing; a high-resolution image file is available as "Document" under the Activities tab of the author's homepage at https://www.ualberta.ca/agriculture-life-environment-sciences/about-us/contact-us/facultylecturer-directory/michael-gaenzle. Modified from reference 36.

COMMUNITY ASSEMBLY IN FOOD FERMENTATIONS

Traditional food fermentations are not controlled by starter cultures; the assembly of microbial communities is controlled by selection of raw materials, product formula, control of the fermentation process, and backslopping, which is the practice of inoculating with a previous batch. Generally, the assembly of animal, plant, or microbial communities is explained by four concepts: diversification/speciation, drift, dispersal, and selection (Table 33.1) (18, 19). Speciation, however, is unlikely to occur in food fermentations. Food fermentations are less than 14,000 years old, and the longest documented continuous use of fermentation microbiota, pit muds for production of baijiu by Luzhou Laojiao in Luzhou, China, is less than 500 years (20). In contrast, bacterial speciation occurs on a time scale of millions of years (21, 22); intraspecies differentiation and host adaptation of pathogens and commensals occur on time scales ranging from dozens to thousands of millennia (22, 23). Accordingly, sourdough isolates of *Lactobacillus reuteri* that have a history of more than 20 years of food use were genetically and physiologically indistinguishable from intestinal representatives of the same species (24). *Lactobacillus delbrueckii* evolved in lactose-rich environments by reduction of genome size, which particularly relates to the silencing of carbohydrate-active enzymes that are active on substrates other than lactose. This was interpreted as adaptation to dairy, but the presence of *L. delbrueckii* in the intestines of suckling but not of weanling piglets suggests that it has adapted to suckling mammals rather than dairy fermentations (25, 26). Comparative genomic analysis of food-fermenting *Aspergillus oryzae* and *Saccharomyces cerevisiae* indicates intraspecies domestication. Both species have been used for several hundred years in koji and brewing, respectively (27, 28). Domestication or speciation is thus of only limited relevance for food fermentations.

Drift, or random events, may shape community assembly in individual fermentation batches (α-diversity) (29), particularly if contamination by microorganisms that have no stable association with the ingredients or the processing environment impacts the fermentation microbiota. Food fermentations are carried outon a much smaller scale and with a much shorter generation time than communities of plants or animals; therefore, β-diversity and γ-diversity are not impacted by drift (Table 33.1). Contamination of fermentation batches with competitive

fermentation organisms is frequent—and then no longer random—or rare and with low impact on overall diversity.

Dispersal limitation is a main factor in community assembly of spontaneous fermentations, and organisms that have a stable association with the raw material, ingredients (including water), or animals or humans that reliably are in contact with the fermentation batch are likely to dominate. The small scale and short fermentation time of food fermentation all but eliminate dispersal limitation in back-slopped fermentations; even an unlikely contamination event will occur eventually if fermentation microbiota are propagated daily under septic conditions for several weeks, months, or years (Table 33.1) (30).

Selection is an important determinant for community assembly of spontaneous fermentations; microbiotas of spontaneously fermented milk, vegetables, and cereals differ substantially from the microbiotas of the raw materials from which the fermentation organisms originated. It is the major determinant of the composition of back-slopped fermentation, because competitive microbes will contaminate the fermentation and outcompete other organisms eventually, even if they have only an indirect association with the raw material, humans, or the processing environment (30) (Table 33.1).

Why are fundamental considerations related to community assembly important? In the section above, the case was made that fermented foods are produced in a virtually unlimited regional variety. The shared patterns of community assembly, however, lead to very similar fermentation microbiotas in comparable products and processes globally. Spontaneous fermentations are dominated by organisms that have a stable association with the raw material or with ingredients. Spontaneous fermentation of raw milk or meat at ambient temperature very likely results in growth of *Lactococcus lactis* and *Lactobacillus sakei*, respectively, as major members of fermentation microbiota. Likewise, spontaneous plant fermentations are initially fermented by plant-associated members of the *Enterobacteriaceae*, including *Enterobacter*, *Cronobacter*, *Klebsiella*, and *Pantoea*¸ followed by *Leuconostoc*, *Weissella*, and *Enterococcus*, until eventually the acid-tolerant organisms *Lactobacillus plantarum* and *Lactobacillus brevis* or *Lactobacillus fermentum* (in cereals) dominate (30, 31). This succession of fermentation microbiotas—referred to here as “the usual suspects”—

reliably occurs in sauerkraut, kimchi, and carrot juice fermentations worldwide but also in spontaneously fermented cereals.

Back-slopped fermentations exert a more stringent selective pressure than spontaneous processes and often select for microbiota that converge at the species level. For example, bakers around the world exert a uniform selective pressure on fermentation microbiota if they use sourdough as the sole leavening agent; the need for dough leavening necessitates rapidly growing and metabolically active organisms. Although the steamed or baked products look, taste, and smell very different, fermentation microbiota are consistently dominated by *Lactobacillus sanfranciscensis* and *Kazachstania humilis* (*Ks. humilis*) or *S. cerevisiae* (Fig. 33.1). Likewise, red smear microbiotas on surface-ripened cheeses are highly comparable worldwide; competitive microorganisms contaminate from human skin wherever red smear cheese is made and are maintained by back-slopping in the dairy. In short, “terroir,” referring to local differences, is relevant with respect to the choice and quality of the raw material, the product formula, and the production processes but not with respect to the composition of fermentation microbiota.

Table 33.1. Concepts in community assembly in food fermentations[a]

Concept	Description	Relevance for food fermentations
Diversification/Speciation	Generation of genetic variation	Speciation of bacteria occurs on a time scale of 1–20 million years and is not relevant for microbiota of fermented foods (21–23). Intraspecies adaptation to food fermentations was proposed for *S. cerevisiae* and *A. oryzae* (23, 24, 28).
Drift	Random events that impact fermentation microbiotas	Drift may shape the α-diversity (diversity in a single sampling unit, e.g., a fermentation batch) but not β-diversity (diversity at a regional level or in a subtype of a food fermentation) or γ-diversity (global microbial diversity in a specific food fermentation).
Dispersal	Movement of fermentation organism across space	Contamination or inoculation of food fermentations from raw materials, the environment, or animals, pests, and humans (30)
Selection	Selection of fermentation organisms with the highest ecological fitness	Together with dispersal, selection shapes community assembly of spontaneous food fermentations. In back-slopped fermentations, selection is the main factor for community assembly (30).

[a] Modified from references 18, 19, and 30.

MAJOR GROUPS OF FERMENTATION ORGANISMS AND METABOLIC PATHWAYS

A catalogue of microorganisms in fermented foods was compiled on the initiative of the International Dairy Federation. The most recent version lists 195 bacterial species representing 18 families and 3 phyla; fungi are represented by 69 species representing 3 phyla (32). The most relevant organisms are lactic acid bacteria, yeasts, acetic acid bacteria, and fungi. The presence of these groups of organisms is highlighted by color coding in Fig. 33.1, and their most relevant properties are briefly presented in this section.

Lactic Acid Bacteria

Lactic acid bacteria are facultative anaerobic Gram-positive bacteria that produce lactic acid as a major product of carbohydrate metabolism (33). Most organisms that are referred to as lactic acid bacteria are classified in the order *Lactobacillales*. Lactic acid bacteria comprise human and animal pathogens, commensal members of human and animal microbiotas, and environmental organisms. Organisms with a safe tradition in food use include only a small fraction of lactic acid bacteria, i.e., *Lactobacillaceae*, *Leuconostocaceae*, *Lc. lactis*, *Streptococcus thermophilus*, and *Tetragenococcus halophilus*. Enterococci are part of fermentation microbiotas in cereals, dairy products, and fermented meats. These organisms do not cause foodborne disease; however, they can cause life-threatening hospital-acquired (nosocomial) infections and contribute to the transmission of antibiotic resistance genes from farm animal microbiota to human pathogens (34). Their safety and their deliberate use in food fermentations are thus subjects of an ongoing debate.

The ecological fitness of lactic acid bacteria in food fermentations relates to their rapid but inefficient metabolism of fermentable carbohydrates (35). An overview of lactic metabolism is provided in Fig. 33.2 (36). Consumption of sugars and oxygen and acidification by accumulation of lactate, acetate, and formate inhibit and eventually kill competing bacteria. Homofermentative lactic acid bacteria metabolize glucose via the Embden-Meyerhof pathway with pyruvate as the central metabolic

intermediate (Fig. 33.2) (36). Heterofermentative lactic acid bacteria convert glucose via the phosphoketolase pathway with pyruvate and acetyl coenzyme A (acetyl-CoA) as central intermediates (Fig. 33.2) (36). Fermentation pathways of lactic acid bacteria relate to their phylogeny: *Leuconostocaceae* and 7 of the 24 phylogenetic groups in the genus *Lactobacillus* are heterofermentative (37), while all other lactic acid bacteria are homofermentative. The metabolic, ecological, and phylogenetic diversity in the genus *Lactobacillus* is equivalent to the diversity that is typically observed in a bacterial family; current efforts toward clarification of the taxonomy of the genus will likely elevate many or most of its phylogenetic groups to the taxonomic rank of genus (37, 38). Many lactic acid bacteria are conditionally respiring; respiratory metabolism is of only limited relevance in food fermentations but is exploited industrially for high-density fermentation of bacterial biomass (36). Organic acids are metabolized to achieve regeneration of reduced cofactors or to contribute to pH homeostasis through intracellular decarboxylation reactions (Fig. 33.3) (36, 39). Organic acid metabolism profoundly impacts the quality of wine and fermented dairy products (36).

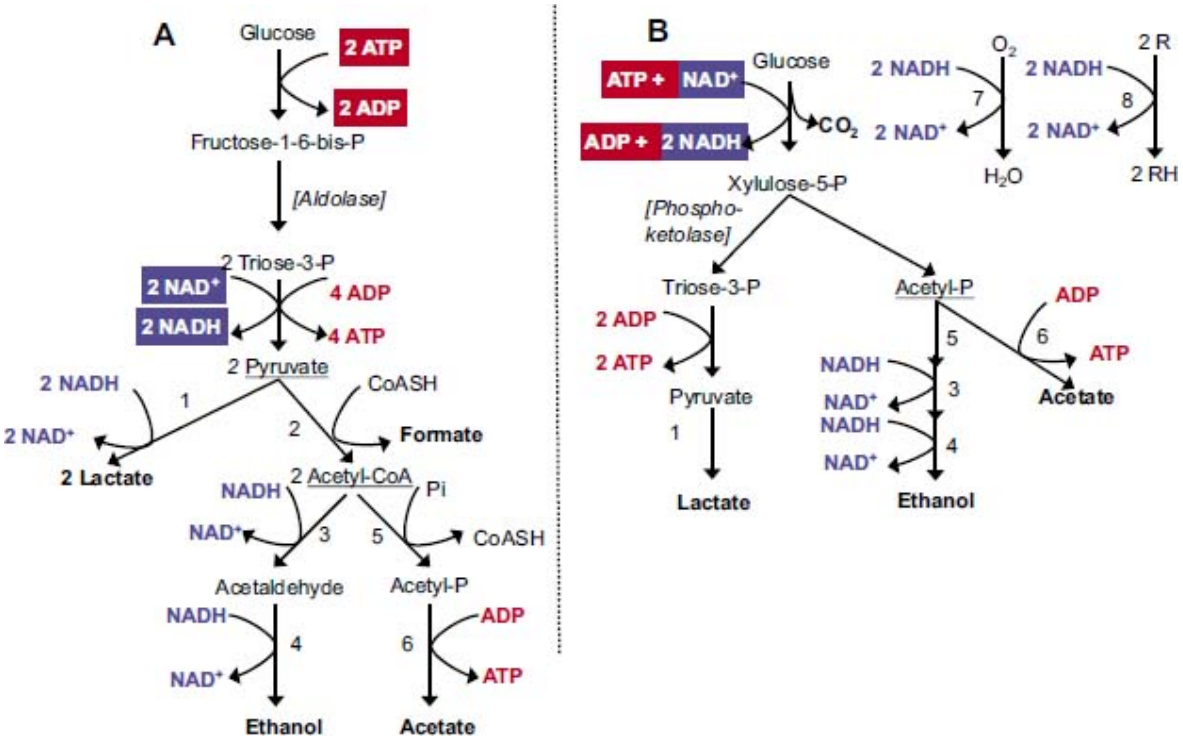


Figure 33.2. Overview of carbohydrate fermentation in lactic acid bacteria. Major end products of metabolism are in boldface type; branching points of metabolism, or metabolic switches, are underlined. The formation of reduced and oxidized cofactors (in white on a blue background and in blue, respectively) is indicated if it occurs upstream or downstream of relevant metabolic branching points; ATP consumption (in white on a red background) and synthesis (in red) are shown to indicate the ATP yield of the metabolic pathway. (A) Homofermentative metabolism of hexoses via the Embden-Meyerhof pathway and alternative fates of pyruvate. The alternative fate of pyruvate depends on the substrate availability, the availability of oxygen, and the pH. (B) Heterofermentative metabolism of hexoses via the phosphoketolase pathway and alternative routes of acetylphosphate. Conversion of acetylphosphate to acetic acid is dependent on alternative routes for regeneration of reduced cofactors (upper right, steps 7 and 8). Enzymes are indicated by numbers as follows: 1, lactate dehydrogenase; 2, pyruvate formate lyase; 3, acetaldehyde dehydrogenase; 4, alcohol dehydrogenase; 5, phosphotransacetylase; 6, acetate kinase; 7, NADH oxidase or NADH peroxidase; 8, mannitol dehydrogenase, glutathione reductase, or alcohol reductase. Modified from reference 36.

Yeasts

Yeasts are unicellular, facultative anaerobic fungi. For yeasts that reproduce sexually, the name referring to the teleomorph state (sexual reproduction) is used, but for many yeasts, only the anamorphic state (asexual reproduction) is known, or the name referring to the anamorphic state remains in use (32). Most food-fermenting yeasts, e.g., *Candida*, *Debaryomyces*, *Kazachstania*, *Pichia*, and *Saccharomyces*, are in the family *Saccharomycetaceae*. Yeasts, in particular *S. cerevisiae*, are responsible for ethanol and CO_2 formation in food fermentations. Glucose is metabolized to pyruvate as central metabolic intermediate by the Embden-Meyerhof pathway. Pyruvate is shunted to the Krebs cycle for respiratory metabolism if oxygen is available or converted to ethanol and CO_2 via pyruvate decarboxylase in fermentative metabolism.

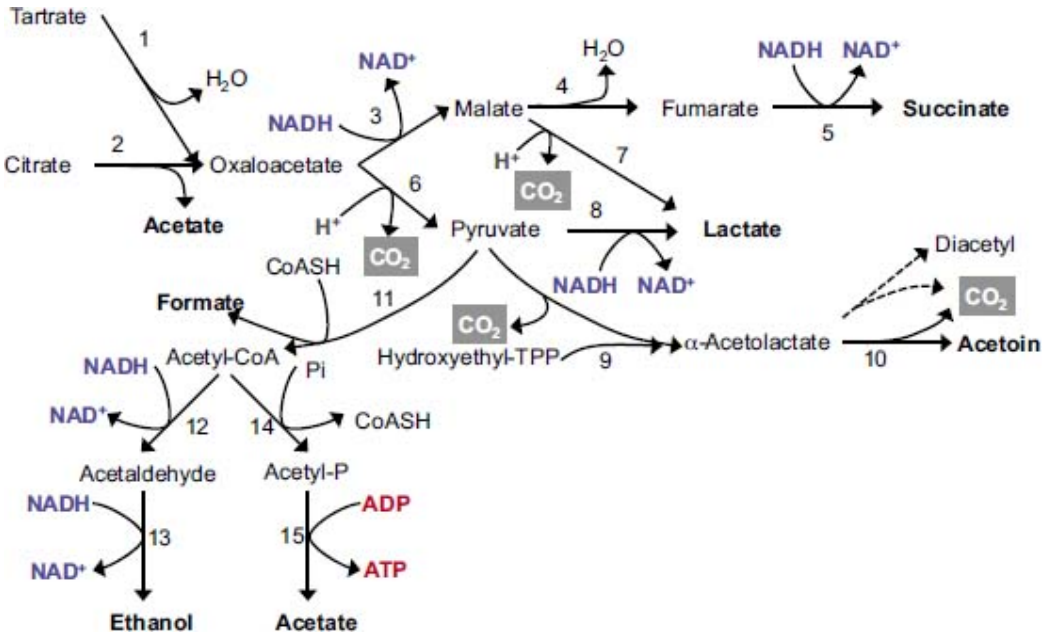

Tartrate
1
H_2O
Citrate
2
Oxaloacetate
Acetate
NAD+
NADH
3
Malate
H_2O
4
Fumarate
NADH NAD+
5
Succinate
H+
CO_2
6
7
H+
CO_2
Pyruvate
8
Lactate
CoASH
11
NADH NAD+
Formate
CO_2
Hydroxyethyl-TPP
9
α-Acetolactate
Diacetyl
CO_2
10
Acetoin
NADH
Acetyl-CoA
Pi
NAD+
12
14
CoASH
Acetaldehyde
Acetyl-P
NADH
13
15
ADP
NAD+
ATP
Ethanol
Acetate

Figure 33.3. Metabolism of organic acids in lactic acid bacteria: alternative fates of citrate and malate in lactic acid bacteria to support pH homeostasis or cofactor regeneration. Major end products of metabolism are in boldface type. The formation of oxidized cofactors is indicated by blue type; ATP synthesis is in red; proton-consuming decarboxylation reactions are on a gray background. Dashed arrows indicate chemical conversions. Tartrate is converted by tartrate dehydratase; citrate is converted to succinate to achieve regeneration of two reduced cofactors or to acetoin to achieve proton consumption and proton motive force generation by two decarboxylation reactions. Tartrate metabolism has been observed only in *L. plantarum*. Citrate conversion to lactate or acetate and ethanol combines oxidation of 1 mol of NADH and one decarboxylation reaction. Lactobacilli convert citrate to succinate or lactate; *T. halophilus* converts citrate preferentially via pyruvate formate lyase acetate and ethanol; *Lc. lactis*, *Leuconostoc* spp., and *Oenococcus oeni* convert citrate to the alternative end product acetoin or lactate. Enzymes are indicated by numbers as follows: 1, tartrate dehydratase; 2, citrate lyase; 3, malate dehydrogenase; 4, fumarate hydratase, 5, succinate dehydrogenase; 6, oxaloacetate decarboxylase; 7, malolactic enzyme; 8, lactate dehydrogenase; 9, acetolactate synthase; 10, acetolactate dehydrogenase; 11, pyruvate formate lyase; 12, acetaldehyde dehydrogenase; 13, alcohol dehydrogenase; 14, phosphotransacetylase; 15, acetate kinase. Modified from reference 36 with information from reference 39.

The competitiveness of *S. cerevisiae* in alcoholic fermentations relates to the specific regulation of fermentative versus respiratory metabolism. In *S. cerevisiae*, respiration is inhibited in the presence of less than 1 g of glucose per liter, even if oxygen is available (Crabtree effect); moreover, sugar is used faster anaerobically than aerobically (Pasteur effect) (40). The regulation of carbohydrate metabolism results in rapid but ineffective conversion of glucose and the accumulation of ethanol as a toxic by-product which inhibits other yeasts and bacteria. After hundreds of years of back-slopping, brewer's yeasts show signs of domestication and exhibit reduced sexual reproduction but more rapid use of maltotriose compared to wild yeasts or wine yeasts that are not back-slopped or are back-slopped on longer (annual) fermentation cycles (28).

A second important metabolic activity of yeasts is the production of diverse flavor volatiles (41). Major pathways for flavor production include the transamination of amino acids (Ehrlich pathway), conversion of phenolic compounds and fatty acids, and synthesis of floral esters (Fig. 33.4). Ester synthesis by yeasts is strain specific and serves the ecological role of attracting insects to overcome dispersal limitation (41). The formation of flavor volatiles by yeasts is a major determinant of the quality of alcoholic beverages but also of soy sauce, bread, and surface-ripened cheeses and meats.

Acetic Acid Bacteria

Acetic acid bacteria are strictly aerobic, Gram-negative members of the *Alphaproteobacteria*. *Acetobacter* and *Gluconacetobacter* are used in vinegar fermentation, in the fermentation of coffee and cocoa, and in kefir or water kefir grains (Fig. 33.1), but they also spoil alcoholic beverages. Metabolism of acetic acid bacteria is characterized by rapid but incomplete oxidation of alcohols to the corresponding aldehydes or organic acids; the resulting low pH and high organic acid concentration create an inhospitable environment for most other microorganisms, including yeasts and fungi (42, 43). The conversion of ethanol to acetic acid is most relevant in food applications, but mannitol and other sugar alcohols or glucose are also oxidized. Acetic acid bacteria also produce exopolysaccharides, including acetan and cellulose (44). These exopolysaccharides form the matrix for the bacterial biofilm on the surfaces of vats used in traditional production of vinegar (“mother of vinegar”) or kombucha and contribute to formation of the exopolysaccharide matrix in kefir grains.

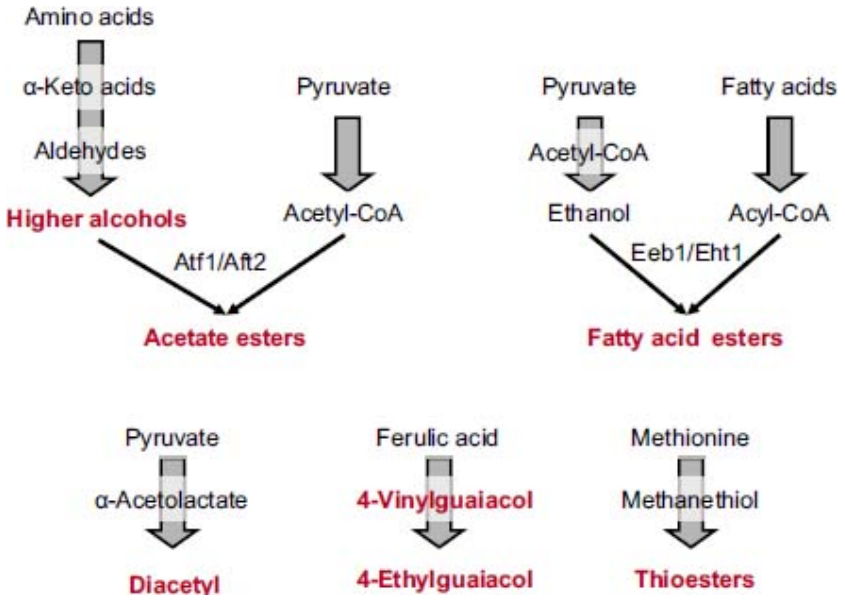


Figure 33.4. Overview of pathways for formation of flavor volatiles by yeasts. Flavor volatiles are shown in red. Drawn using data from reference 41.

Mycelial Fungi

Mycelial fungi are decomposers of organic material that play a critical role in global nutrient cycles. Their extensive array of hydrolytic enzymes is a main motivation for their use in food fermentations. In cereal and soy fermentations in East Asia, fungi are employed as the primary saccharification and proteolytic cultures to degrade starch and proteins prior to fermentation with yeasts, lactic acid bacteria, and/or acetic acid bacteria (27, 45). Fermentations in the European tradition employ fungi as secondary ripening cultures on fermented meats and cheeses to accelerate proteolysis, lipolysis, and flavor formation. Spontaneous fermentations of meats or cereals are brought about mainly by members of the *Ascomycota* in the genera *Aspergillus* and *Penicillium* (32). The Japanese koji mold, *A. oryzae*, has been domesticated by hundreds of years of cultivation on starch substrates. Although it shares a high degree of genetic similarity to its ancestor, *Aspergillus niger*, *A. oryzae* is characterized by a higher expression of amylases and differential expression of asparaginase and glutaminase (27).

Mycelial fungi also are capable of production of secondary metabolites, particularly mycotoxins. Domestication of *A. oryzae* led to silencing of gene clusters that are responsible for mycotoxin production in the ancestral *A. niger* (27). *Penicillium roqueforti* and *Penicillium camemberti* produce the mycotoxins roquefortin C and cyclopiazonic acid, respectively, although their concentrations in cheese are too low to present a food safety risk (32). The formation of red and orange pigments by *Monascus purpureus* is a main reason for its use in production of angkak, or red yeast rice, a red rice produced in Southeast Asia, and in production of red or yellow rice wine (Fig. 33.5) (46). *M. purpureus* also produces monacolin K, or lovastatin, a potent competitive inhibitor of cholesterol synthesis in humans, and citrinin, a nephrotoxic and hepatotoxic mycotoxin. Citrinin, monacolin K, and pigments are synthesized by different polyketide synthases, but their production is regulated by the same signal protein, Mga1; pigment production by wild-type strains is thus generally associated with citrinin production. The level of citrinin in *Monascus* products is regulated in Japan and South Korea; owing to citrinin contamination, *Monascus* products are not used as food colorants in the European Union or the United States (47).

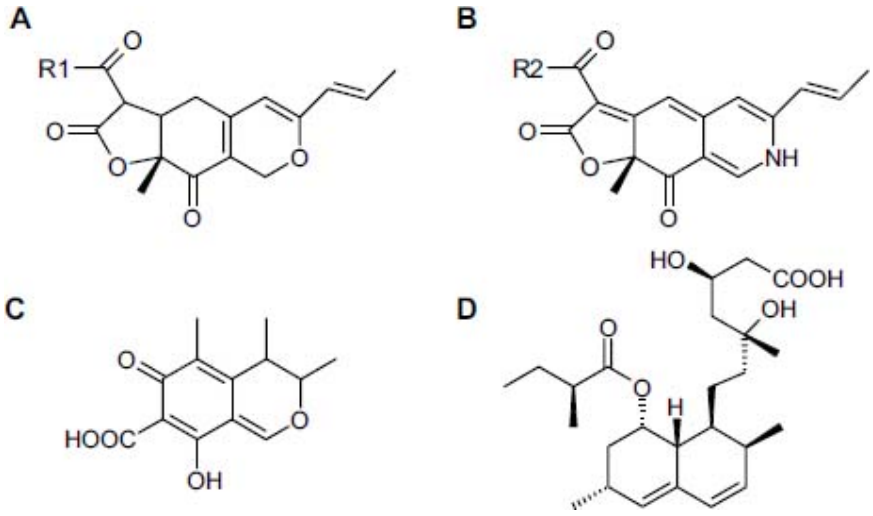


Figure 33.5. Examples of bioactive secondary metabolites of *M. purpureus*. (A) The yellow pigments monascin (R_1 = C_5H_{11}) and ankaflavin (R_1 = C_7H_{15}); B, the red pigments rubropunctamine (R_2 = C_5H_{11}) and monascorubramine (R_2 = C_7H_{15}); C, the mycotoxin citrinin; D, monacolin K (lovastatin), a potent inhibitor of cholesterol synthesis in humans. Drawn with data from references 46 and 47.

In addition to the four major groups of fermentation organisms, members of several other bacterial taxa, including staphylococci, bacilli, and propionibacteria, are relevant in specific food fermentations (Fig. 33.1); their properties and contribution are discussed in the sections related to specific food fermentations.

CEREAL WINES, SPIRITS, AND VINEGAR PRODUCED IN EAST ASIA

Production Processes and Products

In East Asia, alcoholic beverages—" wines" and spirits—as well as vinegar are produced from unmalted cereals. These products belong to very different product categories (Fig. 33.1) but are produced via comparable processes (Fig. 33.6) and fermentation microbiotas (Table 33.2) and are thus presented in the same section. Amylolytic fungal and bacterial cultures are used to produce enzymes for starch saccharification; the alcoholic fermentation is carried out with yeast and lactic acid bacteria in mash (sake and shochu) or in solid-state

fermentations (baijiu and rice vinegar). In Japan, *A. oryzae* has been maintained in solid-state fermentations on cooked rice as a relatively pure saccharification culture for 500 to 700 years, a time span that was sufficient for domestication (27, 48). The term "koji" refers both to the mold and the molded rice cake that is used for hydrolysis of starch and proteins in cereal, soy, and fish fermentations. The Chinese term for all saccharification cultures is "jiuqu." Daqu is most widely used and obtained by spontaneous fermentation (Fig. 33.6); xiaoqu and fuqu are back-slopped (49–51).

In a Japanese fermentation, the succession of fermentation microbiota that is required to convert cooked cereals to vinegar is achieved in a single fermentation vessel that is inoculated with koji (52). Initial growth of *A. oryzae* is followed by alcoholic fermentation with *S. cerevisiae* and lactic fermentation by *Pediococcus acidilactici* (*Pc. acidilactici*), *Lc. lactis*, *L. plantarum*, and *L. fermentum*, and by growth of *Lactobacillus acetotolerans* and *Acetobacter pasteurianus* in the final stages of fermentation (Table 33.2). The fermentation process is completed in 5 months and followed by aging for 1 year. Sake is also brewed with koji as a saccharification culture; *S. cerevisiae* and lactic acid bacteria are maintained as separate starter cultures. Addition of *M. purpureus* to koji allows production of yellow or red rice wines such as Shaoxing wine, which is produced in Zhejiang Province in China (53). *M. purpureus* is a slowly growing organism and is thus grown in association with *A. oryzae* to prevent contamination (53).

Daqu, the saccharification starter for wine, baijiu, and vinegar production, is produced by spontaneous fermentation of ground cereals. Barley, wheat, rice, or sorghum flour is mixed with water to a moisture content of 40%, shaped into bricks, and stacked in fermentation rooms (50, 51). Fermentation microbiotas are controlled by control of the temperature, which increases to 45 to 50°C (low-temperature daqu), 50 to 60°C (mediumtemperature daqu), or 60 to 65°C (high-temperature daqu). During fermentation and subsequent drying, the moisture content is reduced to 14% or less, and daqu is stored dry for up to several months (50).

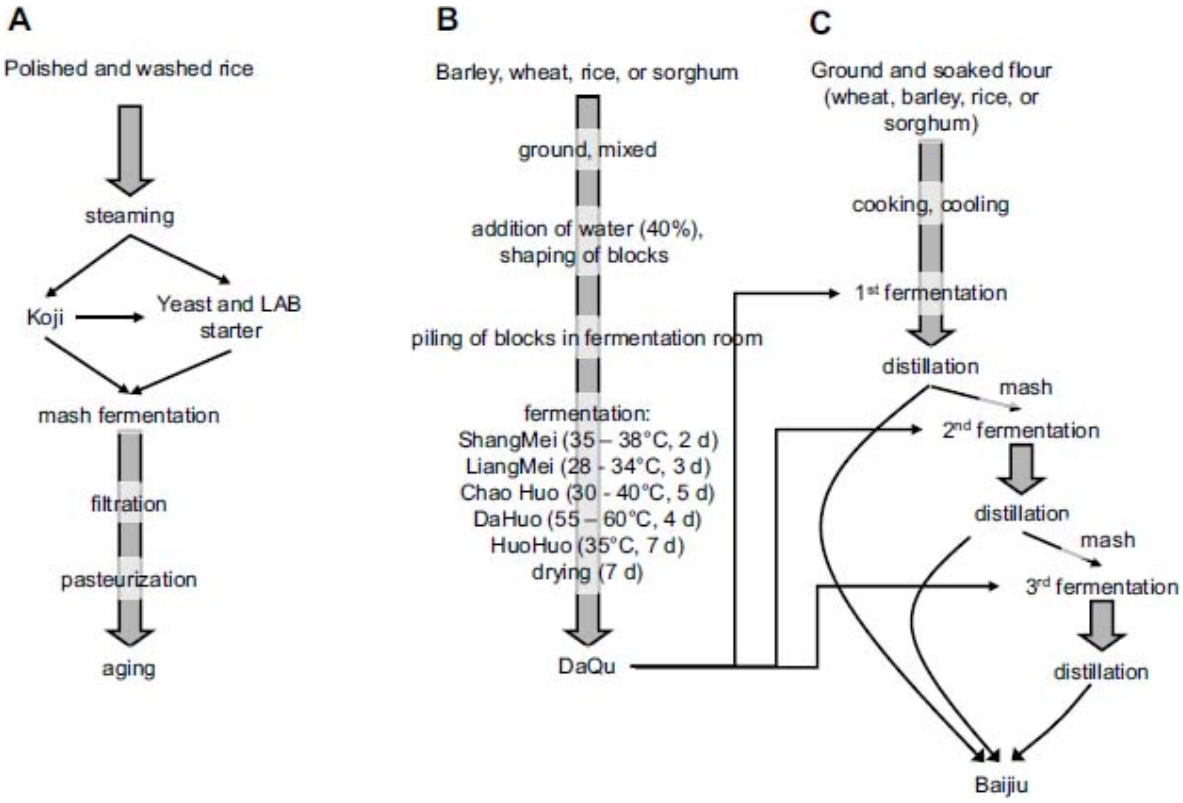

A
Polished and washed rice
steaming
Koji
Yeast and LAB starter
mash fermentation
filtration
pasteurization
aging
B
Barley, wheat, rice, or sorghum
ground, mixed
addition of water (40%), shaping of blocks
piling of blocks in fermentation room
fermentation:
ShangMei (35 – 38°C, 2 d)
LiangMei (28 - 34°C, 3 d)
Chao Huo (30 - 40°C, 5 d)
DaHuo (55 – 60°C, 4 d)
HuoHuo (35°C, 7 d)
drying (7 d)
DaQu
C
Ground and soaked flour (wheat, barley, rice, or sorghum)
cooking, cooling
1st fermentation
distillation
mash
2nd fermentation
distillation
mash
3rd fermentation
distillation
Baijiu

Figure 33.6. Flow chart for production of cereal wines and spirits. (A) Flow chart for production of Japanese rice wine (sake). Rice is polished, washed, and steamed. A part of the raw material is inoculated with koji, the saccharification starter composed of *A. oryzae*; a second part is used with koji for production of the starter containing yeasts and lactic acid bacteria (LAB). Both are combined for the mash fermentation. After completion of the fermentation, the mash is filtered, pasteurized, and aged. (B) Flow chart for production of daqu, the saccharification starter used in Chinese rice wines, spirits, and vinegar fermentations. Cereals are ground, mixed with water to a water content of 40%, and shaped into blocks about 6 by 20 by 30 cm. The blocks are stacked in a fermentation room, and the spontaneous fermentation is controlled by control of the temperature to produce low-temperature daqu (45 to 50°C), medium-temperature daqu (50 to 60°C), or high-temperature daqu (60 to 65°C). After fermentation, daqu is dried, ground, and stored dry. (C) Alcoholic fermentation for production of baijiu. Ground cereals are soaked, cooked, cooled, and fermented after the addition of daqu. Fermentation occurs at ambient temperature in earthen jars or in mud pits that are inoculated with part of the material from the previous batch. For some products, such as sauce-flavored baijiu, a 4- to 5-day aerobic fermentation is conducted prior to anaerobic fermentation in pits. After fermentation, the mash is distilled and the distiller's grains undergo a second fermentation which is again inoculated with daqu and part of a previous batch. The cycle of distillation and fermentation is repeated two to seven times. Drawn with information from references 49–52 and 54.

Alcohol and, where applicable, acetic acid fermentation is performed in earthen jars, or in pit muds with a volume of 5 to 10 m^3. Fermentation microbiota are usually back-slopped or maintained by continuous use of the same pit muds (20). Some products, such as "sauceflavored baijiu," include an aerobic, high-temperature stacking fermentation prior to the anaerobic mash fermentation (49). Chinese liquors are fermented in two to seven cycles of (stacking and) mash fermentation and distillation; after each distillation step, the distiller's grains are mixed with daqu (Fig. 33.6). Traditional vinegar fermentations employ surface aeration with occasional mixing to induce the acetic acid fermentation (12).

Fermentation Microbiotas and Their Impact on Product Quality

An overview of the fermentation microbiotas in Japanese and Chinese wine and spirit fermentations is provided in Table 33.2. In Japan, sake, spirit, and vinegar fermentations are typically controlled by back-slopped (starter) cultures; *A. oryzae* is the main saccharification starter, while *S. cerevisiae* together with lactobacilli dominates the mash fermentation and *A. pasteurianus*, *Gluconacetobacter xylinus*, and *Gluconacetobacter europaeus* carry out the acetic acid fermentation. In sake starters, *Lactobacillus fructivorans* and *Lactobacillus homohiochii* are the dominant lactic acid bacteria (54). Of note, the type strain of the homofermentative *L. homohiochii* has been lost; the current type strain clusters with the heterofermentative *L. fructivorans* group (54).

Table 33.2. Microbiota in Asian wine, liquor, and vinegar fermentations[a]

Product (origin)		Succession of fermentation microbiotas	
Sake (Japan)	*Aspergillus oryzae*	*S. cerevisiae*, *L. fructivorans*, *L. acetotolerans*[b]	
Rice vinegar (Japan)	*Aspergillus oryzae*	*S. cerevisiae*, *Lc. lactis*, *L. casei*, *L. plantarum*, *L. fermentum*	*L. acetotolerans*, *A. pasteurianus*
Rice vinegar (China)	Daqu: *Aspergillus* spp., *Rhizopus* spp., *Bacillus* spp., *Weissella*, *Leuconostoc*, *Enterobacteriaceae* including *Cronobacter* and *Enterobacter*, *Saccharomyces*, *Saccharomycopsis*	Mash: *S. cerevisiae*, lactobacilli	*Acetobacter* spp., *Gluconacetobacter* spp.
Baijiu (China)	Daqu: as above	Stacking fermentation (for sauce-flavored baijiu): *Bacillus* spp., *Weissella* spp., *Pseudomonas* spp., others	Mash: *S. cerevisiae*, *Saccharomycopsis*, lactobacilli Pit mud: Strict anaerobes fermenting proteins and amino acids, including *Clostridiales* and *Bacteroidetes*
Red rice	*M. purpureus* and *A. niger* or *A. oryzae*	Undescribed, likely as above	

wine (Japan and China)			

[a] With data from references 20, 50, 53, and 55–57.

[b] The original isolates were classified as homofermentative *L. homohiochii*. The type strain of this species has been lost but likely represented an organism closely related to *L. acetotolerans* (54).

Baijiu and Chinese rice vinegar are fermented by a succession of two to five different microbial communities representing the aerobic saccharification starter, the anaerobic mash fermentation, and, where applicable, the microbiotas of an aerobic stacking fermentation, the mud of the pit walls, and acetic acid fermentation. This succession likely represents the most diverse fermentation microbiota of any fermented food. Because of this exceptional diversity and because most studies characterized the fermentation microbiota only by sequence-based techniques, current knowledge on the microbial diversity and the key contributors to product quality is still preliminary.

The composition of the Chinese saccharification starter daqu is controlled by the temperature and moisture content (50). Daqu microbiotas are very diverse; bacilli, members of the *Enterobacteriaceae* including *Enterobacter* and *Cronobacter*, and lactic acid bacteria are part of the bacterial microbiota, while the mycobiota is represented by *Aspergillus* spp., *Monascus* spp., *Mucor* spp., and a wide variety of yeasts (50, 55–57). Bacilli and *Enterobacteriaceae* from daqu carry mobile genetic elements that confer heat resistance, suggesting that temperature control is a key factor in community assembly (49, 50, 58). Bacilli as well as fungi produce amylolytic and cellulolytic enzymes that are required for starch saccharification during later stages of the fermentation (59).

Microbiotas of mash fermentations are back-slopped and thus more uniform; *S. cerevisiae* and host-adapted lactobacilli, including *L. acetotolerans*, *Lactobacillus helveticus*, and *Lactobacillus panis*, are dominant organisms. Aerobic stacking fermentation that is included prior to the mash fermentation in sauce-flavored baijiu includes metabolic activity of bacilli, plant-associated lactic acid bacteria, and *Pseudomonas* spp. (Table 33.3). Fermentation in pit muds brings the

mash into contact with strictly anaerobic microbiotas in the mud walls that include *Clostridia*, *Bacteroidetes*, and other strict anaerobes (57, 60).

Fermentation of vinegar is performed in earthen jars to achieve surface aeration with occasional mixing of the mash. Comparable to other vinegar fermentations, *Acetobacter* spp. and *Gluconacetobacter* spp. are the main organisms converting ethanol to acetic acid.

Although the contribution of individual members of daqu microbiotas, particularly bacilli, to the production of amylases and proteases has been demonstrated, the metabolic activities of individual members of the diverse fermentation microbiota involved in production of spirits and vinegar remain unknown. The different process variables—low-, medium-, or high-temperature daqu fermentation, fermentation in mud pits or earthen jars, the number of fermentation and distillation cycles, and the inclusion of an aerobic stacking fermentation—result in a large diversity of products with different flavor characteristics (49). The very diverse fermentation microbiota results in a very intense flavor that is mediated by a diverse set of flavor compounds. In addition to fruity, floral, or malty higher alcohols, aldehydes, and esters that likely result from yeast metabolism and are also found in other yeast-fermented products (Fig. 33.4), baijiu flavor is complemented by sweaty, cheesy, and rotten cabbage odors that are present with high odor activity values (61).

Table 33.3. Representatives of microbiota of cereal porridges and beverages[a]

Organisms	Example(s)
Spontaneous fermentation (the usual suspects)	Ogi, mawe, mahewu, koko, bushera, idli
Early fermentation: *Enterobacteriaceae* including *Enterobacter* spp., *Cronobacter* spp., *Pantoea* spp., and *Klebsiella* spp.	
Intermediate fermentation: *Leuconostoc* spp., *Weissella* spp., *Lc. lactis*, *Enterococcus* spp., *Pc. pentosaceus*, and *Pc. acidilactici*	
Late fermentation: *L. plantarum*, *L. fermentum*, and *L. brevis;* yeasts including *S. cerevisiae*, *I. orientalis*, and *C. glabrata*	
Back-slopped fermentations	
L. paracasei, *Lu. mesenteroides*, and *S. cerevisiae*	Kvass
L. reuteri, *L. vaginalis*, *L. fermentum*, *L. plantarum*, and *L. harbinensis*	Ting, kisra
L. plantarum, *L. paracasei*, *L. brevis*, *Lu. mesenteroides*, *W. confusa*, and *S. cerevisiae*	Boza

[a] Prepared with data from references 67–69 and 77.

TUBERS AND ROOTS

Products and Production Processes

Tubers and roots, including cassava, yams, and sweet potato, are major crops in Africa and, to a lesser extent, in South America and South and East Asia (62). Sliced cassava are fermented in spontaneous solid-state fermentations, e.g., for production of gari and fufu in West Africa, or the whole roots are spontaneously fermented after submersion in water, e.g., for production of lafun in Nigeria. The products are consumed as a paste or after drying and reconstitution to porridges (62). Some cassava

products and elubo (fermented yam roots) are spontaneously fermented after boiling of the tubers (62, 63).

Fermentation Microbiotas and Their Contribution to Product Quality

Cassava and yam fermentations are dominated by the usual suspects, i.e., a succession of fermentation microbiotas initiated by *Enterobacteriaceae* and by acid-sensitive lactic acid bacteria, including *Leuconostoc* spp., *Weissella* spp., and *Enterococcus* spp., followed by *Lactobacillus* spp., particularly *L. plantarum* and *L. brevis* (62–64). In addition to the usual suspects, *Lactobacillus manihotivorans* and *Bacillus* spp. were identified in some of the fermentations. Tubers and roots have a low content of fermentable carbohydrates and low amylase activity; many *Bacillus* spp. and cassava isolates of *L. manihotivorans* and *L. plantarum* express extracellular amylase activity. Extracellular amylases are exceptional in lactobacilli but frequently found in isolates from tubers or cereal fermentations in tropical climates (35, 65). Extracellular amylases of lactobacilli are homologous to intracellular α-glucosidases in other lactobacilli but differ by the presence of a signal peptide directing protein export (35). Although several species of lactobacilli were named after amylolytic strains, the expression of extracellular amylases is strain specific rather than species specific.

A second key metabolic activity of lactic acid bacteria in cassava fermentations is β-glucosidase activity, which hydrolyzes the toxic cyanogenic glycoside linamarin to acetone and hydrogen cyanide (Fig. 33.7) (66), which can be washed away or evaporates prior to consumption. Cassava also exhibits linamarase activity, but fermentation and permeabilization of the plant cell wall are required to give the enzyme access to the substrate.

CEREAL FOODS

Nonalcoholic Beverages and Porridges

Production Processes and Products

Fermented porridges and nonalcoholic beverages are widely produced in Africa from corn, millet, or sorghum. A majority of the products are produced by spontaneous fermentation at the household level with a corresponding diversity of production processes; an overview is provided by Nout (67), Sekwati-Monang (68), and Franz et al. (69). Cereal beverages in Europe include kvass (Russia) and boza (Turkey and Balkan countries); lactic acid-fermented oat porridges are produced in Scotland (sowans) and Finland (9). Comparable to cultured dairy products, nonalcoholic cereal beverages often contain viable fermentation organisms and are a suitable carrier for probiotic bacteria (9, 69).

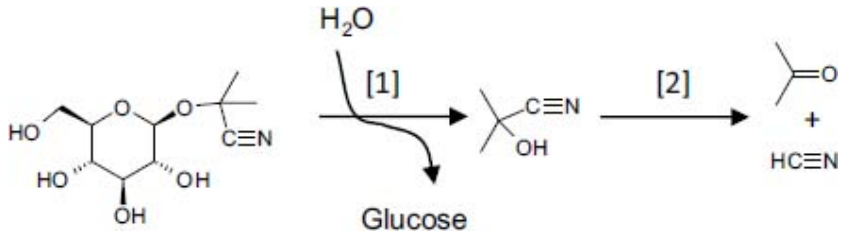


Figure 33.7. Degradation of linamarin in cassava fermentations. Linamarin is a toxic cyanogenic β-glucoside that is degraded by linamarase activity of the raw material or by β-glucosidases of lactobacilli (1). The degradation product decomposes spontaneously (2) to the volatile compounds hydrogen cyanide and acetone.

Fermented cereal beverages have a solid content of about 10%; malt from millet, sorghum, or barley is added as saccharification agent and as source of fermentation microbes. The balance of sweet taste derived from starch hydrolysis, sour taste derived from lactic fermentation, and carbonation from yeast metabolism typically provides a refreshing beverage. If fermentation microbiotas are not inactivated, cereal beverages spoil by alcoholic fermentation. Boza is produced in Turkey and Bulgaria by boiling a thin slurry of wheat or millet flour; after boiling, the slurry is filtered, cooled, and fermented by addition of 20% sucrose and a portion of a previous batch (70). Kvass is produced from stale rye bread in Russia and other Eastern European countries (Fig. 33.8) (71). Commercial kvass production employs rye malt and boiled rye flour as alternative ingredients (71). The color and the balance of sweet and sour taste of kvass resemble those of cola products, and it has therefore also been referred to as “Russian cola.” Bushera is produced in

Uganda by boiling a thin slurry of flour from sorghum malt, followed by cooling, addition of sorghum malt, and spontaneous fermentation (Fig. 33.8) (72). Mahewu (Zimbabwe) and obiolor and kunun-zaki (Nigeria), kwete (Uganda), and togwa (Tanzania) are produced by comparable procedures with millet or sorghum malt (Fig. 33.8) (68).

Cereal porridges are produced by fermentation of thick slurries of cereal flour or by soaking of grains, followed by wet milling (Fig. 33.9). Soaking prior to milling of grains greatly reduces the energy input required for milling (9). Porridges are generally cooked or steamed prior to consumption and thus do not contain viable fermentation microbiota (68) (Fig. 33.9). Ting is produced in Botswana and South Africa from sorghum flour; the fermented slurry is boiled, diluted to a soft or stiff consistency, and eaten after addition of sucrose, milk, or condiments (73). Idli is produced in India and Sri Lanka by separate soaking of black beans and rice, followed by wet milling, mixing, and fermentation (74). Koko is produced in Nigeria by steeping, wet milling, and spontaneous fermentation of millet. After fermentation, the bottom layer is boiled to a porridge; the top layer, koko sour water, is added to adjust the consistency of the porridge or consumed as a beverage (Fig. 33.9). Ogi (Nigeria and West Africa), mawe (Benin), ben saalga (Burkina Faso), and kirario (Kenya) are produced from maize or pearl millet by similar procedures involving soaking, wet milling, spontaneous fermentation, and cooking prior to consumption (68, 69).

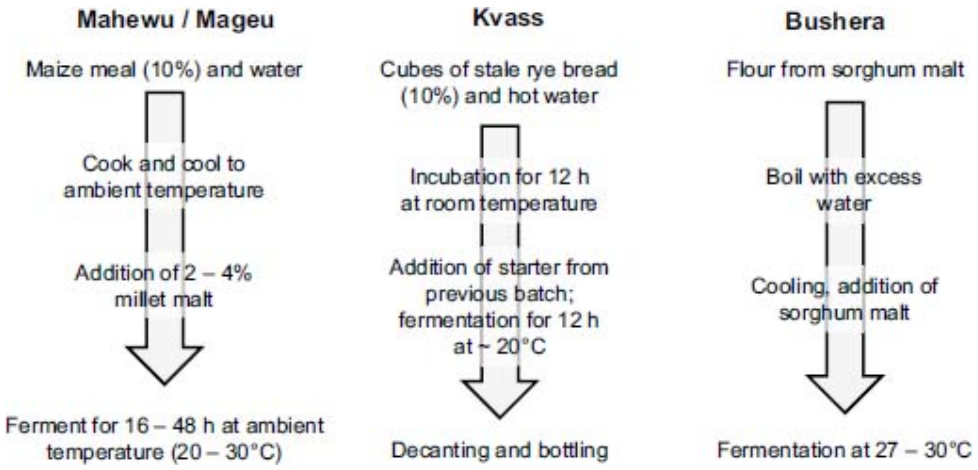


Figure 33.8. Flow charts for production of selected cereal beverages. Shown are processes for production of mahewu (Zimbabwe and South Africa), kvass (Russia), and bushera (Uganda). Kvass is alternatively produced with rye malt and boiled rye flour. Drawn with information from references 68, 71, and 72.

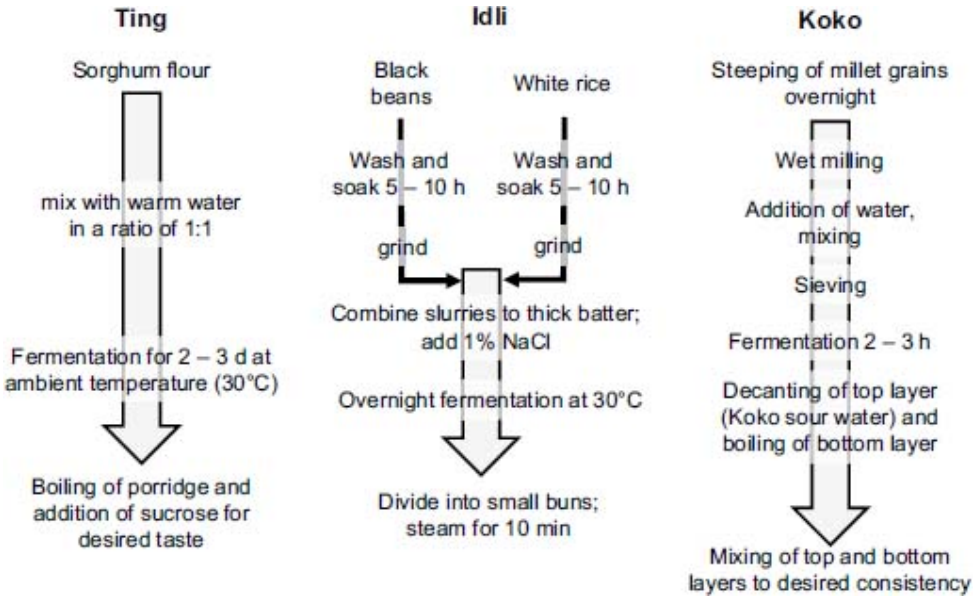


Figure 33.9. Flow charts for production of selected cereal porridges. Shown are processes for production of ting (Botswana and South Africa), idli (India and Sri Lanka), and koko (Ghana). Drawn with information from references 68, 73, and 74.

Fermentation Microbiotas and Their Impact on Product Quality

The microbiota of cereal fermentations used for production of beverages and porridges consists predominantly of lactic acid bacteria and yeasts; community assembly follows the same pattern as assembly of the sourdough microbiota, which has been studied in more depth (see below). The use of alternative cereal substrates—sorghum, millet, or maize—in many of the fermentations selects for strain-specific metabolic properties rather than specific species of lactic acid bacteria or yeasts. Spontaneous cereal fermentations are dispersal limited and reliably undergo fermentation by the usual suspects, i.e., a suc plant-associated *Enterobacteriaceae*, followed by plantassociated lactic acid bacteria, including *Leuconostoc*, *Weissella*, *Lactococcus*, and *Enterococcus*, which are displaced eventually by more acid-tolerant lactobacilli (Table 33.3) (25, 74–76). Because fermentations are carried out with different inocula and for different lengths of time, all representatives of this continuum of fermentation microbiota, including *Enterobacteriaceae*, have been isolated from cereal products (68, 69, 77). Malt that is used in production of fermented cereal beverages has a higher bacterial cell count than flour or grains and thus accelerates fermentation (78). Fully fermented products obtained by spontaneous fermentation are generally dominated by *L. plantarum* and *L. fermentum*. Back-slopping is practiced for production of kvass, boza, ting, and kisra, among others; in these fermentations, host-adapted lactobacilli, including *L. reuteri* and *Lactobacillus vaginalis*, occur (Table 33.3). The high ambient temperature in many African countries and South Asia favors thermophilic lactic acid bacteria over mesophilic organisms. Likewise, thermophilic yeasts, including the opportunistic pathogens *Candida glabrata* and *Issatchenkia orientalis*, contribute to the fermentation alongside *S. cerevisiae*.

The composition and endogenous enzymes of the fermentation substrates select for strain-specific properties of fermentation microbiota. Resting grains of C_4 cereals, including maize, millet, and sorghum, have low amylase activity compared to wheat, rye, and barley (79); accordingly, many lactobacilli from maize, millet, and sorghum fermentations express extracellular amylase activity (35).

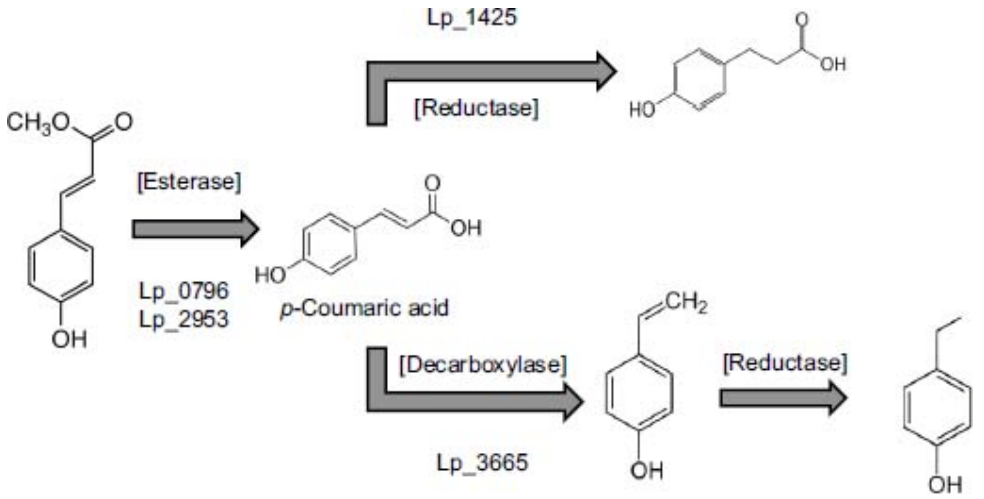


Figure 33.10. Metabolism of phenolic acids by lactic acid bacteria using the example of coumaric acid metabolism by *L. plantarum*. Protein numbers refer to the genome sequence of *L. plantarum* WCFS1 (GenBank accession number NC_004567.2).

Millet and sorghum also differ from wheat, rye, and barley by a high content of phenolic compounds, including tannins and phenolic acids. Phenolic compounds are of agronomic relevance, as they contribute to the drought and pest resistance of sorghum and millet varieties; however, they also decrease the nutritional value and impart a bitter taste (79, 80). Lactic acid metabolism in cereal fermentations modifies phenolic compounds through glycosyl hydrolases, which hydrolyze flavonoid glycosides, tannases, phenolic acid esterases, and phenolic acid decarboxylases or phenolic acid reductases with specific activity on hydroxyl benzoic and hydroxyl cinnamic acids (Fig. 33.10) (81). Metabolism of phenolic compounds in lactobacilli is strain specific and particularly frequent in *L. plantarum* (82, 83). Tannins and phenolic acids in sorghum and millet fermentations have antimicrobial activity, which selects for fermentation organisms with high resistance to phenolic acids (84). The conversion of phenolic compounds during sorghum and millet fermentations likely also improves the palatability of fermented porridges and beverages produced from these cereals.

Bread and Steamed Bread

Production Processes

Bread is generally fermented with mixed cultures of yeast and lactic acid bacteria. Traditional processes for production of bread and steamed bread use sourdough as the sole leavening agent. Sourdough is fermented by continuous propagation or back-slopping. The use of sourdough as the sole leavening agent requires multiple fermentation steps in bread production to ensure sufficient leavening capacity by sourdough yeasts and lactic acid bacteria (Fig. 33.11). The production of yeast biomass dedicated for use as a leavening agent in baking started in the 19th century; baker's yeast all but replaced sourdough as the leavening agent in wheat baking and supplemented the use of sourdough in rye baking. In rye baking, the use of sourdough remained necessary to improve water binding and gas retention through solubilization of rye pentosans and to inhibit endogenous amylases by acidification. The shift of technological aims also resulted in altered fermentation processes. Sourdoughs that are used as leavening agents are propagated frequently to maintain the sourdough microbiota in the exponential phase of growth; these doughs are referred to as type I sourdoughs (Fig. 33.11). Sourdoughs used for dough acidification are fermented for longer times and at higher temperatures to enhance acid and flavor production; these sourdoughs are referred to as type II sourdoughs (85) (Fig. 33.11).

Type I sourdoughs are fermented at the site of bread production; few bakeries manage to scale type I fermentation processes to a large industrial scale. In contrast, type II sourdoughs are produced at bakeries or at specialized ingredient suppliers for distribution in a stabilized form (dried, pasteurized, or refrigerated) (86). The fermentation of type II sourdoughs is readily scaled and integrated into automated processing lines. The scalability of type II sourdough fermentations and their beneficial impact on bread quality contributed to the widespread reintroduction of sourdough processes in industrial baking in recent decades. Sponge doughs represent an intermediate between straight doughs (fermentation with baker's yeast only) and sourdoughs. Sponge doughs are started only with baker's yeast; depending on the contamination of the yeast preparation and the flour, a lactic microbiota develops after 6 to 12 h and acidifies the dough. Freeze-dried starter cultures for use in baking are commercially available but are of little relevance compared to refrigerated sourdoughs containing active yeasts

and lactic acid bacteria or to dried or pasteurized sourdough products that do not contain viable microorganisms (86).

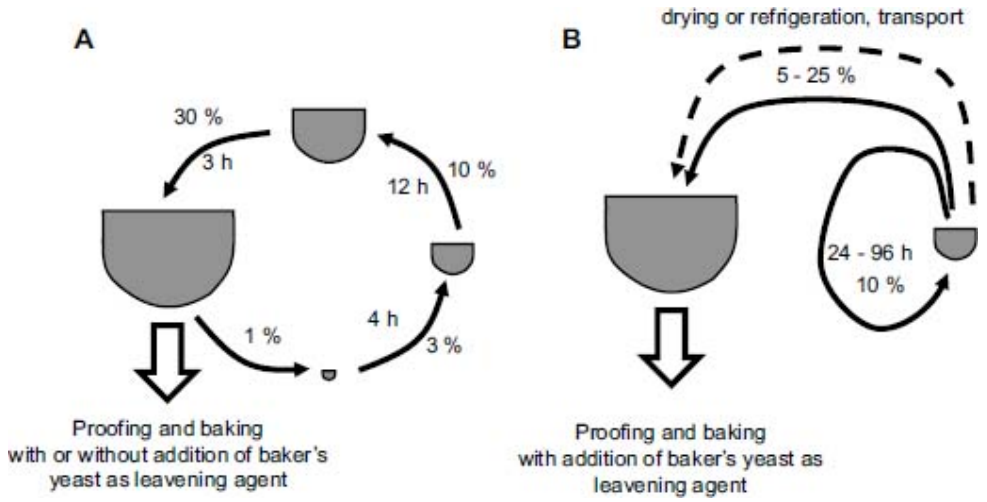


Figure 33.11. Schematic representation of type I (A) and type II (B) sourdough fermentation processes. Times indicate typical fermentation times for each fermentation step; the percentage values indicate the percentage of the flour in the bread recipe that is fermented at a specific step. Type I sourdoughs are propagated for use as the sole leavening agent; this necessitates the use of two to four fermentation cycles per batch of bread, which uses an increasing proportion of the flour. Fermentations are conducted at an ambient temperature of 15 to 30°C. Sourdoughs are maintained by use of a small proportion of the last sourdough or the bread dough as the inoculum for the next fermentation cycle. Type II sourdoughs are generally fermented at a higher temperature (30 to >40°C); if the fermentation is carried out by ingredient suppliers, type II doughs are pasteurized, dried, or refrigerated prior to use in the bakery.

Products

Bread or steamed bread is a staple of the diet in most regions of the world where wheat or rye is cultivated, including Europe, North America, China, and the Middle East. While fermentation processes (type I or type II sourdough) and the bulk raw material (wheat or rye) are comparable throughout the world (87), local differences in the use of ingredients and production processes account for a large variety of products. Examples are shown in Fig. 33.1 (groups 7 and 8). Baking of

bread in an oven or on hot stones heats the surface to tempera temperatures of more than 100°C, leading to crust formation and flavor generation in the Maillard reaction. Steamed bread is widely consumed in Southeast Asia (mantou) and in Central Europe (Dampfnudel or Germknödel); steaming of bread avoids crust formation during baking (88). Leavened flat bread (e.g., naan, pita, and kisra) is produced in Central Asia, the Middle East, eastern Mediterranean countries, and Sudan.

The most common cereal used in bread production is wheat. Wheat prolamins have the unique property of forming a polymeric protein network, the gluten macropolymer. The gluten macropolymer mediates dough hydration and gas retention during fermentation and baking (89) and supports production of bread with a much larger volume than bread from other cereals. The gluten proteins are polymerized by disulfide bonds. Additives, ingredients, or microbial metabolites that impact intramolecular disulfide cross-linking also impact the volume and texture of wheat bread (89).

Examples of wheat breads include the French baguette, which is produced with type I sourdoughs or by sponge doughs; San Francisco sourdough bread (type I sourdough); and the Italian panettone, the French brioche, and the Suebian Mutschel, which are produced with type I sourdoughs with the addition of eggs, butter, and sugar. In rye dough, dough hydration and gas retention are mediated by arabinoxylans (pentosans), which are solubilized by partial enzymatic hydrolysis of waterinsoluble pentosans during sourdough fermentation (90). Rye breads are darker and denser than wheat breads, particularly when whole flour and whole grains are the major ingredients. Rye bread is common in regions of Northern, Eastern, and Central Europe where the growing season is too short, cold, or wet for wheat; examples include the Westphalian pumpernickel, the Finnish reikäleipä, and crispbread (Fig. 33.1).

Cereals other than wheat and rye have a low content of structure-forming proteins or polysaccharides and do not allow the production of leavened bread unless structure-forming ingredients such as proteins or hydrocolloids are added. Traditionally, millet and sorghum are used for production of leavened flatbread. In recent years, gluten-free breads have become popular in North America and Europe. A gluten-free diet

that is free of prolamins of wheat, rye, and barley is the only treatment for celiac disease; gluten-free breads also cater to consumers with wheat or gluten intolerance (91). Commercial recipes for gluten-free breads are based on purified starches, structure-forming proteins, and hydrocolloids and gluten-free cereals or pseudocereals. The use of sourdough in gluten-free baking does not follow traditional procedures but generally follows type II technology to achieve improved flavor, texture, and shelf life (92).

The use of ingredients other than flour and water further contributes to the diversity of products. Examples of such ingredients are sugar, eggs, butter or other oils and fats, seeds, nuts, dried fruits, and insects; examples of products with a substantial proportion of noncereal ingredients are panettone, brioche, and hamburger buns.

Sourdough Fermentation Microbiotas

Sourdough microbiota have a high γ-diversity; more than 50 species of lactic acid bacteria and more than 20 species of yeasts have been identified in sourdoughs. However, the α-diversity (diversity in a single sourdough) is relatively low. Overviews of key organisms in sourdough are provided in references 87, 93, and 94 and in Table 33.4.

Type I sourdoughs used as sole leavening agents have a consistent composition of microbiota throughout the world. This uniformity relates to the technological aim of type I fermentations, which is leavening. In straight dough processes, the biomass of the added baker's yeast accounts for about 1% of the dough dry weight; type I sourdoughs achieve a comparable level of CO_2 production, although microbial biomass accounts for less than 0.1% of the dough dry weight. Dough leavening is achieved by fermentation protocols that maintain sourdough microbiotas perpetually in a metabolically active and exponentially growing state; this is achieved by multiple daily fermentation steps and uninterrupted propagation. Even short interruptions, e.g., by weekends or by extensive baking practices on farms or in households, reduce the leavening power and shift the fermentation microbiota (87, 95). Type I sourdoughs are continuously maintained over decades or centuries (95, 96). Anecdotal evidence dates

the origin of sourdoughs that are currently used in San Francisco to the 1849 gold rush.

Table 33.4. Composition of sourdough microbiotas[a]

Fermentation Type	Key organism(s)	Other organisms
Type I Sourdoughs	*L. sanfranciscensis*, *Ks. humilis*, *Ks. exigua*	*L. plantarum*, *L. brevis*, *L. alimentarius*, *Lu. mesenteroides*
Type II sourdoughs	*L. pontis*, *L. amylovorus*	*L. fermentum*, *L. panis*, *L. reuteri*, *L. crispatus*, *L. helveticus*, *I. orientalis*
Spontaneous sourdoughs and sponge doughs	*L. plantarum*, *L. fermentum*, *S. cerevisiae*	*Enterococcus* spp., *Pc. pentosaceus*, *Weissella* spp., *Lu. mesenteroides*, *L. casei*, *L. brevis*, *L. plantarum*, and *L. fermentum*

[a] Data are from references 87, 93, and 94.

The continuous use of type I sourdoughs eliminates dispersal limitation because contamination events, even from unlikely sources, will occur eventually. The consistent selective pressure in type I sourdoughs selects for the most rapidly growing organisms. Type I sourdoughs typically contain two to four strains; in over 90% of type I sourdoughs, *L. sanfranciscensis* is the most abundant organism. *L. sanfranciscensis* is often associated with organisms of the *Lactobacillus alimentarius* and *L. brevis* groups or with *Lactobacillus pontis* and *L. panis* (87). All isolates of *L. sanfranciscensis* described to date were isolated from type I sourdoughs, but the phylogenetic position of the strain, its metabolic features, and its detection in insects by culture-independent methods suggest an association with flowers or insects (21, 25). Comparable to other insect-associated lactobacilli, *L. sanfranciscensis* has a very small genome (1.3 Mbp), a very restricted metabolic potential, and a low acid tolerance but a high tolerance to oxygen (97). The prevalence of *L. sanfranciscensis* in type I sourdough is explained by its efficient use of sucrose and maltose, the main carbohydrate sources in sourdough, and the high coding density of rRNA operons, which support rapid growth

(36, 97). *L. sanfranciscensis* invariably coexists with sourdough yeasts, particularly *Ks. humilis* (previously *Candida milleri* or *Candida humilis*), *Kazachstania exigua* (previously *S. exiguous*), or *S. cerevisiae* (98). Yeasts and lactobacilli in sourdough do not compete for the main nutrients; *L. sanfranciscensis* preferentially metabolizes maltose over glucose, while *Ks. humilis* and *Ks. exigua* are maltose negative. Likewise, lactobacilli satisfy requirements for amino acids by uptake of peptides, while sourdough yeasts preferentially metabolize free amino acids (99). Yeast invertase activity supports growth of *L. sanfranciscensis* by releasing fructose from cereal fructans that are not accessible to lactobacilli (100).

The α-diversity and the β-diversity in type II sourdoughs are higher than those in type I sourdoughs, which reflects the fact that the technological aim, dough acidification, accommodates a higher variability in the fermentation processes (25, 85). Common characteristics of type II fermentation processes, which are often conducted on a large industrial scale, are long fermentation times, high levels of dough acidity at the end of the fermentation, and high fermentation temperatures (87, 101, 102). Type II sourdough microbiotas are dominated by vertebrate-host-adapted, acid-tolerant, and thermophilic organisms of the *L. reuteri* group (*L. pontis*, *L. panis*, and *L. reuteri*) in association with organisms of the *L. delbrueckii* group (*Lactobacillus amylovorus*, *Lactobacillus crispatus*, and *Lactobacillus acidophilus*). Yeasts are often absent in type II sourdoughs; in addition to yeast species that are also found in type I sourdoughs, thermophilic yeasts and particularly *I. orientalis* (anamorph: *Candida krusei*) and *C. glabrata* occur (98).

Sponge doughs and spontaneous sourdoughs are characterized by a larger diversity of fermentation organisms than that in the back-slopped type I and type II sourdoughs.

In spontaneous sourdough fermentations, fermentation is initiated by the usual suspects, the succession of fermentation microbiota that also occurs in other spontaneously fermented plants (25). The addition of baker's yeast in sponge sourdough suppresses the initial growth of *Enterobacteriaceae* that is observed in other spontaneous cereal fermentations, resulting in dominance of environmental and nomadic lactic acid bacteria, e.g., *L. plantarum*, *L. fermentum*, *Leuconostoc* spp., and *Pediococcus* spp. (25, 87, 98).

Impact of Sourdough Microbiotas on Product Quality

The beneficial impact of sourdough fermentation on bread quality is dependent not only on the metabolic activity of fermentation microbiota but also on the modulation of the activity of cereal enzymes (Table 33.5). Wheat and rye flours have amylase, protease, pentosanase, and phytase activities; of these, amylase activities are inhibited by low pH, while proteases, pentosanases, and phytases are more active at the pH of sourdough, 3.5 to 4.5, than at the pH of flour or yeast-fermented dough, 5.5 to 6.5. The extended time of sourdough fermentations compared to straight dough processes also supports conversion of proteins and polysaccharides by cereal enzymes (90).

Type I sourdough fermentations leave little room for optimization of product quality by strain selection because leavening power is the overriding selection criterion (87). Proteolysis during sourdough fermentation provides amino acids as flavor precursor compounds in yeast metabolism or the Maillard reaction during baking (103, 104) but also weakens gluten structure (104, 105). The negative impact of gluten hydrolysis and depolymerization is partially compensated for by the recruitment of pentosans as water-binding polysaccharides; solubilization of water-insoluble arabinoxylans during sourdough fermentation increases their water-binding capacity (90, 104, 105). Phytate hydrolysis during sourdough fermentation improves the mineral bioavailability (90). Sourdough yeasts improve bread flavor by conversion of amino acids to flavor volatiles and by strain-specific formation of volatile esters (28, 103). Specific contributions of *L. sanfranciscensis* to bread quality all relate to cofactor regeneration supporting rapid growth. Acetic acid is a main flavor volatile in bread and also a major contributor to the inhibition of fungal growth during bread storage (36, 103, 106, 107). Acetate formation by *L. sanfranciscensis* depends on the availability of fructose, oxidized glutathione, and aldehydes. These compounds are used to oxidize reduced cofactors (NADH) to NAD^+ with concomitant reduction to mannitol, reduced glutathione, and the corresponding aldehydes and thus enable acetate production in heterofermentative metabolism (36). The reduction of aldehydes originating from lipid oxidation by *L. sanfranciscensis* alters the flavor profile because the corresponding alcohols have a much lower flavor threshold (88, 103). The reduction of

oxidized to monomeric glutathione interferes with gluten polymerization and reduces bread volume (87, 105).

Type II sourdough fermentations leave more room for selection of cultures that improve bread quality through production of specific metabolites. An overview is provided in Table 33.5; examples include the production of exopolysaccharides for improved bread volume and reduced staling (108), the conversion of amino acids to taste-active compounds and flavor precursors (103, 109), and the formation of antifungal metabolites (107).

Table 33.5. Impact of sourdough fermentation on product quality[a]

Metabolite or metabolic pathway	Impact on quality	Organism(s)
Carbohydrate metabolism		
Formation of lactic acid	Dough acidification	All LAB
Formation of acetic acid	Flavor and enhanced mold-free shelf life	Heterofermentative LAB
Formation of CO_2	Leavening	Heterofermentative LAB and yeasts
Formation of exopolysaccharides by LAB	Improved bread volume, improved texture, and reduced staling	*L. reuteri*, *Leuconostoc* spp., and *Weissella* spp.
Formation of mannitol	Sweet taste	Heterofermentative LAB except *Weissella* spp.
Protein and amino acid metabolism		
Release of amino acids from peptides	Taste compounds and flavor precursor compounds	All LAB
Conversion of glutamine to glutamate by LAB	Umami taste	All LAB
Conversion of arginine to ornithine by LAB	Precursor to formation of 2-acetyl-2-pyrroline during baking	Species-specific trait of LAB, particularly in type II fermentations
Conversion of glutamate to γ-butyric acid	Bioactive	*L. reuteri*, *L. plantarum*, *L. brevis*, others
Reduction of oxidized glutathione	Reduced gluten quality and bread volume,	*L. sanfranciscensis*

	accelerated degradation of allergens	
Formation of γ-glutamyl peptides	Enhanced taste	*L. reuteri*
Conversion of amino acids to flavor volatiles	Improved flavor	Yeasts
Lipid metabolism		
Reduction of aldehydes originating from lipid oxidation to alcohols	Altered flavor	*L. sanfranciscensis*
Hydration of unsaturated fatty acids to hydroxyl-fatty acids	Enhanced mold-free shelf life	*Lactobacillus hammesii*
Others		
Conversion of phenolic acids	Flavor, bioavailability of bioactive compounds	*L. plantarum*, *L. brevis*
Ester synthesis	Flavor	Yeasts

[a] Modified from reference 90. LAB, lactic acid bacteria.

FERMENTED SOY AND BEAN PRODUCTS AND CONDIMENTS

Products and Production Processes

East and Southeast Asia have a rich tradition of fermented beans and soybeans (110–112). Beans and soybeans are typically fermented after a cooking step and without milling: examples include tempe, obtained by fermentation of cooked soybeans; natto, *Bacillus*-fermented soybeans; and stinky tofu, which is produced by fermentation of tofu (a curd prepared by precipitation of soy proteins) with lactic acid bacteria and bacilli. Beans and soybeans are also used for production of condiments.

The most widely known condiment is soy sauce, which is produced by fermentation of soy or soy and wheat (Fig. 33.12). Miso paste is produced in Japan and Korea from soybeans and wheat or rice and is used as a soup base. Dochi is a condiment obtained by fermentation of black beans in China (113). Gochujang, a condiment used in Korean cuisine, is produced by fermentation of hot chili peppers, soy, spices, and salt with *A. oryzae* and *Bacillus* spp. (112).

Fermentation Microbiotas and Their Contribution to Product Quality

The fermentation microbiotas in production of soy sauce are controlled by use of *A. oryzae* as a saccharification culture at the koji stage and by the addition of 17 to 25% NaCl to the moromi stage, which selects for the halophilic organism *T. halophilus* and yeasts (Fig. 33.12) (27, 110). During the 2- to 3-day koji fermentation, *A. oryzae* consumes about 20% of the starch and produces proteases, amylases, and glutaminase for hydrolysis of starch and proteins during the subsequent mash fermentation. *T. halophilus* produces lactate, while yeasts including *Zygosaccharomyces rouxii* and *Candida versatilis* produce flavor volatiles from amino acids and phenolic acids (Fig. 33.4) (27, 110). Pentoses are more reactive in the Maillard reaction than hexoses; therefore, pentose metabolism by *T. halophilus* indirectly impacts the color and flavor of soy sauce. The finished product contains 17 to 25% NaCl, 1% glutamate and lactic acid, and numerous additional umami and kokumi tastants and flavor volatiles that are produced by proteolysis, peptide conversion, or the Maillard reaction (109). Miso is produced from cooked soybeans and steamed rice; miso fermentations employ a lower salt concentration (10 to 12%) but they also use *A. oryzae* at the koji stage and salt-tolerant lactic acid bacteria, including *T. halophilus*. Dochi is produced by solid-state fermentation of cooked black beans with *A. oryzae*, followed by a high-salt (15%) mash fermentation (113).

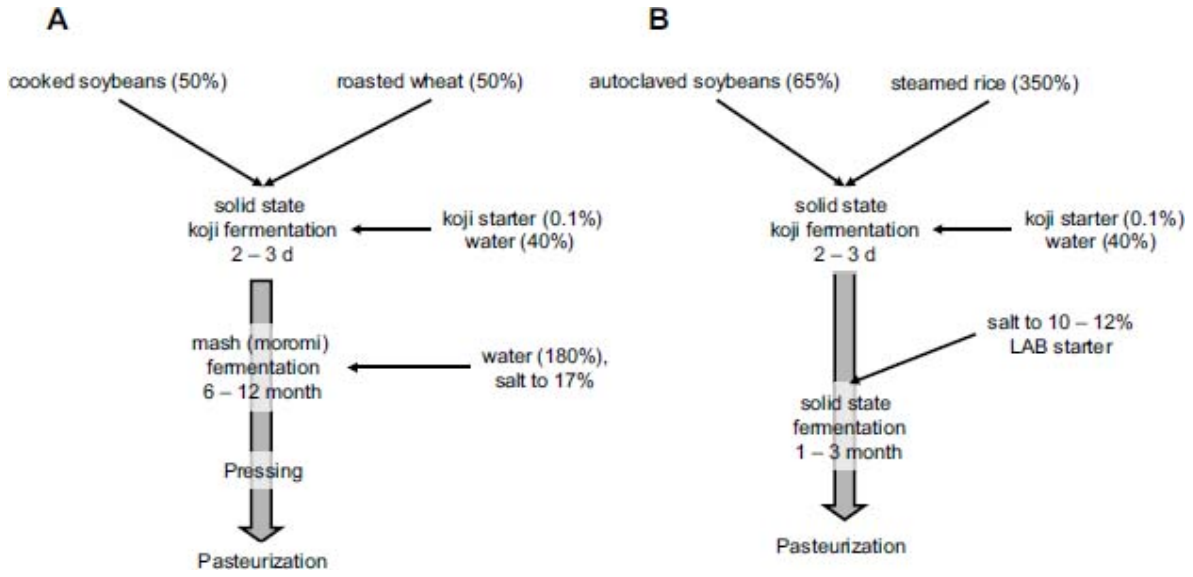


Figure 33.12. Flow chart for production of Japanese soy sauce (A) and miso (B). Koji fermentation by *A. oryzae* generates saccharolytic and proteolytic enzymes that hydrolyze starch and proteins in the subsequent mash (moromi) fermentation. The high salt concentration selects for the halophilic organism *T. halophilus* as the dominant bacterial representative of the moromi microbiota; *Z. rouxii* and other halophilic yeasts contribute to ethanol and flavor formation. Products in other East Asian countries use cooked soybeans as the sole ingredient and employ salt concentrations of up to 27%. Drawn with information from reference 110.

Tempe is a soybean cake fermented by *Rhizopus stolonifer* or *Rhizopus oligosporus*; the fungal mycelium produces a white mat on the surface of the product; the mycelium penetrates the cake and secretes extracellular proteases and carbohydrate hydrolases (114).

Natto is fermented in Japan by inoculation of cooked soybeans with *Bacillus subtilis* var. natto. *B. subtilis* produces hydrolytic enzymes but also contributes to the slimy or ropy appearance of the natto by synthesis of polyglutamate and fructans (110, 115).

FERMENTED VEGETABLES

Products and Production Processes

Vegetables are preserved by fermentation in most regions of Asia, Europe, and North America. The most common fermented vegetables are cabbage, which is used to produce sauerkraut and kimchi, pickle cucumbers, and olives; carrot juice, mustard or mustard leaves, and radish roots are also subjected to fermentation (Fig. 33.1) (112,116–118). Sauerkraut, kimchi, pickles, and olives are fermented on an industrial scale; most other products are fermented at the artisanal or household level.

Sauerkraut is prepared from finely sliced white cabbage, which is dry salted to a concentration of 1.8 to 2.2% and spontaneously fermented in covered containers at ambient temperature (15 to 20°C) (119). Refrigerated sauerkraut is shelf stable for several weeks, but it is most commonly sold after pasteurization. Kimchi is prepared from coarsely cut Chinese cabbage with addition of radish, onions, garlic, and spices, including black pepper and mustard. The raw material is brined in a 10% salt solution to achieve a final NaCl concentration of 2.5% and fermented in closed containers at temperatures ranging from 5°C to 20°C (112). Kimchi is usually consumed fresh. Depending on the fermentation temperature, sauerkraut and kimchi fermentations are completed in 2 to 4 weeks.

Pickles are fermented by brining in 5 to 8% NaCl solution; the brine is typically acidified with vinegar to a pH of 4.5 to prevent growth of CO_2-producing *Enterobacteriaceae* and *Leuconostoc* species. Spontaneous fermentation is carried out without temperature control at ambient temperature (15 to 30°C). After about 2 to 3 weeks of fermentation, pickles are seasoned, cut as desired, and usually pasteurized after packaging (119). Fermentation of other vegetables after cutting and dry salting or brining is done in a comparable manner, with ambient temperatures ranging from 5°C to 30°C, depending on the region (112, 116).

Green and black olives are preserved by treatment with lye (1.8 to 2.5% NaOH), chemical acidification, and pasteurization, by fermentation with lactic acid bacteria, or by a combination of these processes (118). Fermentation is completed after 30 to 60 days at ambient temperature (119).

Fermentation Microbiotas and Their Impact on Product Quality

Vegetables are generally fermented spontaneously without addition of starter cultures; fermentation microbiotas thus derive from the microbiota of the raw material, and the evolution depends on the substrate availability, the NaCl concentration, the pH, and the presence of plant-derived antimicrobial compounds. Cabbage and carrots contain about 2 to 4% glucose and fructose (31, 119). Vegetables harbor 10^5 to 10^7 CFU of bacteria per g; aerobic bacteria including *Pseudomonas* and *Enterobacteriaceae* dominate. Lactic acid bacteria are present, with a cell count of 10^2 to 10^4 CFU/g; *Lactococcus*, *Leuconostoc*, *Weissella*, *Enterococcus*, and lactobacilli are generally present (116).

On the basis of the consistent microbiota of the raw material, vegetable fermentations are characterized by a consistent succession of fermentation microbiotas (the usual suspects) (Fig. 33.13). Fermentation microbiotas are stratified by oxygen requirement and acid tolerance; growth of plant-associated *Enterobacteriaceae* is followed by that of enterococci, lactococci, *Leuconostoc*, and *Weissella* spp. until, at later stages of the fermentation after the pH drops to 4.5, *L. plantarum* and *L. brevis* dominate and acidify to a pH of ~3.5 (Fig. 33.13) (31, 120). The succession of fermentation microbiota is not specific to the vegetable (31, 119, 120) and closely resembles the succession of microbiota in spontaneous cereal fermentations (Table 33.3). The addition of salt favors growth of lactic acid bacteria over *Enterobacteriaceae* (121). Gas production is undesirable in production of pickles or olives, because CO_2 may disrupt the plant tissue; in these fermentations, acidification with vinegar or lactic acid shortcuts the deployment of the usual suspects by inhibition of *Enterobacteriaceae* and, if the pH is 4.5 or less, of the heterofermentative organisms *Leuconostoc* and *Weissella* (119). The ambient temperature also impacts fermentation microbiota; the psychrotrophic organisms *L. sakei* and *Lactobacillus curvatus* complement the usual suspects in low-temperature batches, while pediococci may occur in high-temperature (30°C) fermentations. Fermentation microbiotas of olives are additionally impacted by the high content (1 g/kg) of phenolic compounds; free phenolic acids exert antimicrobial activity at this

concentration (122). The use of starter cultures for fermentation of vegetables has been described; however, starter cultures do not have a significant influence on the evolution of fermentation microbiotas (116, 120). Decarboxylase-negative starter cultures were reported to decrease formation of biogenic amines during sauerkraut fermentation, particularly tyramine, putrescine, and cadaverine (123).

Early spoilage of pickles and olives occurs by gasproducing *Enterobacteriaceae*; late spoilage by yeasts or acetic acid bacteria occurs if the fermentation batch is not maintained under anaerobic (tightly sealed) conditions. Lactate-consuming bacteria, including clostridia and *Lactobacillus buchneri*, also contribute to spoilage (Fig. 33.13) (36, 117).

Acid production represents the main contribution of fermentation microbiota to product quality; formation of exopolysaccharides and excessive production of acetate are considered spoilage events (116, 117). Product flavor is determined by properties of the raw material and the addition of spices and therefore not dependent on flavor production by fermentation microbes.

Olives are unpalatable unless the main bitter compound, oleuropein, is removed. Oleuropein is a β-glucosylated phenolic compound that can be removed by lye treatment or by fermentation. During fermentation, the β-glucosidase activity of *L. plantarum* hydrolyzes oleuropein to its aglycone, which does not impart a bitter taste (118, 124).

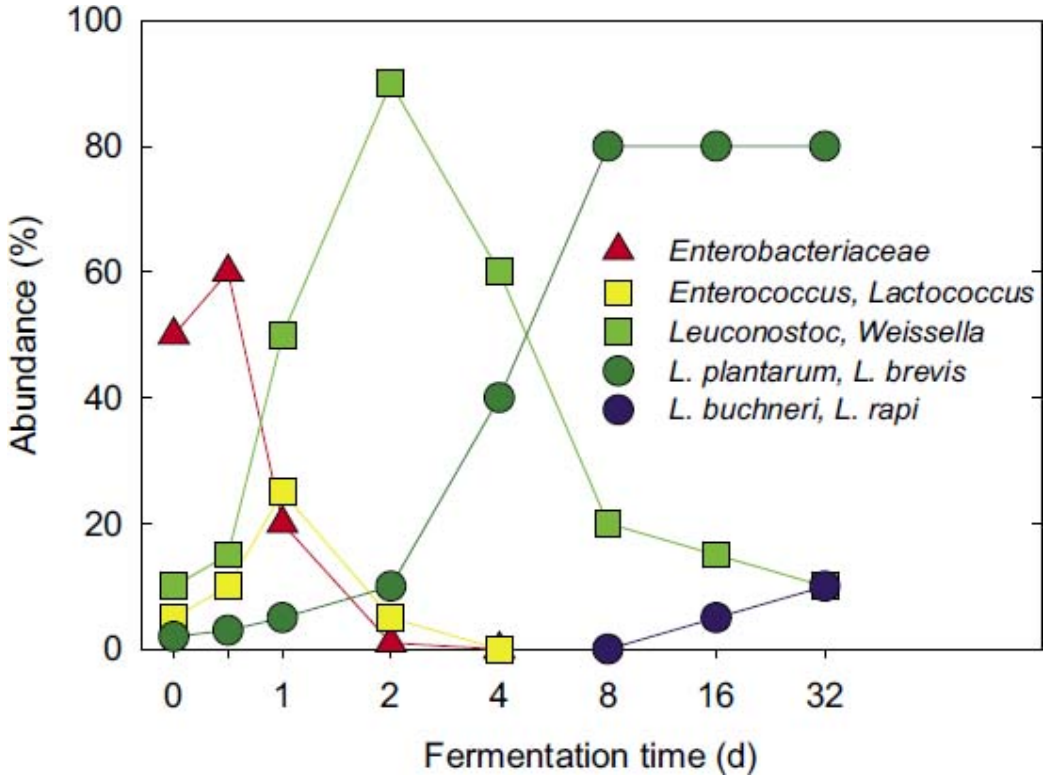


Figure 33.13. Schematic representation of the typical succession of fermentation microbiota in spontaneous plant fermentations, including most cereal fermentations, sauerkraut or kimchi, and olives (the usual suspects). Plant-associated *Enterobacteriaceae* include *Enterobacter*, *Klebsiella*, *Cronobacter*, and *Pantoea*; among lactococci and enterococci, *E. faecium*, *Enterococcus faecalis*, and *Lc. lactis* are most frequently observed. Later fermentation stages include growth of *Weissella cibaria*, *Weissella confusa*, *Weissella kimchi*, and *Lu. mesenteroides*. *L. buchneri* and *Leuconostoc rapi* are lactate-utilizing spoilage organisms. Drawn with information from references 31, 116, and 120.

FERMENTED (CULTURED) DAIRY PRODUCTS

Products and Production Processes

Microbiotas of raw milk acidify the substrate if it is stored at ambient temperature. Acidification coagulates casein to form a weak gel or a highly viscous liquid. Raw milk contains a highly diverse microbiota originating from the animals and their housing, bedding, feed, and water (125). Lactococci, lactobacilli, streptococci, and enterococci are generally

present but are not necessarily major components of the raw milk microbiota; one or more of these organisms become dominant after spontaneous fermentation and inhibit the growth of pathogens or spoilage microbes. Control of fermentation by backslopping supports development of reproducible mesophilic or thermophilic fermentation microbiotas and consistent product quality.

Yogurt originated in the Fertile Crescent and is currently produced from pasteurized milk by fermentation at 40 to 45°C. Yogurt is produced with identical cultures globally, but product properties, particularly the acidity, flavor, and gel structure, can vary widely. Aseptic packaging prior to fermentation in retail containers leads to set-style yogurt with a firm texture; fermentation in bulk prior to aseptic packaging in retail containers for production of stirred yogurt alters the gel structure of the product (126). The fermentation organisms *Sc. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* differ in their acid tolerance and growth temperature; *Sc. thermophilus* grows optimally at 36 to 42°C, while *L. delbrueckii* subsp. *bulgaricus* grows optimally at 44 to 45°C (127). Adjusting the ratio of cocci to rods by appropriate composition of the starter culture, by the use of attenuated, less acid-tolerant strains of *L. delbrueckii*, or by the choice of fermentation time and temperature results in products with low acidity and mild flavor (streptococci dominate) or in more acidic and flavorful “Balkan style” yogurts (lactobacilli dominate). Increasing the solid content by the addition of milk solids prevents syneresis; alternatively, hydrocolloids can be used to prevent expulsion of whey during refrigerated storage (126). Yogurt is often flavored or filled with a fruit preparation as a flavoring agent.

Sour cream, cultured butter, and buttermilk are produced from cream after adjustment of the fat content to 30 to 40% and fermentation with mesophilic starter cultures. Churning of the cultured cream separates the butter, with a fat content of about 80%, from the buttermilk, which contains the aqueous phase and the phospholipids of the milk fat globule membrane. Phospholipids of the milk fat globule membrane and the low pH make traditional buttermilk from cultured sour cream highly prone to oxidation (128, 129). Commercially available buttermilk with a higher oxidative stability is often produced by fermentation of sweet cream buttermilk or skim milk with mesophilic starter cultures.

Kefir is fermented with a mixture of thermophilic and mesophilic lactic acid bacteria, yeasts, and acetic acid bacteria to obtain a sour, carbonated beverage with an alcohol content of < 0.5 to 2% (130). Fermentation microorganisms are not homogenously dispersed in the product but are embedded in the kefir grains, granular structures that resemble cauliflowers and are composed of insoluble exopolysaccharides and microbial biomass (131, 132). Koumiss (or "airag" in Mongolian) is produced from mare's milk in Central Asia. Fermentation with lactic acid bacteria and yeasts yields a beverage with less than 1% ethanol (sweet koumiss) or up to 3% ethanol (strong koumiss) (133).

Fermentation with mesophilic or thermophilic cultures results in soured and set milk products; examples include viili (Finland), leben (or leban or lben; Middle East and North Africa), and diverse products from the milk of cows, goats, sheep, camels, or buffalo; an overview of African fermented milk products has been provided by Jans et al. (134).

Fermentation Microbiotas and Their Role in Product Quality

Yogurt is fermented by *Sc. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* (Table 33.6); the use of both organisms is mandated by the Codex Alimentarius for products called "yogurt." The two fermentation organisms form a symbiotic relationship and grow faster in coculture than in single culture. Initially, *Sc. thermophilus* grows, and the reduction of the pH, the consumption of oxygen, and the formation of CO_2 and formate by pyruvate-formate lyase improve conditions for growth of *L. delbrueckii*. Conversely, the extracellular protease activity of *L. delbrueckii* provides amino acids that stimulate growth of the generally protease-deficient *Sc. thermophilus* (135, 136). Flavor formation is also enhanced by coculture of the two organisms (137). Major flavor volatiles in yogurt include acetaldehyde, diacetyl, acetoin, and acetic acid. Acetaldehyde and acetate are predominantly produced from glucose via pyruvate and acetyl-CoA; threonine aldolase additionally contributes to acetaldehyde production (138). Diacetyl and acetoin production is dependent on the use of citrate as alternative source of pyruvate (Fig. 33.14) (139). In homofermentative lactic acid bacteria, pyruvate formation from glucose necessitates further metabolism by lactate dehydrogenase or pyruvate formate lyase to

regenerate reduced cofactors (Fig. 33.9) (36, 139). Pyruvate formed from citrate is not burdened with reduced cofactors and can thus be partially converted to α-acetolactate; this conversion is also observed during growth of lactococci under aerobic conditions. Cultured butter, sour cream, and traditional buttermilk are fermented with mesophilic DL cultures (see below), including citrate-utilizing *Lc. lactis* and *Leuconostoc mesenteroides* (*Lu. mesenteroides*) (Table 33.6). Diacetyl produced from citrate is the character impact compound of the cultured-butter flavor.

Table 33.6. Examples of cultured dairy products and the corresponding fermentation microbiota

Product (origin)	Major fermentation organisms	Metabolites relevant for product quality
Yogurt (Bulgaria, global)	*Sc. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*	Diacetyl, acetaldehyde, exopolysaccharides
	L. acidophilus, *Bifidobacterium* spp.	Added as probiotic cultures without contribution to sensory quality of yogurt
Viili (Finland)	*Lc. lactis*, *Lu. Mesenteroides*	Diacetyl, exopolysaccharides
Kefir (north Caucasus, global)	*Lactobacillus kefiri*, *Lactobacillus parakefiri*, *L. paracasei*, *Lu. mesenteroides*, *Lc. lactis*, *Bifidobacterium breve*, *Acetobacter orientalis*, *Candida kefyr*, *K. marxianus*, *S. cerevisiae*	Exopolysaccharides, acetic acid, CO_2, ethanol
Cultured butter and buttermilk (global)	*Lc. lactis* subsp. *lactis* biovar diacetylactis, *Lu. Mesenteroides*	Diacetyl, contribution to lipid peroxidation in sour cream and buttermilk
Koumiss (Central Asia, Mongolia)	3–7 species of lactic acid bacteria, including *L. helveticus*, *L. casei*, *L. plantarum*, and *Lc. lactis*; *S. unisporus* and *K. marxianus*	CO_2, ethanol
Suusac (Sudan)	*E. faecium*, *L. helveticus*, *Sc. infantarius*, *W. confusa*, *Candida famata*	

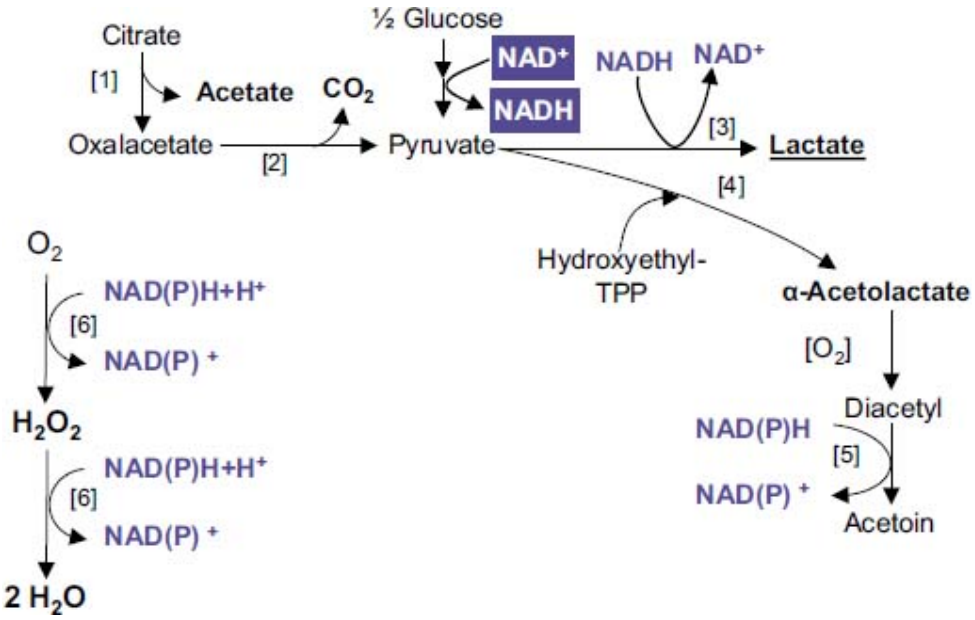


Figure 33.14. Production of diacetyl by lactic starter cultures. Enzymes are indicated by numbers as follows: 1, citrate lyase; 2, oxaloacetate decarboxylase; 3, lactate dehydrogenase; 4, acetolactate synthase; 5, diacetyl/acetoin reductase; 6, NADH oxidase or NADH peroxidase. The formation of reduced and oxidized cofactors (in white on a blue background and in blue, respectively) is indicated if it occurs upstream or downstream of relevant metabolic branching points. Drawn with information from references 36 and 139.

Acid coagulation of milk casein generates only weak gels that are prone to syneresis during refrigerated storage; in yogurt and other cultured dairy products, exopolysaccharide production by the fermentation microbiota is a key determinant of the texture and rheology of the products (140). Lactic acid bacteria produce exopolysaccharides (EPS) through extracellular dextransucrases or fructansucrases or by intracellular glycosyltransferases (141). Extracellular EPS-producing enzymes generate large quantities (1 to 20 g/liter) of EPS but require sucrose as a substrate and are thus relevant in cereal fermentations (108) but not in dairy products unless sucrose is added. Synthesis by intracellular glycosyltransferases generates relatively low concentrations of EPS (less than 1 g/liter). These EPS are typically heteropolysaccharides that are composed of repeating units with one to eight sugar moieties.

EPS from *Sc. thermophilus* and *Lc. lactis* are typically composed of glucose, galactose, and rhamnose as major constituents; ribose, fucose, and *N*-acetylglucosamine are also constituents of some EPS (for reviews, see references 141–143). The constituent monosaccharides are synthesized as nucleotide-activated precursors; the repeating unit of the EPS is assembled intracellularly through successive activity of dedicated glycosyl transferases (Fig. 33.15). During assembly, the repeating unit is linked to the membrane-bound undecaprenyl phosphate, the lipid carrier that also mediates synthesis and export of the peptidoglycan (Fig. 33.15). Export and subsequent polymerization yield polysaccharides with a relative molecular weight ranging from 4.0×10^4 to 6.0×10^6. Strains of *Sc. thermophilus* are typically more copious EPS producers than *L. delbrueckii* strains; the size, charge, and linkage type of EPS produced by *Sc. thermophilus* strongly impact yogurt texture (140). Ropy EPS produced by *Lc. lactis* also account for the viscous texture of viili, a cultured milk produced with *Lc. lactis* and *Lu. mesenteroides* (Table 33.6).

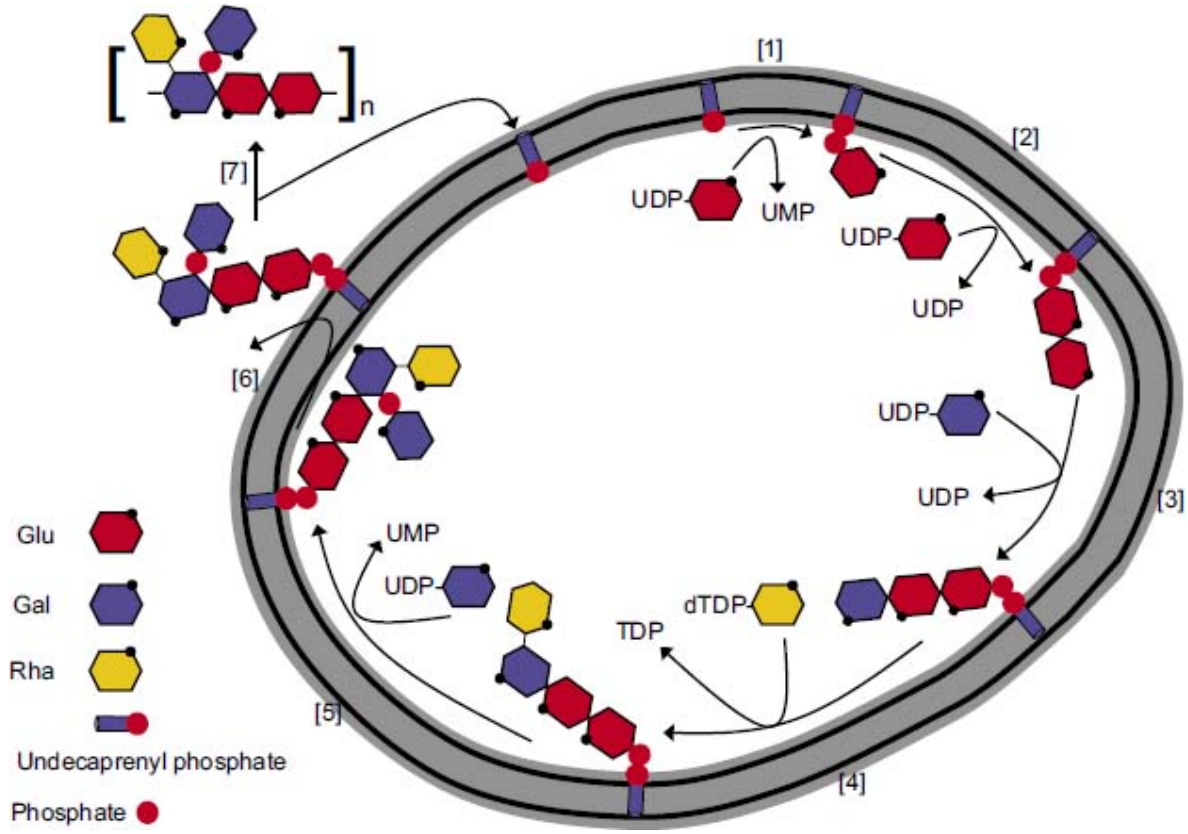


Figure 33.15. Synthesis of exopolysaccharides by intracellular glycosyltransferases. Shown is the example of exopolysaccharide synthesis in *Lc. lactis* B40. (1) The priming glycyltransferase links glucose to the membrane-bound lipid carrier undecaprenyl phosphate using UDPglucose as the substrate. (2 to 5) Successive activity of the glycosyltransferases EpsE, EpsF, EpsG, EpsH, EpsH, and EpsJ assembles the repeating unit of the EPS, with UDP-glucose, UDP-galactose, and dTDP-rhamnose as substrates. (6) EpsK translocates the repeating unit, which is then (7) polymerized by EpsI with EpsA and EpsB, determining the chain length. The lipid carrier is retranslocated and dephosphorylated. Drawn using data from reference 142.

Kefir grains consist of microbial cells embedded in an extracellular polysaccharide matrix. The matrix consists of kefiran, an EPS produced by *Lactobacillus kefiranofaciens* (131, 132). Homofermentative and heterofermentative lactobacilli are the dominant organisms in kefir grains and coexist with *Lc. lactis* and *Lu. mesenteroides* as well as acetic acid bacteria and yeasts. A core microbiome of kefir has not been established, but *L. kefiranofaciens* or related kefiran-producing

lactobacilli appear to be invariably present. Yeasts in kefir include the lactose-utilizing *Kluyveromyces marxianus* but also lactose-negative *Saccharomyces unisporus* and *S. cerevisiae*, indicating metabolic cooperativity between yeasts and lactose-utilizing lactic acid bacteria. The composition of the microbiota of kefir grains differs from that of the microbiota of the bulk liquid (132), demonstrating that some organisms are preferentially associated with the EPS matrix.

The fermentation microbiota of yogurt, sour cream, and cultured butter is controlled by back-slopping or the use of starter cultures and is uniform globally. Fermented milk products in Africa exhibit a much larger diversity of fermentation organisms, which also include *Streptococcus infantarius*, *Enterococcus* spp., pediococci, and *Weissella* spp. (134). It is unclear, however, whether this diversity reflects the use of milk from species other than cows, different production practices, or the use of spontaneous fermentation.

CHEESE

Production Processes

Cheese is produced by precipitation of casein from milk; 90% of the lactose, whey proteins (20% of protein in cow's milk), and 10% of the fat are removed with the whey. Cheese making converts perishable milk to a stable and tradable commodity; moreover, it removes most of the lactose from milk. Cheese has been produced in the Mediterranean for over 7,200 years (144) and thus predates the spread of lactase persistence in Europeans (145). Most cheese is made from the milk of cows, but milk from goats, sheep, and water buffaloes is also used in cheese production (Fig. 33.1 and 33.16). Cheese production involves four basic processing steps: coagulation of casein by acidification and/or the use of rennet, cutting of the curd to drain the whey, salting and shaping of the cheese, and ripening (Fig. 33.16). Ultrafiltration is additionally employed to concentrate cheese milk prior to coagulation. Process variables and the use of diverse fermentation microbiotas result in a large variety of cheeses. Comprehensive descriptions of processes and fermentation microbiotas that are used in cheese making have been provided by Fox et al. (146) and McSweeney (147).

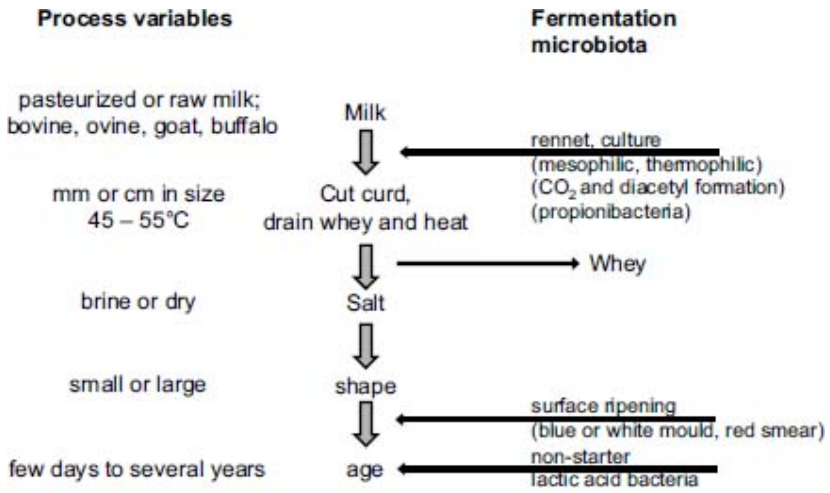


Figure 33.16. Overview of basic processing steps during cheese production. Variables related to processing steps are on the left; variables related to fermentation microbiota are on the right.

The different compositions of bovine, ovine, and caprine milk also relate to different cheese compositions; ovine milk has a higher fat and protein content than bovine milk (146). Milk pasteurization eliminates vegetative cells of pathogens that are present in the milk, particularly *Salmonella*, enterohemorrhagic *Escherichia coli*, and *Staphylococcus aureus*. Pasteurization also eliminates nonpathogenic milk microorganisms that may impact cheese quality as nonstarter adjunct cultures and inactivates enzymes present in raw milk. In addition to proteases and lipases, bovine milk harbors γ-glutamyltransferase activity, which produces taste-active peptides during ripening (148). As an alternative to pasteurization, milk destined for the production of long-ripened cheeses is treated at 60°C for 30 min to reduce contaminants.

Coagulation of casein, which constitutes 80% of the protein in bovine milk, is achieved by acidification, treatment with rennet, or a combination of these. Casein micelles in milk are aggregates of several thousand molecules of α_{s1}-, α_{s2}-, β-, and κ-caseins with an average size of 150 to 200 nm (149). Casein micelles are stabilized by colloidal calcium phosphate nanoclusters and κ-casein, which has a glycosylated and hydrophilic C terminus. κ-Casein is found almost exclusively at the surface of the micelle, and the glycomacropeptide “hairs” on the surface mediate steric stability of the micelles (149). Acidification to the isoelectric point of casein, pH 4.6, solubilizes the calcium phosphate

nanoclusters and collapses the κ-casein layer on the surface, allowing formation of a weak gel. Heating of milk to more than 70°C denatures whey proteins, which associate with κ-casein and destabilize the casein micelles.

Rennet is produced from calf stomach and contains chymosin. Chymosin is an acid protease that specifically cleaves κ-casein between Met_{105} and Phe_{106} and thus removes the glycomacropeptide. Cleavage of κ-casein during cheese making occurs optimally at pH 6 to 6.5 and 45 to 48°C (146). Removal of the glycomacropeptide destabilizes the casein micelles and supports gel formation. Rennet from calf stomach can be replaced by recombinant chymosin, or by microbial and plant proteases. Proteases other than rennet, however, also cleave other milk proteins and may reduce the cheese yield and favor formation of bitter peptides during ripening.

After the cheese curd is set by acid- or rennet-induced gelation, whey removal is initiated by cutting of the curd to cubes with a volume of about 1 ml. Cutting to a smaller size facilitates whey removal for production of hard cheeses. Cooking of the curd to temperatures ranging from 30 to 55°C further improves whey removal; high temperature and low pH are complementary with regard to their effect on draining of the whey. Light pressing of the cheese curd can be applied to further expel whey. Heating of the cheese curd to >65°C plasticizes it; it can be stretched and molded into its shape (pasta filata).

Cheese is salted to provide taste, to enhance syneresis, and to contribute to preservation. Salt is applied directly to the curd, e.g., in the case of Cheddar, by dry salting of the surface after shaping or by placing the cheese in brine. The salt concentration in the water phase of cheese ranges from 1% for fresh, acid-coagulated cheeses to more than 9% for long-ripened cheeses, such as Parmigiano Reggiano.

Aging of cheeses is one of the most variable production steps. Acid-coagulated cheeses are generally not ripened; the ripening time for rennet-coagulated cheeses ranges from a few weeks to a few years (146) (Fig. 33.1). Aging of cheese achieves dehydration, degradation of proteins and lipids to taste-active peptides, amino acids, and fatty acids, and their further conversion to flavor volatiles. Microbial contaminants that may be present in raw milk and grow during initial stages of cheese

production are inactivated during ripening; cell counts of *Salmonella*, *E. coli*, and *St. aureus* are decreased by more than 5 log CFU/g if the ripening time exceeds 60 days (146). During aging of internally bacterially ripened cheese, the primary acidification cultures are replaced or complemented by nonstarter lactic acid bacteria and, in some cheese varieties, propionibacteria. Ripening with surface microbiota accelerates proteolysis and lipolysis; however, the respiratory metabolism of surface microbiota increases the pH and increases hygiene risks.

Products

Cheese is produced traditionally in Central Asia, the Mediterranean, and European countries. European migration to the Americas and Oceania introduced cheese making to those areas. Although cheese is not traditionally produced in Southeast Asia, industrial development and the associated changes in lifestyle have resulted in increased cheese consumption. A few types of cheese, particularly Cheddar and mozzarella, account for a majority of the global cheese production, but the number of cheese varieties is virtually unlimited. The former French president Charles de Gaulle asked in frustration, “How can you govern a country that has two hundred and forty-six varieties of cheese?” As the number of cheese varieties in other countries with a long tradition of cheese making is comparable, hundreds of different cheese varieties have been recognized (150), and Fig. 33.1 provides only a very selective overview. Numerous cheeses are protected by a “protected designation of origin” scheme and are produced only in specific geographic regions (147). Cheeses can be classified based on the method of coagulation (rennet, acid, or acid and heat) and subdivided on the basis of technological parameters and ripening microbiota (146, 147). Acid-coagulated cheeses account for about 25% of cheese production and are typically produced without ripening. Examples include Greek yogurt, quark, cottage cheese, and cream cheese (Fig. 33.1). Ricotta and paneer are examples of fresh cheeses that are produced in Italy and South Asia, respectively, from skim milk or whey by heat and acid coagulation of proteins. Owing to the high pH, these products are highly susceptible to spoilage. Some acid-coagulated cheeses, e.g., queso blanco, are acidified by addition of acetic or citric acid rather than microbial acidification.

Mysost, geitost, and related products in northern Europe are produced by concentration of cheese whey to a solid content of more than 80%, followed by cooling, kneading, and packaging. The taste of these whey products is determined by sweetness imparted by the high lactose content and Maillard products that are formed during whey concentration (146).

Rennet-coagulated cheeses account for a majority of the global cheese production; these products are ripened for weeks or up to several years. Most cheeses, including Cheddar and Gouda, are ripened internally by bacterial activity; in these products, the development of a surface microbiota is inhibited by regular washing or dehydration of the rind. Rennet-coagulated cheeses contain small irregular holes if the cheese curd is imperfectly packed, e.g., in the case of Tilsiter. The use of citrate-utilizing or heterofermentative starter cultures leads to formation of small regular holes in the cheese matrix, e.g., in Gouda and Edam cheeses. Gas formation by propionibacteria in Emmental cheese and, to a lesser extent, in Jarlsberg and Leerdammer leads to formation of large, regular holes.

The development of surface microbiota fundamentally changes the color, texture, and flavor of cheeses. Red smear cheeses are ripened by a surface biofilm consisting of yeasts and bacteria; these produce orange and red pigments and characteristic flavor volatiles. Flavor production is more pronounced in soft cheeses, e.g., Oka (Quebec, Canada), Tilsiter (Tilsit, East Prussia; now produced in Poland, Germany, and Switzerland), Limburger (Limburg, Belgium), and Münster (Alsace, France), than in hard cheeses (e.g., Gruyère and Tête de Moine, Switzerland) and increases during ripening. Camembert and Brie, which originate from Normandy in France, are ripened with surface mycobiota that form white mycelia on the surface of the cheese and soften the cheese curd through production of extracellular proteases and lipases. Roquefort is the archetypical blue cheese; it is a raw-milk cheese produced from ovine milk in Roquefort-sur-Soulzon in France. Roquefort is ripened in caves surrounding Roquefort-sur-Soulzon which are also inhabited by the blue mold *P. roqueforti*. Poking of holes in the cheese curd transfers fungal spores as well as oxygen into the interior. Other traditional blue cheeses include Gorgonzola (Italy) and Stilton (England). Contemporary cheese varieties combine ripening with white

surface mycobiota and internal ripening with *P. roqueforti*, e.g., Cambozola and Danablu.

Fermentation Microbiotas

Table 33.7. provides an overview of cheese microbiotas. Acidification of cheese milk to achieve coagulation of casein or to support rennet-mediated coagulation is mediated by addition of mesophilic or thermophilic starter cultures. Mesophilic cultures grow optimally at around 30°C but can tolerate heating of the cheese curd to a temperature of up to 45°C; thermophilic cultures grow optimally at 37 to 45°C and resist higher temperatures used to treat the cheese curd to expulse whey.

Mesophilic cultures consist primarily of the homofermentative organisms *Lc. lactis* subsp. *cremoris* and/or *Lc. lactis* subsp. *lactis*; cultures containing only these organisms are termed “O cultures.” *Lc. lactis* subsp. *cremoris* is often associated with *Lc. lactis* subsp. *cremoris* biovar diacetylactis (D cultures), which converts citrate to diacetyl and CO_2 (Fig. 33.14), and with the heterofermentative organisms *Lu. mesenteroides* (L cultures) (151). Citrate utilization, the defining criterion for *Lc. lactis* subsp. *cremoris* biovar diacetylactis, is plasmid encoded. *Lc. lactis* subsp. *lactis* is differentiated from *Lc. lactis* subsp. *cremoris* by the presence of the arginine deiminase pathway and glutamate decarboxylase (151). Undefined multistrain starter cultures are maintained by backslopping at the farm level or in dairies; these cultures contain multiple strains with complementary phage resistance patterns and exhibit a remarkable functional stability over years of back-slopping (151). Phage predation on individual strains temporarily reduces individual strains in the mixture without compromising starter functionality. Milk contains lactose as the sole fermentable carbohydrate and is a substrate with a low concentration of peptides and amino acids; microbial growth thus requires extracellular proteinase activity (152). Lactococci and *Leuconostoc* spp. are plant-associated organisms and populate raw-milk microbiota through contamination from feed or straw; in *Lc. lactis*, lactose utilization and proteolytic activity are plasmid-encoded traits. Some but not all strains in the starter mixtures express extracellular proteases to satisfy the demand of other strains for peptides and amino acids (151).

Thermophilic starter cultures consist of *Sc. thermophilus* in combination with *L. helveticus*, *L. delbrueckii* subsp. *lactis*, or *L. delbrueckii* subsp. *bulgaricus*. *L. helveticus* is a commensal member of chicken intestinal microbiota (21, 153); *L. delbrueckii* occurs in the lactose-rich intestines of suckling mammals (26). *Sc. thermophilus* is closely related to oral streptococci but readily distinguished from pathogenic members of the genus (154). Thermophilic starter cultures hydrolyze lactose through intracellular β-galactosidase and preferentially metabolize the glucose moiety and excrete galactose (35, 154). *L. delbrueckii* and *L. helveticus* generally express extracellular proteases, while most strains of *Sc. thermophilus* rely on other components of the starter mixture to supply amino acids and peptides (154).

Undefined, mixed-strain starter cultures have been available since the end of the 19th century; single-strain defined starter cultures were developed in New Zealand in the 1930s (146). Currently, defined strains that are directly added to the cheese vat (referred to as “direct vat set” or “direct vat inoculation”) are routinely used in industrial operations. The use of single strains has the advantage of defined and reproducible metabolic properties; however, single strains rapidly succumb to phage predation unless appropriate measures are taken (155, 156). Phage control relies on hygienic design and sanitation of cheese making operations to prevent dispersal of phages to the cheese milk and the buildup of phage populations in the cheese plant. Because phages of *Lc. lactis* and *Sc. thermophilus* are highly specific for their target strains, culture rotation prevents culture failure due to phage attack. After several consecutive days of using strain (or strain mixture) A, phages that prey on these strains are likely to accumulate; timely switching to strain (or strain mixture) B with a different phage sensitivity prevents starter failure. Defined strains are additionally selected for phage resistance, making use of the mechanisms of phage resistance in lactic starter cultures (Fig. 33.17) (155, 156). Restriction-modification systems and phage resistance by abortive infection are often plasmid encoded and transferable; phage resistance mediated by clustered regularly interspaced short palindromic repeats (CRISPR)-Cas systems is considered the bacterial “immune system” against phage attack and is trained by successive exposure to diverse phages (Fig. 33.17) (155, 156).

CRISPR spacers that direct the Cas endonuclease to hydrolyze incoming phage DNA are added after a phage attack and provide resistance to subsequent attacks by the same phage (156).

Table 33.7. Microbiotas of cheese fermentations[a]

Process	Organism	Characteristics	Products (examples)
Primary acidification Mesophilic	*Lc. lactis* subsp. *Lactis*	Citrate utilizing (biovar diacetylactis D cultures) or not (all other biovars, O cultures)	Gouda, Cheddar, Camembert
	Lu. mesenteroides	Heterofermentative and citrate utilizing (L cultures)	
Thermophilic	*Sc. thermophilus*, *L. delbrueckii*, *L. helveticus*	Homofermentative and thermophilic	Parmesan, Emmental, mozzarella
Secondary/adjunct cultures Propionibacteria	*Pr. freudenreichii*, *Pr. acidipropionici*	Formation of CO_2, acetate, and propionic acid	Emmental (Swiss cheese), other cheeses with eyes
Molds	*P. roqueforti*	Blue mycelium, formation of methylketones	Roquefort, Stilton
	P. camemberti	White mycelium	Camembert and Brie
	G. candidum	White mycelium, formation of *S*-methylthioesters	Camembert, red smear cheese
		All are lactate utilizing, proteolytic, and lipolytic	
Nonstarter lactic	*L. plantarum*, *L.*	Proteolysis and	Long-

acid bacteria	*paracasei*, *L. rhamnosus*, *Enterococcus* spp., others	peptide hydrolysis, conversion of glutamine to glutamate; glutamate dehydrogenase, formation of diacetyl, strain-specific amino acid decarboxylation	ripened cheeses, including aged Gouda, Cheddar, Parmigiano Reggiano, Pecorino
Rind biofilms	*Br. linens*, *Staphylococcus* spp., *Corynebacterium* spp.	Proteolysis, lipolysis, formation of red pigmented biofilm	Limburger, Münster, Oka, Tête du Moine
Spoilage microbiota Early blowing	*Enterobacteriaceae*	Gas production and formation of small, irregularly shaped holes early during cheese ripening	
Late blowing	*Clostridium butyricum*	Gas formation and rupture of the cheese curd late in cheese ripening	Swiss-type cheese
	L. wasatchensis	Gas formation and rupture of the cheese curd late in cheese ripening	Cheddar

[a] Organisms that are used in starter cultures are underlined.

During cheese ripening, the viable cell counts of the primary mesophilic or thermophilic acidification culture decrease, and other lactic acid bacteria—nonstarter lactic acid bacteria (NSLAB)—grow in the cheese curd to high cell counts (157). Growth of NSLAB occurs more rapidly in cheese made from raw milk than in cheese made from pasteurized milk (146). The composition of NSLAB is more heterogeneous than the

composition of starter cultures; nomadic organisms of the *L. plantarum* and *L. casei* groups are frequently encountered in high cell counts, but *L. curvatus*, heterofermentative lactobacilli, including *L. brevis*, and *Enterococcus faecium* also occur (157). Owing to their important role in cheese quality, NSLAB can be included as adjunct cultures in starter culture preparations.

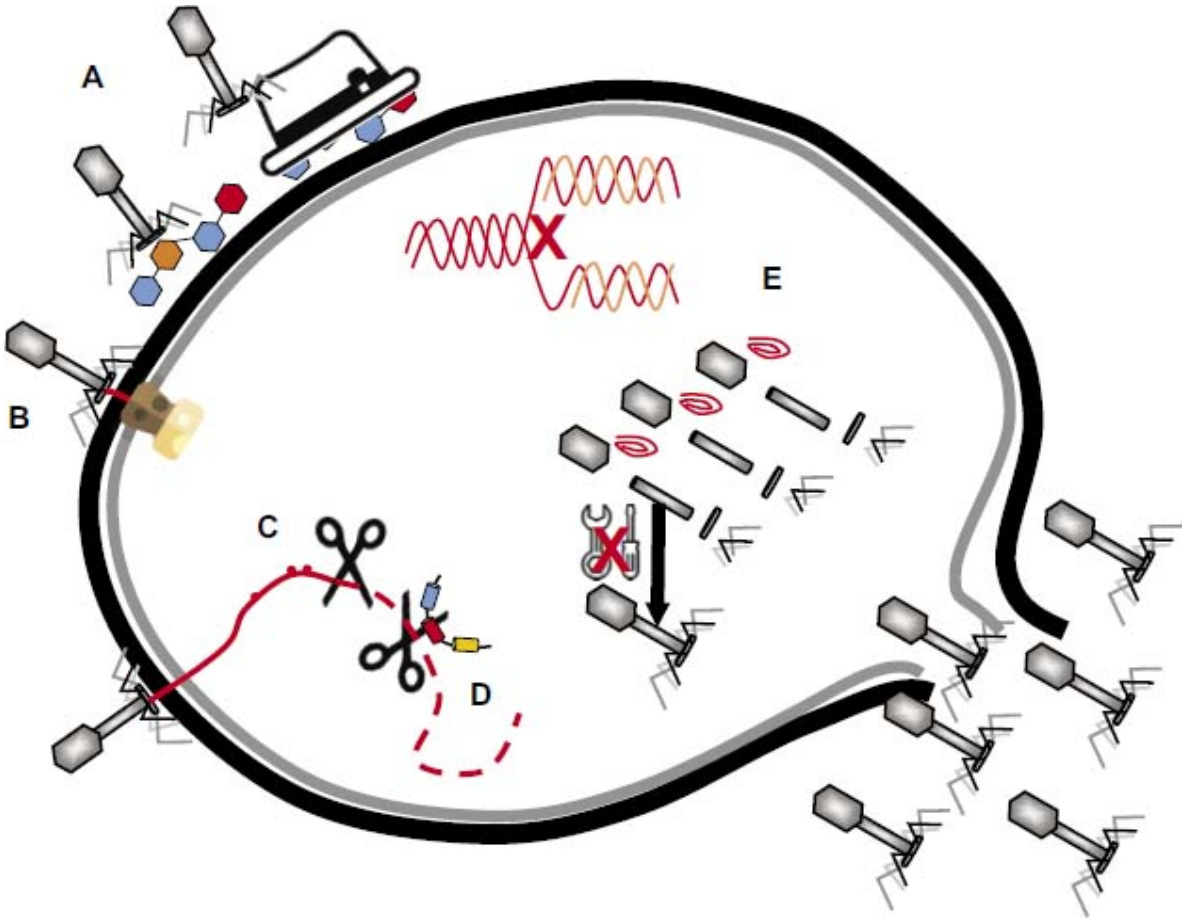


Figure 33.17. Mechanisms of phage resistance in dairy starter cultures. (A) Inhibition of adsorption by masking or modification of the phage receptor, which is usually a surface polysaccharide. (B) Inhibition of transfer of phage DNA into the host cytoplasm by membrane-associated Sie-like proteins (*Lc. lactis* and *Sc. thermophilus*). (C and D) Cutting of phage nucleic acids by restrictionmodification systems, which recognize DNA methylation patterns, or by CRISPR-Cas systems, which recognize proto-spacer sequences of the phage DNA. (E) Inhibition of replication of the phage DNA or of phage assembly by abortive infection (Abi) systems. In *Lc. lactis*, 23 different Abi systems have been described; these are generally plasmid encoded and interfere with DNA replication, RNA transcription, or packaging of the phage DNA. Abi systems also lead to the death of the infected cell. Drawn with information from references 155 and 156 and icons from https://www.flaticon.com.

Gas formation in Swiss-type cheeses, including Emmental, is mediated by *Propionibacterium freudenreichii* (*Pr. freudenreichii*) and/or

Propionibacterium acidipropionici. Propionibacteria are present in low cell counts in raw milk or added as a minor component of the starter culture. These organisms are thermotolerant but not acid or salt resistant; accordingly, heating of the cheese curd to high temperatures and a low acid (pH >5) and salt (NaCl < 1%) content favor their development. *Pr. freudenreichii* lacks protease activity, and its growth is thus dependent on the proteolytic activity of the primary acidification culture (158). During production of Swiss cheese, development of propionibacteria is stimulated by ripening at 20 to 25°C in the first weeks of ripening.

Production of red smear cheeses includes ripening with a red-colored surface biofilm composed of yeasts, including *Debaryomyces* spp. and *Geotrichum candidum*, and bacteria, including staphylococci, members of the *Actinobacteria*, such as *Brevibacterium linens* and *Corynebacterium* spp., and halotolerant members of the *Gammaproteobacteria*, such as *Halomonas*, *Vibrio*, and *Pseudoalteromonas* (159–161). These biofilms develop naturally unless the cheese rind is washed regularly or dried to a low water activity. Red smear cheeses are inoculated by smearing on the surface of the cheeses. Yeasts are the trailblazers of red smear communities, as they consume lactate; the concomitant increase of the pH supports growth of bacterial members of red smear communities (161). Community assembly of red smear microbiotas is not influenced by geography but is dependent on the moisture and, to a lesser extent, the pH of the rind (161). Halophilic Gram-negative bacteria may be introduced into the cheese rind from sea salt (161); many Gram-positive members of biofilm microbiotas, including staphylococci, *Brevibacterium*, and *Corynebacterium*, are commensal or opportunistic pathogenic members of human skin microbiota (162, 163).

Ripening with surface mycobiotas is achieved by inoculation with *P. roqueforti* or *P. camemberti* and incubation at 10 to 15°C and high relative moisture; both fungi may occur in association with *G. candidum*. Spores or the fungi are added directly to the cheese milk or applied to the surface of the cheese.

Growth of *Enterobacteriaceae* in the first 24 to 48 h of ripening of white brined cheese from raw milk leads to gas formation (early blowing defect). Early blowing usually relates to slow acidification by the starter

culture (147). Late blowing is attributed to growth of *Clostridium butyricum* or *Clostridium tyrobutyricum*. Conditions that favor growth of propionibacteria (high temperature, high pH, and low NaCl levels) also favor growth of clostridia; therefore, this defect is most common in Swiss cheese (164). Contamination of cheese milk with clostridial endospores is prevented or reduced by excluding silage from the feed of dairy cows or by centrifugation of cheese milk to remove spores; alternatively, nitrite or lysozyme is added as a preservative to prevent clostridial growth (146, 147). Late blowing in Cheddar cheese is caused by the heterofermentative organism *Lactobacillus wasatchensis*, which uses residual galactose and pentoses to produce CO_2 and acetic acid. Growth of *L. wasatchensis* in cheese is related to contamination of silage and to the use of *Sc. thermophilus* as an adjunct culture in cheese making. Different from *Lc. lactis*, lactose metabolism by *Sc. thermophilus* accumulates galactose, which is later used by *L. wasatchensis* (165).

The low pH and water activity of ripened cheeses inhibit growth of *Listeria monocytogenes* (166); however, respiratory metabolism of surface mycobiota consumes lactate and increases the pH of the cheese surface, providing an opportunity for *L. monocytogenes* to grow if it is present as a contaminant from raw milk or the cheese processing environment (147).

Impact of Fermentation Microbiotas on Product Quality

Unripened cheese curd has a neutral or slightly acidic taste and odor; these are determined by proteolysis and lipolysis during cheese ripening and by microbial formation of odorants (146, 147).

Carbohydrate metabolism early during cheese ripening reduces the lactose concentration to negligible levels; after a few weeks of ripening, cheese is virtually lactose free. Conversion of citrate by DL cultures or by adjunct cultures results in gas formation and production of diacetyl and acetoin, which are major flavor compounds in most long-ripened cheeses. Diacetyl production by adjunct cultures exceeds the production by *Lc. lactis* (167). Acetate accumulates to lower concentrations than lactate; however, because acetate also has a lower taste threshold, its contribution to the sour and salty taste is approximately equivalent to

that of lactate (168). Propionibacteria in Swiss-type cheeses metabolize lactate to propionate, acetate, and CO_2; cometabolism of aspartate and lactate enhances growth and CO_2 production and accumulates succinates (Fig. 33.18) (169). Succinate as well as propionate contributes to the umami taste perception in Swiss-type cheeses.

In contrast to most other food fermentations, cheese milk has a low proteolytic activity. Plasmin is present in milk and cathepsins are present in somatic cells, but milk-derived enzymes are inactivated by pasteurization (147). Accordingly, microbial enzymes play a major role in protein degradation during cheese ripening. The proteolytic system of lactic acid bacteria consists of an extracellular protease; its expression is repressed by high concentrations of peptides in the growth substrate. Peptides are transported by di- or oligopeptide transporters and hydrolyzed by strain-specific intracellular peptidases (Fig. 33.19) (104, 170, 171). Lysis of the primary acidification culture during cheese ripening releases intracellular peptidases and thus increases their activity on casein and casein-derived peptides. Starter lysis and consequently proteolysis are accelerated by a dose of bacteriophages or bacteriocins that lyse the cells without interfering with acidification of the cheese milk. The use of proteolytic surface microbiota also accelerates proteolysis.

Amino acids are the major contributors to the sweet (Met), umami (Glu and Asp), and bitter (Ile) tastes of cheese (109, 168). α-Glutamyl peptides contribute to umami taste; γ-glutamyl dipeptides enhance the activity of sweet, salty, and umami tastants by interaction with the calcium-sensing receptor proteins that mediate signal transduction from the taste receptors to the nerve cells (109, 168, 172). γ-Glutamyl dipeptides are not released by proteolysis but produced by γ-glutamyl-transferases from milk, fungi, or heterofermentative lactobacilli (109, 148). Taken together, taste-active peptides provide a strong umami and salty taste to long-ripened cheeses, such as Parmigiano Reggiano and pecorino. Bitter-tasting peptides accumulate if the peptide accumulation and hydrolysis are unbalanced. The specificity of the protease used for treatment with rennet, the specificity of the extracellular protease of the starter cultures, and the extent of starter lysis during ripening influence the accumulation of bitter peptides (147, 173). The bitter taste defect of cheese is also impacted by the accumulation of umami tastants, which

can mask bitter taste (174). Lipase activity in cheese is derived from surface cultures, i.e., *P. roqueforti* or *P. camemberti*, or from traditional rennet pastes that are used in the manufacture of certain traditional cheese varieties, including Parmigiano Reggiano and Pecorino (147). Free fatty acids do not accumulate to concentrations above their taste threshold (168) but play a role as precursor compounds for flavor volatiles.

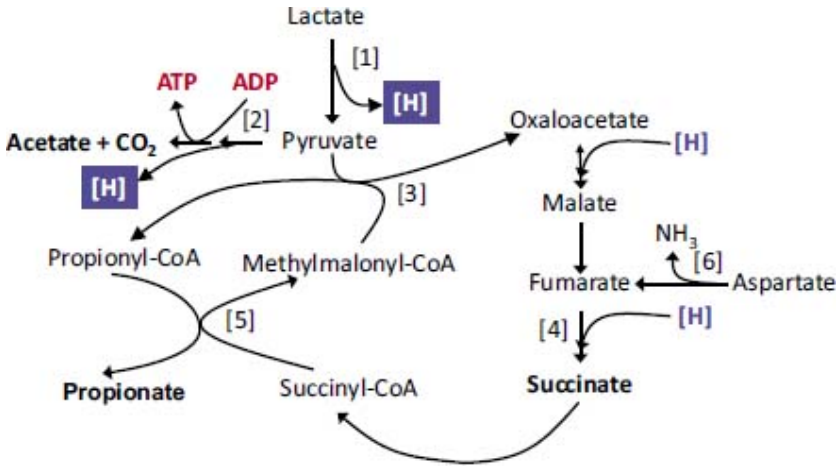


Figure 33.18. Lactate and aspartate metabolism by propionibacteria. The oxidizing branch of the pathway converts lactate to acetate and CO_2 via lactate dehydrogenase (1), pyruvate dehydrogenase (2), and phosphotransacetylase/acetate kinase. This branch generates 2 mol of NADH (indicated as [H]) and ATP. The reducing branch of the pathway (Wood-Werkman cycle) consumes 2 mol of NADH by conversion of pyruvate to propionate via oxaloacetate transcarboxylase (3), fumarate reductase (4), and CoA transferase (5). Fumarate reductase may be linked to an anaerobic electron transfer chain that contributes to ATP synthesis. Cometabolism of aspartate and lactate via aspartate-ammonia lyase (aspartase) (6) provides an alternative route for cofactor regeneration and accumulates succinate as the end product. The formation of reduced and oxidized cofactors (in white on a blue background and in blue, respectively) is indicated if it occurs upstream or downstream of relevant metabolic branching points; synthesis (in red) is shown to indicate the ATP yield of the metabolic pathway. Drawn using data from references 158 and 169.

Glutamate is a key intermediate for the conversion of amino acids to flavor volatiles (Fig. 33.20) (175). Transamination of amino acids with α-

ketoglutarate as the amino acceptor converts hydrophobic amino acids to yield glutamate and the corresponding α-keto-acids. Glutamate is recycled to α-ketoglutarate by glutamate dehydrogenase activity of lactobacilli; α-keto acids are decarboxylated to form organic acids, aldehydes, or alcohols, which contribute to cheese flavor. For example, leucine and isoleucine are converted to 2- or 3-methylbutanal and 2- or 3-methylbutanoic acid, which are major contributors to the flavor of long-ripened cheeses (176–178). In addition, conversion of methionine and cysteine by cystathionine-γ-lyase yields volatile sulfur compounds, including dimethyltrisulfide, with significant contribution to cheese flavor. The strain-specific decarboxylation of amino acids by NSLAB generates biogenic amines, particularly histamine and tyramine (147).

Short-chain (C_4 to C_6) fatty acids that are released from milk fat by lipolysis and their ethyl esters also contribute to cheese flavor. Metabolism of *P. roqueforti* converts fatty acids to methylketones—e.g., 2-heptanone, 2-nonanone, and 2-undecanone—which impart the characteristic flavor of blue cheeses. Surface ripening with red smear microbiota substantially expands the metabolic capacity of fermentation microbiota to convert amino acids to potent odorants, including short-chain fatty acids and *S*-methylthioesters (179, 180). The red to orange color of red smear cheese is imparted by carotenoids produced by *Brevibacterium* spp. (181).

FERMENTED EGGS AND FISH

Products and Production Processes

Fermented fish products are common in coastal regions of East and Southeast Asia and in Scandinavian countries. Fish and eggs have a relatively high pH and a low content of fermentable carbohydrates; additionally, deepsea animals accumulate high concentrations of lowmolecular-weight nitrogen compounds, such as urea and trimethylamine oxide, to protect proteins against cold and pressure denaturation (182). Microbial growth in eggs and fish thus leads to putrefaction and spoilage unless fermentation microbiota are controlled by addition of fermentable carbohydrates or salt or by reduction of the water activity.

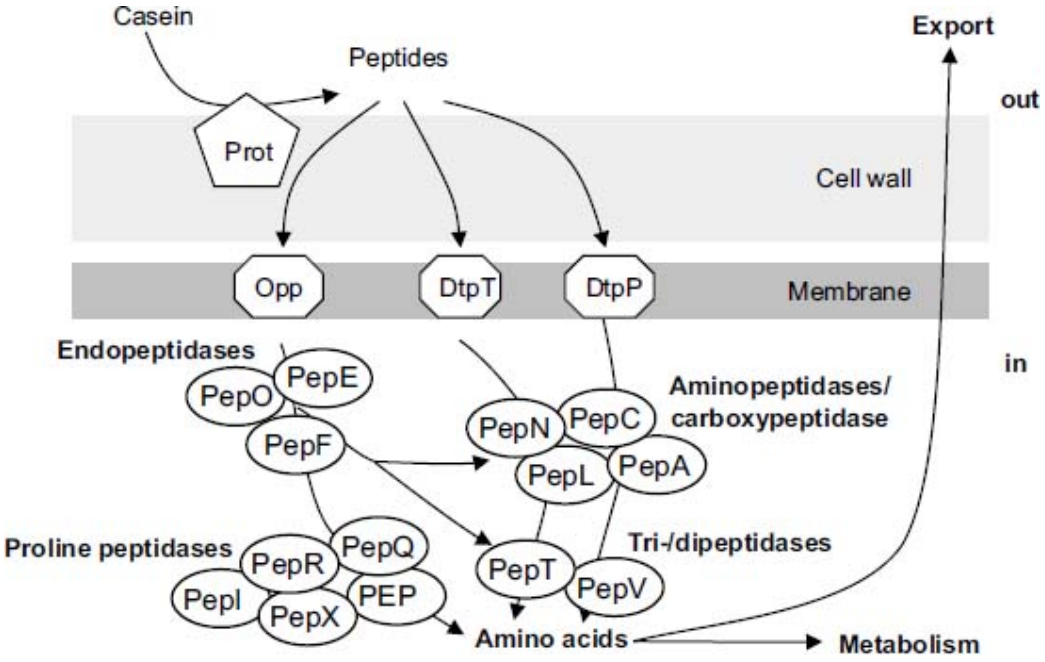


Figure 33.19. Proteolytic system of lactic acid bacteria. Protein degradation is initiated by extracellular and cell wall-bound proteases that are present in some lactic acid bacteria, particularly strains that dominate dairy fermentations, but absent in most species of lactobacilli. Peptides are transported by the dipeptide transporters DtpT and DtpP or the oligopeptide transporter Opp and hydrolyzed by a strain-specific array of intracellular peptidases. Drawn according to information from references 37, 104, and 170.

Fermentation of whole fish or fish pieces is achieved by mixing cleaned and gutted fish, fish pieces, or minced fish with cooked rice or corn and sucrose. Salt is added to a concentration of 3 to 5%; many products additionally include 5 to 8% garlic. Fermentation occurs at ambient temperatures of 20 to 24°C in Japan and China and about 30°C in Thailand, the Philippines, and Indonesia for days or weeks (183–186). Aji-no-susu produced in Japan has a higher salinity, 6 to 7%, and is fermented for several months (187).

In ancient Rome, a sauce called garum was produced from fish, including the intestines and the blood, by addition of salt and fermentation over several months (188). At the time, the price of high-quality garum was equivalent to that of expensive perfume. Its production has gone out of fashion since the fall of the Roman Empire (189), and it is unclear whether contemporary reproductions of garum

match the quality of the antique original (190). Fish sauces in Southeast and East Asia are produced by addition of 15 to more than 20% NaCl to control putrefaction; the production of fish sauce in Japan additionally includes barley koji as a source of carbohydrates and of proteolytic enzymes. Fermentation takes place for several months at ambient temperature; the resulting sauce or paste is used as a condiment (191–193).

Fermented fish products in Iceland, Norway, and Sweden include hákarl, rakfisk, and surströmming, respectively (194). For production of hákarl, washed pieces of shark meat are fermented in gravel pits for 3 to 6 weeks, followed by drying for several weeks or months. Rakfisk is produced from salmonoid freshwater fish, which is gutted, cleaned, brined to a salt concentration of 4 to 6%, and fermented and ripened at 4 to 10°C for several weeks. Surströmming is produced from Baltic herring, which is salted to a concentration of 5 to 10% NaCl and fermented at 15 to 18°C for 3 to 4 weeks before it is transferred to cans without a heating step (194).

Pidan, alkaline-fermented duck eggs produced in China, are produced by coating the eggs with a mud consisting of calcium oxide, water, and sodium carbonate, followed by fermentation for 20 to 30 days at room temperature (195). The alkaline pH (9.0) and the high osmotic pressure lead to gelation of egg white and egg yolk and to formation of FeS, which provides the characteristic green color (195).

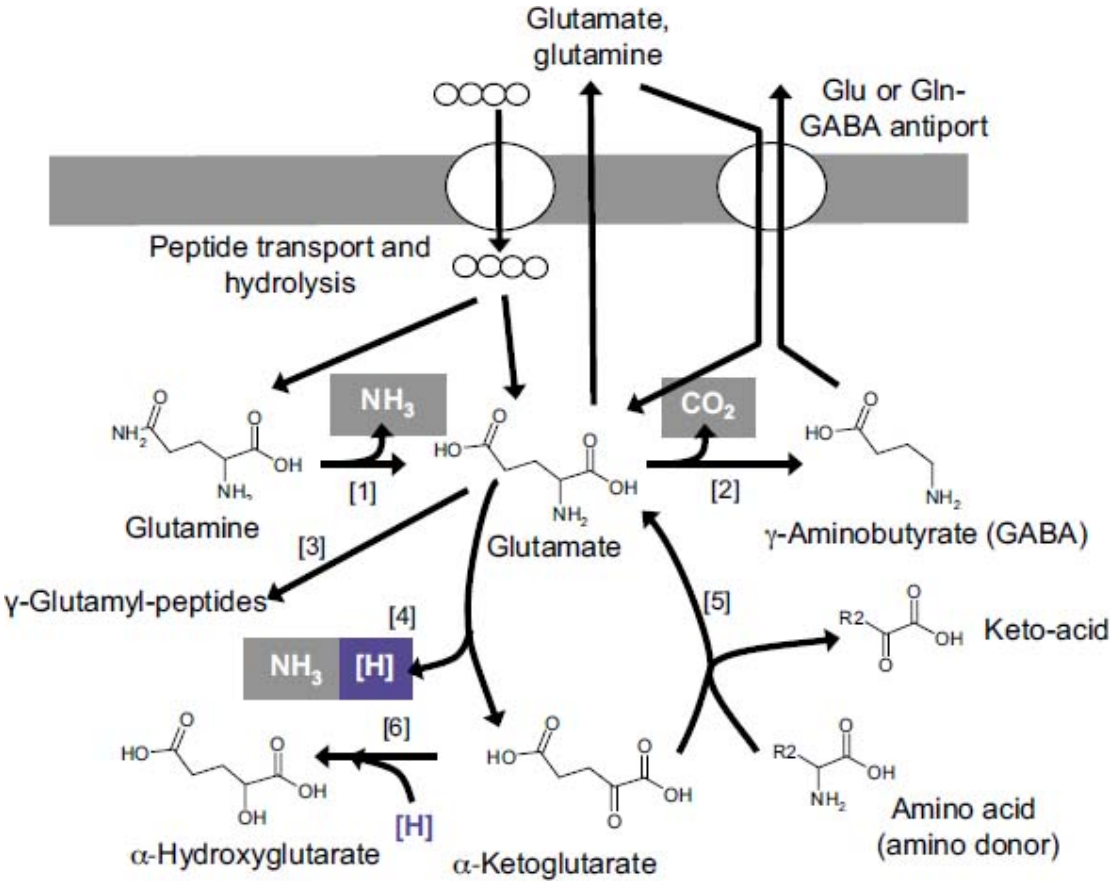

Glutamate, glutamine
Glu or Gln-GABA antiport
Peptide transport and hydrolysis
NH3
CO2
Glutamine
[1]
Glutamate
[2]
γ-Aminobutyrate (GABA)
[3]
γ-Glutamyl-peptides
[4]
NH3 [H]
[5]
Keto-acid
[6]
[H]
α-Hydroxyglutarate
α-Ketoglutarate
Amino acid (amino donor)

Figure 33.20. Glutamine and glutamate metabolism in lactic acid bacteria. "[H]" indicates NAD(P)H-consuming or -generating reactions; individual enzymes are indicated by numbers and discussed below. Amino acids are taken up as peptides and cleaved by an array of intracellular peptidases; glutamate and glutamine are additionally transported by the Gln- or Glu-γ-aminobutyrate (GABA) antiporter GadC. (1) Glutamine is converted to glutamate by glutaminase of glutamine amidotransferases. (2) Strainspecific conversion of glutamate to GABA. (3) Heterofermentative lactobacilli convert glutamate to γ-glutamyl peptides via glutamyl-cysteine ligase. (4) NADH-dependent conversion of glutamate to α-ketoglutarate (αKG) by glutamate dehydrogenase is a strain-specific property of *L. plantarum*, *L. paracasei*, and other LAB. (5) LAB generally exhibit transaminase activity and convert αKG to glutamate in the presence of other amino acids as amino donors. (6) Heterofermentative lactobacilli convert αKG to α-hydroxyglutarate. The formation of reduced and oxidized cofactors (in white on a blue background and in blue, respectively) is indicated if it occurs upstream or downstream of relevant metabolic branching points; proton-consuming decarboxylation of deamidation reactions is on a gray background. Modified from reference 175.

Fermentation Microbiotas and Their Impact on Product Quality

An overview of fermentation microbiota in fermented fish products is provided in Table 33.8. Bacilli and staphylococci have been isolated from pidan, but their cell counts are too low to make a relevant impact on product quality (195). Hákarl is fermented by lactobacilli and organisms of the *Acinetobacter* and *Moraxella* groups. The organisms break down urea and trimethylamine oxide with a concomitant increase in pH; during the drying period, the bacterial counts decrease. The breakdown of urea and trimethylamine oxide is a main contribution of fermentation to improve the palatability of shark meat (194). Rakfisk is fermented with *L. sakei*; the changes in odor and color are attributed to autolytic enzymes (194). Fermentation of surströmming is attributed to strict anaerobic halophiles, including *Halanaerobium* spp., which produce odor volatiles, including propionic acid, hydrogen sulfide, butyric acid, and acetic acid (194).

Fermentation microbiotas of lightly salted fish with addition of starch, sugar, or garlic resemble the fermentation microbiotas of fermented meats (Table 33.8) with *L. plantarum* and *Staphylococcus xylosus* as dominant members of fermentation microbiota. Depending on the ambient temperature, thermophilic (*L. reuteri* and *L. fermentum*) or mesophilic (*L. brevis*) organisms are additionally present. Acid production in conjunction with addition of salt determines the safety of the fish products (185).

Table 33.8. Microbiota of fish fermentations[a]

Raw material	Control of fermentation microbiota	Products (examples)	Major fermentation organisms
Whole gutted fish or fish fillet	Addition of NaCl (3–5%), rice, sugar, and/or garlic	Suan yu (sour fish), narezushi, som fug, plaa-som	*L. plantarum*, *L. fermentum*, *L. alimentarius*, *Pc. pentosaceus*, *L. brevis*, *St. xylosus*, *D. hansenii*
Fish sauce from diverse raw materials	Addition of NaCl (15–26%); addition of koji (Japan)	Naam pla, shottsuru	*T. halophilus*, *Lentibacillus salicampi*, staphylococci, *Z. rouxii*
Trout	Addition of NaCl (3–6%), temperature (0–10°C)	Rakfisk	*L. sakei*
Shark	Low temperature, drying	Hákarl	*Acinetobacter*, lactobacilli

[a] Data are from references 183–187 and 194.

Fermentation microbiotas of fish sauce are controlled mainly by addition of salt; accordingly, the halophilic organism *T. halophilus* and the salt-tolerant organism *Z. rouxii* together are considered dominant members of fermentation microbiota (191–193); aerobic halophilic

bacteria, including *Lentibacillus*, may also be present (196). Protein degradation to generate taste-active peptides and amino acids is predominantly an autolytic process that is catalyzed by enzymes derived from the raw material; the addition of koji accelerates the ripening process (191).

FERMENTED MEATS

Production Processes

Fermented meats are produced from whole-muscle meat, ham, comminuted meat, or dry-cured sausages (Fig. 33.21). Dry-cured ham is produced predominantly from pork. Whole-muscle meat is initially preserved by dry salting with curing salt, brining, or injection of brine. The meat is maintained at refrigeration temperature until the salt concentration is equilibrated through the muscle to prevent growth of toxigenic clostridia. Nitrite or nitrate is used as a curing agent. After a NaCl concentration of about 4.5% has been reached throughout the product, corresponding to a water activity of < 0.96, the products are ripened at a low temperature (12 to 15°C). Depending on the climatic conditions, preservation and drying are supported by cold smoking; this step is usually omitted in Mediterranean countries with a dry climate. After the water activity is reduced to levels below 0.91, further drying and ripening are conducted at ambient temperature. Surface ripening with molds is used for some products, such as Tyrolean bacon. Process innovation in the production of dry-cured hams particularly relates to the salting/brining process. The use of pulsed electrical fields, pulsed pressure brining at 150 to 300 MPa, tumbling, or freezing of meat prior to salting or brining damages tissue integrity and thus accelerates salt diffusion during brining and proteolysis during ripening (197).

Dry-cured sausages are produced with comminuted meat and fat with addition of 2.5 to 3% NaCl, nitrite or nitrate, and spices. Ascorbic acid is added to reduce the formation of toxic nitrosamines from nitrite (198); the addition of glucono-δ-lactone reduces the pH without microbial activity (Fig. 33.21). The content of fermentable carbohydrates in meat is generally low, as glucose and glycogen are consumed in postmortem conversion to lactate by muscle tissue. Therefore, glucose or other

fermentable carbohydrates are added to control acidification during fermentation. The meat is comminuted at a low temperature, often with frozen ingredients, to avoid melting of the fat and coagulation of protein; removal of oxygen during meat cutting prevents lipid oxidation. The sausage batter is then filled into casings. The particle size of meat and fat after cutting and the diameter of the casing influences the water loss during ripening and the texture of the final products. The addition of NaCl and fermentable carbohydrates favors growth of lactic acid bacteria over other organisms that are present in the sausage batter. Lactic metabolism reduces the pH to values ranging from 4.8 to 5.3; acidification reduces the risk of growth of pathogenic bacteria, coagulates meat proteins to generate a solid sausage matrix, and reduces the water binding capacity. After a 2- to 3-day fermentation step at temperatures ranging from 18 to 30°C, products are dried at controlled water activity and temperature over a period of 2 to more than 6 weeks. During drying, the pH of the sausages increases due to amino acid metabolism of the fermentation organisms. If the surface microbiota contributes to ripening, lactate consumption by yeasts and fungi growing on the surface additionally increases the pH. Products are microbiologically stable when the water activity is 0.91 or less, or at a water activity of less than 0.94 and a pH of less than 5.3.

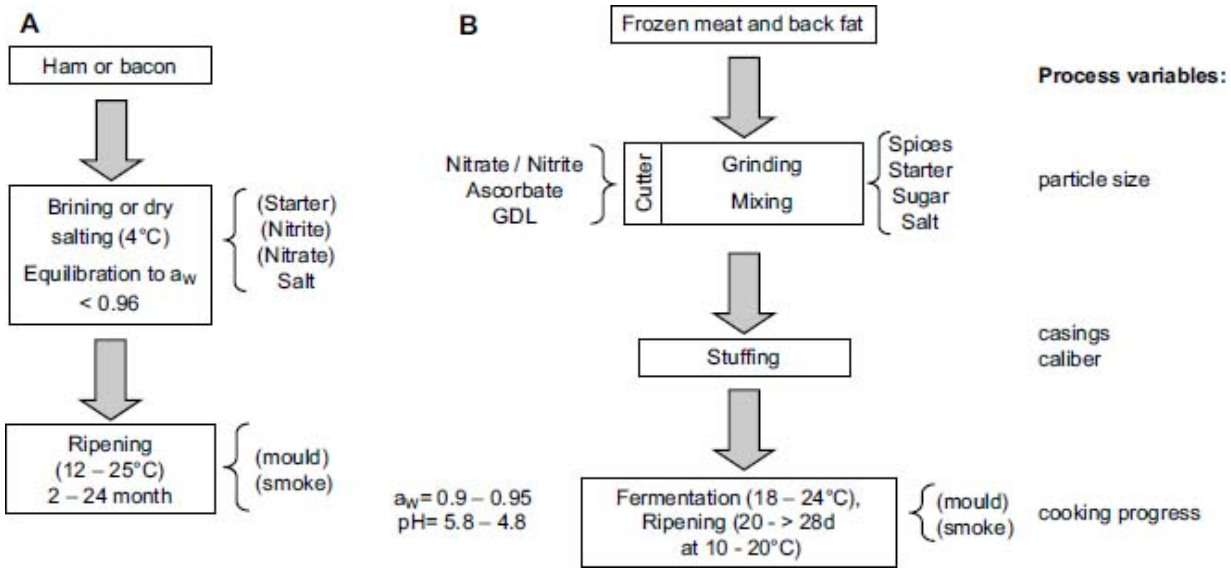


Figure 33.21. Basic processing steps for production of dry-cured ham (A) and dry-cured sausage (B). Ham is produced from whole-muscle pork meat; pork is also a major ingredient in sausage production, but product formulas may additionally include beef and/or horse and donkey meat. Sausages are generally cured with nitrite or nitrate, but not all hams are cured with nitrite or nitrate. Fermentation microbiotas in sausage production are introduced by back-slopping or by use of starter cultures or derived from house microbiota of the production facility.

Dry-cured sausages are generally not heated prior to consumption; therefore, pathogenic microorganisms, including *L. monocytogenes* and pathogenic strains of *E. coli*, *Salmonella*, and *St. aureus*, represent a hygiene risk. Control of pathogenic microorganisms during meat fermentations relies predominantly on the use of NaCl, nitrite, and competitive starter cultures to prevent growth. The risk of *L. monocytogenes* is additionally controlled by bacteriocin-producing starter cultures (199, 200); however, bacteriocins of lactic starter cultures are inactive against Gram-negative organisms (199, 201, 202). Enterohemorrhagic *E. coli* resists the fermentation and initial ripening steps; its safe elimination requires clean raw material, process control to prevent initial growth, and long ripening times (199, 203, 204). If ripening times are not sufficient to eliminate enterohemorrhagic *E. coli* (e.g., in production of summer sausage, which is fermented and ripened

at a relatively high temperature for a short time), a heating step is included after fermentation to control hygiene risks.

Products

Fermented meats are produced throughout Europe, the Middle East, North Africa, Southeast Asia, and North America. An excellent overview of dry-cured hams and bacon has been provided by Bosse et al. (197). The preservation of bacon and ham produced in Central and Northern Europe usually relies on smoking and relatively long ripening times of several weeks to a few months; examples include Tyrolean bacon, Black Forest ham, and jambon d'Ardenne. Reindeer ham is produced in the north of Finland by dry salting and smoking of boneless cuts of reindeer. Mediterranean products are typically ripened for 12 to 18 months and often cured without addition of nitrite or nitrate; typical examples include Parma ham (prosciutto di Parma), San Daniele ham (prosciutto di San Daniele), and Serrano ham (Jamón Serrano). Dry-cured meat products in Islamic countries of North Africa and the Middle East are produced from beef.

Dry-cured sausages produced in Mediterranean countries (Italy, Spain, and France), Hungary, and Turkey are typically dried to a water activity of less than 0.9, corresponding to a water loss of 25 to 40%. A majority of products are ripened with a surface mycobiota composed of yeasts and molds; the pH of these products can increase to 6.0 during ripening (205). Semidry sausages produced in Central and Northern Europe are ripened for a shorter time, 3 to 4 weeks. The water activity in these products ranges from 0.9 to 0.95, corresponding to a water loss of 10 to 20%, and the final pH of the products is typically below 5.2 (205). Surface treatment often includes smoking, and only a few products are mold ripened. Summer sausage in the United States, nham in Thailand, and nem chua in Vietnam are produced with short fermentation times at ambient temperature (25 to 40°C) (205, 206). The final pH of the products is between 4.5 and 5.0. Because the short fermentation times do not provide sufficient pathogen control, hygiene risks are controlled by a heating step after fermentation (summer sausage) or by inclusion of liberal amounts of garlic in the sausage formula (186, 206). Cured sausages with minimal or no fermentation and ripening are available in Germany (Mettwurst), Italy (sopréssa), and Spain (sobrasada); in these

products, the pH remains above 5.3 to prevent coagulation of meat proteins and the moisture loss is less than 10%. Hygiene risks in these products are controlled only by the procurement of clean, uncontaminated meat (205, 206).

Fermentation Microbiotas

Microbial growth on dry-cured ham is restricted to the surface of the product, where bacterial cell counts reach 10^8 CFU/cm2 (197, 207). The microbial diversity is reduced during ripening, and *Micrococcaceae* and *Staphylococcus* spp., including *Staphylococcus equorum*, *St. xylosus*, and *Staphylococcus saprophyticus*, account for a majority of the bacterial microbiota during ripening. The diversity of mycobiota increases during ripening and includes salt-tolerant yeasts such as *Debaryomyces hansenii*, *Candida* spp., *Penicillium chrysogenum*, and *Penicillium nalgiovense* (197, 207).

Dry-cured sausages are fermented with a combination of lactic acid bacteria and staphylococci or *Kocuria varians* (207) (Table 33.9). Rapid growth of lactic acid bacteria to cell counts ranging from 10^8 to 10^9 CFU/g is decisive for product quality and safety. Dominating lactic acid bacteria include, in decreasing order of importance, *L. sakei*, *L. curvatus*, and *L. plantarum;* high fermentation temperatures favor *Pediococcus pentosaceus* and *Pc. acidilactici* (207). *E. faecium*, *Lactobacillus paracasei*, *L. alimentarius*, *L. brevis*, *Leuconostoc* spp., and *Weissella* can also be present as minor components (207–209). The competitiveness of *L. sakei* in meat fermentations relates to its rapid growth at 15 to 25°C in the presence of NaCl (207); in addition, metabolism of pentoses, nucleic acids, and sialic acid and formation of heme-dependent oxidoreductases and bacteriocin have been suggested to contribute to its competitiveness (210–212). Growth of staphylococci in the meat matrix remains restricted to the outer layer, where oxygen remains available throughout ripening; cell counts range from 10^6 to 10^7 CFU/g and decline during ripening (205, 207). *St. xylosus* and, less frequently, *St. equorum* or *St. saprophyticus* are the dominant catalase-positive organisms, but numerous other coagulase-negative staphylococci, including *Staphylococcus carnosus*, *Staphylococcus*

epidermidis, *Staphylococcus sciuri*, and *Staphylococcus warneri*, are also present at lower cell counts (213).

Table 33.9. Major fermentation organisms in meat fermentations[a]

Group	Organism(s)
Lactic acid bacteria	Key organisms: *L. sakei*, *L. curvatus*, *L. plantarum*, *Pc. acidilactici*, *Pc. pentosaceus*
	Other organisms: *Enterococcus* spp., *L. paracasei*, *L. alimentarius*, *L. brevis*, *Leuconostoc* spp., *Weissella* spp.
Actinobacteria	*Kocuria varians*
Staphylococci	Key organisms: *St. xylosus*, *St. equorum*, *St. saprophyticus*
Other organisms: *St. carnosus*, *St. epidermidis*, *Staphylococcus pasteuri*, *St. sciuri*, *St. warneri*	
Yeasts	*D. hansenii*, *C. famata*
Molds	*P. nalgiovense*, *P. chrysogenum*, *P. camemberti*

[a] Organisms that are used in starter cultures are underlined. Modified from reference 207.

In traditional meat fermentations, fermentation organisms are derived from the raw material, ingredients, or "house microbiota" (i.e., organisms that persist in the production environment). Most industrial fermentations and many small-scale, artisanal fermentations are controlled by starter cultures to minimize hygienic risks, to prevent growth of fermentation organisms that decarboxylate amino acids to biogenic amines, and to ensure a uniform product quality (205, 207, 213). Starter cultures are added as freeze-dried preparations directly to the sausage batter. Their use enables control of fermentation microbiota throughout ripening (212, 214); however, the reduced bacterial diversity of sausages produced with starter cultures also relates to a reduced flavor intensity (212–214).

Surface microbiotas consisting of yeasts and mycelial fungi develop during ripening unless fungal growth is controlled by smoking or surface treatment with potassium sorbate. Yeast microbiotas are typically dominated by *D. hansenii*, an acid-tolerant and osmotolerant yeast, but other yeasts, including diverse *Candida* spp., have also been identified (215, 216). Mycobiotas on dry-cured sausages include *Penicillium* spp. and, less frequently, *Aspergillus* spp. (215, 216). Mycobiotas include mycotoxin-producing strains; the use of *D. hansenii* or the mycotoxin-negative *P. nalgiovense* as a starter culture controls growth of mycotoxin-producing fungi during ripening (204, 218).

Impact of Fermentation Microbiotas on Product Quality

Biochemical conversions during ripening of dry-cured ham are mainly attributable to muscle proteases and lipases. Hydrolysis of proteins to peptides is attributable to activity of cathepsins, calpains, and the 20S proteasome; of these enzymes, cathepsin D is the major contributor to proteolysis during ripening (219, 220). Muscle peptidases further convert peptides to amino acids; the umami-tasting glutamate and aspartate as well as alanine and proline (sweet taste) accumulate to concentrations exceeding the taste threshold (109). Peptides also contribute to the taste of ham; in particular, peptides with umami and kokumi taste activity contribute to the taste of the final product (109). Lysosomal lipases and phospholipases remain active throughout ripening and contribute to lipolysis (220). Surface mycobiotas contribute to proteolysis, lipolysis, and flavor formation (197). Bacterial metabolism is relevant mainly during brining. Growth of salt-tolerant and nitrite reductase-positive staphylococci in brine and on the surface of the ham increases nitrite concentration and enhances color formation by formation of nitroso-myoglobin (197, 221). Staphylococci also influence flavor formation through conversion of amino acids to flavor volatiles (197).

Acidification is the key contribution of lactic acid bacteria to the quality and safety of dry fermented sausages (Table 33.10). Rapid acidification coagulates meat proteins; therefore, it confers a firm texture to the sausages, favors nitrite disproportionation to nitrate and NO, and thus improves formation of the characteristic color of cured meats by nitroso-myoglobin and controls hygienic risks by inhibition of the growth of

pathogens and spoilage microbiota (198, 199, 205). Acid formation is controlled by fermentation parameters and the addition of fermentable carbohydrates to the sausage batter. Rapid acidification with pediococci in summer sausage or in Central and Northern European-style semi-dry fermented sausages (Fig. 33.1) also leads to acidity as a dominant part of the taste profile; in contrast, Mediterranean-style, long-ripened products have a less acidic taste (222). The formation of bacteriocins by lactic acid bacteria in meat fermentations contributes to control of *L. monocytogenes* and increases the competitiveness of cultures (199, 200, 212).

Staphylococci also express catalase and thus prevent lipid oxidation by peroxides that are formed by meat enzymes or in metabolism of lactic acid bacteria. Nitrate reductase activity of staphylococci or *Kocuria varians* is essential when nitrate is used as the curing agent but also supports formation of the typical color of cured meats if nitrite is used as the curing agent (Table 33.10). Nitrite reacts to nitrate and NO, which has a high affinity for the oxygen-binding site of myoglobin; the resulting formation of nitroso-myoglobin is responsible for the characteristic color and oxidative stability of cured meats (198, 205). NO also has strong inhibitory activity against clostridia, including *Clostridium botulinum*, while growth of other microorganisms, including lactic acid bacteria and *Listeria*, is not affected by nitrite. The specific inhibitory activity of NO against clostridia also relates to the high affinity of NO for iron proteins (223), which are essential components of the clostridial anaerobic electron transport chain (224).

Table 33.10. Contribution of microbial conversions to the safety and quality of fermented meats

Organisms	Metabolic pathway	Technological role
Lactic acid bacteria	Acid formation	Coagulation of meat proteins; enhanced disproportion of HNO_2 to NO and HNO_3; control of hygienic risks and spoilage microbiota
	Production of bacteriocins	Increased competitiveness; control of *L. monocytogenes* and spoilage microbiota
	Peptidase activity	Release of taste-active amino acids from peptides
	Decarboxylation of amino acids	Undesirable formation of biogenic amines from histidine, tyrosine, and phenylalanine
Staphylococci	Nitrate reductase	Reduction of HNO_3 to HNO_2
	Catalase	Removal of H_2O_2 to control lipid oxidation
	Amino acid metabolism	Conversion of amino acids to flavor volatiles
	Decarboxylation of amino acids	Undesirable formation of biogenic amines from histidine, tyrosine, and phenylalanine
Surface mycobiotas	Release of lipases and proteases	Formation of taste-active peptides and flavor volatiles
	Mycotoxin formation	Undesirable formation of mycotoxins
		Product-specific appearance is improved by formation of even and stable mycelium of desirable color.

Flavor formation during fermentation of dry-cured sausages also relies on muscle proteases and lipases as primary degraders of meat proteins and lipids (219, 220). The accumulation of amino acids in aseptic batches of meat and in lactic acid-fermented sausages is comparable, but strain-specific peptidase activities of lactic starter cultures contribute to the accumulation of amino acids (Fig. 33.19) (225, 226). The taste of dry-cured meats is mediated by organic acids and taste-active amino acids and peptides, particularly amino acids and peptides with umami and kokumi taste activity (109, 219, 220). Lipolysis, lipid oxidation, and the conversion of amino acids to flavor volatiles shape the odor of dry-cured sausages. Conversion of amino acids to flavor volatiles through the transaminase pathway (Fig. 33.20) is primarily attributed to staphylococci; the increased diversity of staphylococcal communities in traditionally fermented sausages thus corresponds to an increased production of flavor volatiles. Lactic acid bacteria produce organic acids, including acetate, and diacetyl (214, 222, 227, 228). Surface mycobiotas consume lactic acid, increase the sausage pH, and contribute to flavor generation by proteolysis and lipolysis; in addition, surface ripening controls lipid oxidation and leads to formation of flavor volatiles (227, 229).

Decarboxylation of amino acids by contaminants of the raw material prior to sausage production or by the fermentation microbiota results in accumulation of biogenic amines (204, 217). The presence of amino acid decarboxylases in lactic acid bacteria is strain specific; tyramine is usually the most abundant biogenic amine in dry-cured sausages. Staphylococci have a lower potential for amino acid decarboxylation (213). Fermentation control by decarboxylase-negative starter cultures reduces the formation of biogenic amines during sausage ripening (207, 217).

CONCLUDING REMARKS

Owing to the diversity of fermented food products, a chapter on fermented foods is invariably incomplete. Moreover, the study of food fermentations and fermenta fermentation microbiotas is closely linked to economic activity; therefore, production processes and fermentation organisms of many products that are produced in developing countries

are not well represented in the scientific literature and in this chapter. Many spots on the periodic table of fermented foods were filled in the last decade. Moreover, newly available sequencing technologies that allow a rapid assessment of the composition and activity of food microbiotas by high-throughput sequencing of bacterial 16S rRNA genes, genomes, and transcriptomes are a very powerful complement to classical culture-based methods. Sequencing technologies can guide the tedious but indispensable culture-dependent experimentation and application trials that provide the link between the presence and metabolic activity of bacteria in food fermentations and the quality and safety of fermented foods. This will not only maintain the diversity of fermented foods as affordable, nutritious, and tasty foods and as part of our cultural heritage but also allow us to exploit processes and fermentation organisms from traditional fermented foods in novel food and nonfood applications.

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VII
Current Issues and Advances in Food Microbiology

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34
Antimicrobial Resistance, Gut Microbiota, and Health

Antimicrobials have been broadly used in human medicine, agricultural production, and food processing. They are essential for ensuring human and animal health, as well as the productivity and safety of our food supply. Besides antibiotics, many natural or synthetic compounds, such as salt, alcohol, acids (e.g., lactic acid and acetic acid), bacteriocin, common synthetic preservatives, sanitizers, etc., also exhibit antimicrobial activities. The term "antimicrobial resistance," in a broad sense, describes the decreased susceptibility of a multitude of microbes to a broad spectrum of single or multiple agents, including the temporary or permanent ability of an organism and its progeny to remain viable and/or multiply under conditions that would otherwise destroy or inhibit the susceptible members of such organisms (1). Various antimicrobial mechanisms of resistance, whether to antibiotics, food antimicrobials (e.g., acids and bacteriocin), or disinfectants, due to permanent genetic and structural alterations or to temporary adjustment of metabolic machinery such as stress responses, formation of biofilms and spores, etc., directly contribute to the persistence of the microbes in the environment or, for pathogens, to infection in their host.

The rapid rise of antibiotic-resistant (ART) pathogens negates effective prevention and treatment of infections by antibiotics, causing serious losses in public health and social economy and societal panic on antibiotic resistance (AR). Despite the fact that antibiotics are not used in food processing, their broad applications in food animal, crop, and aquaculture production still impact the

food chain. For decades, it has been a common belief that the use of antibiotics in agriculture and medicine inevitably led to the AR problem seen today. However, scientific breakthroughs in the past decade have illustrated that AR is a more complicated issue, and a comprehensive understanding is essential for targeted strategies with productive outcomes. It thus becomes necessary to equip professionals in food science, microbiology, and public health with a comprehensive scope and updated knowledge, to better combat the major challenges to human society. This chapter summarizes important concepts and knowledge breakthroughs regarding AR and microbiota. This is an update of the chapter "Antibiotic Resistance" in the previous edition of this book (2) and thus includes content adopted directly without further citation.

SCIENTIFIC SCOPE

Pathogens, Commensals, and Microbiota

The immediate threat to medical therapy and the ultimate concern, with the public perception of an upcoming postantibiotic era, is the failure of ART pathogens to respond to normal antibiotic treatment. However, limiting the scope of research on AR to ART pathogens in past decades turned out to be a big mistake, as it missed a big part of the picture. Actually, pathogens represent only a very small percentage of the microbial population (microbiota), as commensal bacteria dominate in the host, food, and environmental microbiotas. It is now recognized that commensal bacteria have key roles in AR ecology, including emergence, enrichment, dissemination, and persistence (3–6). Due to their dominance in populations, it is more likely that AR-encoding genes emerged in commensals first and then spread to pathogens. Even though it was the general belief that after removal of the antibiotic selective pressure, the corresponding AR trait(s) would gradually be lost, scientific data obtained in the past couple of decades from studies of humans, animals, food, and the environment illustrated that this was not the case. Various

molecular stabilization mechanisms and niche fitness advantages have led to the persistence and even dominance of certain ART commensals and pathogens in the ecosystem. Thus, once ART pathogens have emerged and been detected, it is already too late to get rid of them. Therefore, shifting the role of the current government AR surveillance system, i.e., the National Antimicrobial Resistance Monitoring System (NARMS), by the Food and Drug Administration (FDA), Centers for Disease Control and Prevention, and U.S. Department of Agriculture (USDA) from ARTpathogen-oriented disaster reporting to a commensal microbiota-associated AR risk forecast system has become a pressing need (6, 7).

Foodborne Microbes, Gut Microbiota, and Health

Besides nutrients from digested foods, the human gastrointestinal (GI) tract is also home to as many as 10^{14} microbes, almost equivalent to the total number of cells in a human body. Conventional food intake, through foodborne microbes and food ingredients, serves as the most important avenue bridging the host and environment, constantly shaping the host gut microbiota. It is now recognized that in addition to helping digest foods, the gut microbiota has more important roles in host health. For instance, the gut bacteria modulate the maturation and maintain the normal function of the host immune system (8–11) and produce small molecules affecting brain function (12, 13). The profiles of gut microbiota further impact potentially serious infections by opportunistic pathogens such as *Clostridioides* (formerly *Clostridium*) *difficile* and vancomycin-resistant enterococci (14, 15), as well as energy metabolism of the hosts, contributing to growth promotion in food animal production and obesity in human beings (16–19).

Foodborne Antibiotic Residues, AR, and Gut Microbiota Dysbiosis

It is important to recognize that antibiotic residues and AR are two different concepts. The presence of trace amounts of antibiotics and other chemical residues in food animal, seafood, vegetable, fruit, and grain products is sometimes inevitable due to the environment to which products are exposed (e.g., water, soil, and air) and production practices. To ensure the safety of the U.S. food supply, government agencies have established practices to limit hazardous compounds in edible food products. The FDA has established tolerance levels for chemical residues including antibiotics (20), and the USDA's Food Safety Inspection Service and Agriculture Research Service have established standard practices and procedures for sampling and assessing the products.

The primary concern about the presence of chemicals, including antibiotic residues, in food products is the potential public health hazard posed by the compound or its derivative(s). For instance, the antibiotic chloramphenicol may increase the risk of childhood leukemia (21), and it has been detected in certain foreign seafood products stopped by the FDA from entering the food chain in the United States (22).

Although in theory, foodborne antibiotic residues might inhibit or kill susceptible bacteria and accordingly select for ART bacteria and disrupt the integrity of the gut microbiota, in reality, the levels of antibiotic residues in USDA-certified meat and poultry are very low. For instance, the FDA's established tolerance level for penicillin is 0.05 ppm (or 0.05 mg/kg) in uncooked, edible tissue of cattle, 0.01 ppm (or 0.01 mg/kg) in uncooked, edible tissue of turkey, and 0 ppm in all other meat, egg, and dairy products; the value is 0.01 ppm for negligible residues of ampicillin, a derivative of penicillin, in uncooked edible tissues of swine and cattle and in milk (20). Meanwhile, the MIC_{50}s of ampicillin are around 0.12 to 8 μg/ml for susceptible and 16 μg/ml for resistant strains of *Enterococcus* and 0.35 to 8 μg/ml for susceptible and 32 μg/ml for resistant Gram-negative bacteria, respectively (23). The antibiotic exposure that occurs through a normal, single-meal intake with 1 lb of meat is no more than 0.023 mg. For an adult with 5 liters of blood in the

circulation system, the resulting maximum plasma concentration is 0.0046 μg/ml, far below the effective concentration to inhibit sensitive bacteria and select for resistant populations.

In the GI tract, once the antibiotic residue is mixed with other foods as well as fluid in the stomach and intestine, its concentration in the food matrices also decreases to significantly below the MICs for susceptible bacteria. In addition, many antibiotics are heat sensitive; thus, their activities would be reduced by cooking. On the other hand, the therapeutic dosages of ampicillin, for instance, are 250 to 3,500 mg by the oral route or 250 to 500 mg by intramuscular or intravenous injection every 6 h, depending on the nature of the infection(s) (24). If fully absorbed, the therapeutic drug concentrations (50 to 700 μg/ml of plasma) are up to thousands of times of the MICs of susceptible *Enterococcus* and Gram-negative bacteria and 3 to 43 and 2 to 22 times of the MICs of ART *Enterococcus* and Gram-negative bacteria, respectively. While sufficient to inhibit and kill pathogens, the therapeutic drugs, especially when given by oral administration, also massively kill susceptible bacteria and further wipe out even the resistant subpopulation of bacterial groups with lower MICs, causing irreparable gut microbiota dysbiosis. The exposure through medical treatment by oral antibiotics is 4 to 5 log (10,000 to 100,000 times) greater than the exposure from conventional food intake. The data suggest that therapeutic antibiotic intake, especially oral administration, which directly exposes the gut microbiota to unnecessarily high concentrations of drugs, is the dominant, key driver for massive AR and gut microbiota dysbiosis, while the impact of the foodborne antibiotic residues is minimal. This conclusion is further supported by a ground breaking study by Zhang et al. (25), detailed below.

Drug Administration and Excretion Routes and Fecal Microbiota

Through a carefully designed experiment in a well-controlled mouse system, the Wang team discovered that mainstream oral antibiotic intake and drugs excreted through the gut are key drivers for the rapid surge of AR in feces as well as the disruption of gut microbiota integrity (gut microbiota dysbiosis). In mice that had been inoculated with a bacterial cocktail with three *Escherichia coli* strains carrying the ampicillin resistance-encoding gene bla_{CMY2} and then given the therapeutic dosage of ampicillin by mouth, the targeted marker bla_{CMY2} gene pool in feces increased very quickly and remained high throughout the treatment period. However, when the same drug dosage and 5-day treatment duration were used but the ampicillin administration was changed from oral to injection, the bla_{CMY2} gene pool remained same as those in mice in the placebo group throughout the treatment period, which was only 1/100,000 of the AR gene pool seen in mice receiving oral ampicillin. The data revealed that oral antibiotic administration, instead of the application of antibiotics itself, was the direct driver for massive AR in feces. Since the ART-bacteriumrich feces from billions of human beings and animals released to the environment represent the most impactful contributor to the environmental AR gene pool, the finding in fact suggested that the mainstream oral antibiotic intake is a key driver for the rapid surge of AR in the ecosystem. Consistently, the profile of the gut microbiota was disrupted by oral ampicillin but spared by intravenously injected ampicillin throughout the 5-day treatment period. Furthermore, certain changes in the gut microbiota (dysbiosis) due to oral ampicillin remained even 10 days after the antibiotic treatment was halted (25).

Drugs also differ in pharmacological properties. Thus the Wang team further tested tetracycline, a drug with significant excretion through the bile/gut and feces, versus ampicillin, which is mostly excreted through the kidney and urine. As expected, changing tetracycline administration from oral to injection reduced the side effects of AR and gut microbiota dysbiosis in mice, but the

mitigation was less than that in the case of ampicillin, since part of the injected tetracycline still ended up in the gut.

The knowledge breakthrough regarding key drivers was further supported by historical data. Penicillin has been broadly used worldwide since World War II, but massive resistance was not a problem until after the 1970s in industrialized countries and the 1990s in China, correlating well with the switch of mainstream penicillin administration from muscle injection to oral (derivatives) in the corresponding countries. Although this time point also overlapped with the broad applications of antibiotics in food animal production, the antibiotics have been given to animals predominantly by mouth through feed or water. The historical trends of many modern diseases associated with gut microbiota dysbiosis are further quite similar to that of AR.

The Wang team further illustrated the main contributions of the food chain to AR using the same mouse model. For mice without prior inoculation of the bla_{CMY_2}+ *E. coli*, the marker gene pool was not detected in feces through out the 5-day ampicillin treatment period, regardless of whether it was given orally or by injection (25). The result illustrated that without the foodborne ART bacteria as the seed, the 5-day antibiotic treatment was not sufficient for the development of the AR gene.

Therefore, the food chain primarily impacts AR ecology through (i) the emergence, enrichment, and spread of ART bacteria in the food production environment and (ii) the role of conventional food or feed intake as a critical avenue for spreading ART bacteria and AR genes to the human and animal hosts. Once ingested, the foodborne ART bacteria serve as the seed in the host GI tract, facilitating additional evolution, enrichment, dissemination, and persistence of AR genes and ART bacteria, independent of clinical antibiotic exposure. The AR-rich feces further affect the AR gene pool in water, soil, air, etc., and accordingly contaminate food and feed, swelling the AR gene pool through continued circulation among the

food (feed), hosts, and the environment, as different components of the ecosystem are closely connected (Fig. 34.1).

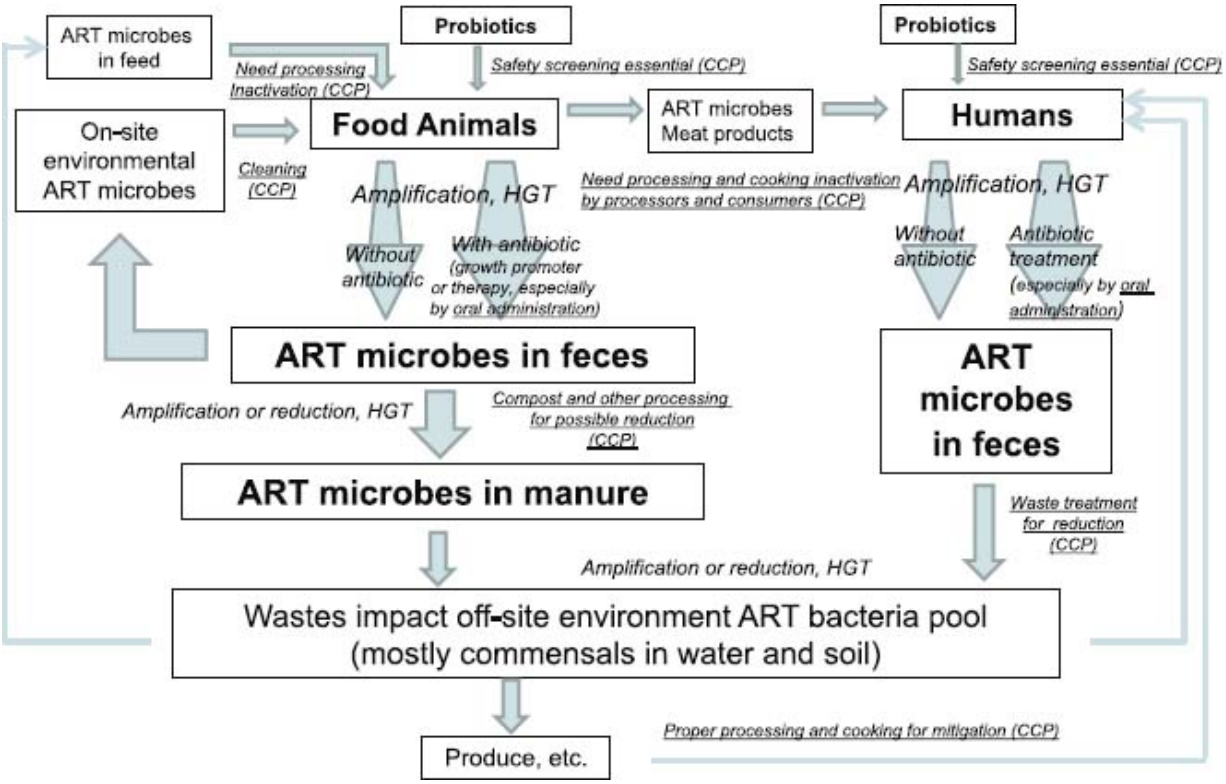


Figure 34.1. Flow of ART microorganisms from farm to fork. CCP, critical control point for mitigation.

Antibiotic Resistance, Health Care-Related Infections, and Biofilms

Difficult-to-treat hospital-acquired infections caused by ART pathogens and opportunistic pathogens, such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci, have captured public attention. In 2003, over one-quarter of nosocomial (health care-related) enterococcal infections were resistant to vancomycin, and almost 60% of health care-related *S. aureus* infections were caused by MRSA. These represent 12% and 11% increases, respectively, in resistance compared to the previous 5-year averages (26).

However, it is essential to recognize that the failure of health care-related infections to respond to antibiotic treatment in the clinical environment is not necessarily due to the direct application of antibiotics during the treatment. In fact, data from Massachusetts General Hospital suggested that improving sanitation significantly reduced health care-related infections, including those caused by ART pathogens. Thus, health care-related infection is more of an issue of cross-contamination followed by selective enrichment, and simply limiting the uses of antibiotics in disease prevention and treatment is not the right solution. Knowing the difference is essential for targeted controls.

It is further important to recognize and differentiate multiple mechanisms which contribute to the lack of response of bacteria to various antimicrobial treatments, including antibiotics. For instance, when microorganisms attach to surfaces and tissues, they can form complex and protective structures called biofilms. While planktonic (floating) cells are easy to suppress or kill with antimicrobial agents, biofilms are resistant to adverse environmental factors, including antibiotic treatment. The formation of biofilms is a leading cause of many persistent and even deadly diseases, regardless of whether AR genes are present. Therefore, it is much more productive to use antibiotics promptly for disease prevention and treatment, instead of waiting until minor infections turn into serious diseases involving biofilms that are naturally resistant to any treatment, including antibiotics.

RELATED BACKGROUND AND IMPORTANT CONCEPTS

Types of Antibiotics

Depending on the mode of action, antibiotics can be categorized into bactericidal and bacteriostatic agents. Bactericidal antibiotics normally target key cell structures and activities, such as the biosynthesis of cell wall (β-lactam antibiotics) and DNA

(fluoroquinolones), whereas bacteriostatic antibiotics commonly interfere with protein synthesis, inhibiting the proliferation of bacterial cells (27, 28). The three types of commonly used bactericidal antibiotics include the members of the β-lactam antibiotics family, the aminoglycosides, and the quinolones, whereas macrolides (including telithromycin), sulfonamides, and tetracycline are normally bacteriostatic antibiotics (29).

Bactericidal Antibiotics

β-Lactams

β-Lactam antibiotics are most commonly used in clinical therapy for infections. β-Lactam antibiotics are antibiotic agents containing a β-lactam in the molecular structure and are sometimes β-lactamase inhibitors (although β-lactamase inhibitors are not true antibiotics). β-Lactam antibiotics are bactericidal, interfering with the biosynthesis of the peptidoglycan layer in bacterial cell walls by irreversibly blocking penicillin-binding proteins, including carboxypeptidases, endopeptidases, and transpeptidases. Penicillin-binding proteins are a group of proteins that facilitate cross-linking of newly synthesized peptidoglycan to the existing cell wall structure (30, 31). Once treated with β-lactam antibiotics, susceptible bacterial cells develop a weak cell wall and are eventually subject to cell lysis. Four major classes of antibiotics and their derivatives belong to the β-lactam antibiotic category, including penicillin, cephalosporins, carbapenems, and monobactams. Based on their antimicrobial activities and antibacterial spectra, they can be further divided into different groups.

β-Lactamase inhibitors

Apart from the four families mentioned above, β-lactamase inhibitors are also recognized as β-lactam antibiotics. One of the most common mechanisms of resistance to β-lactam antibiotics in bacteria is furnished by genes encoding β-lactamase, which

hydrolyzes intracellular β-lactam molecules. To be most effective, β-lactamase inhibitors are administered together with β-lactam antibiotics to minimize the effects of bacterial resistance. β-Lactamase inhibitors are not true antibiotics but rather antibiotic enhancers (32, 33).

Aminoglycosides

Aminoglycosides are another type of bactericidal antibiotic. Their key structure includes an aminocyclitol ring, with different glycosidic linkages and side chains distinguishing the members of this family (34). Aminoglycosides affect bacterial cells by displacing cations such as Mg^{2+} and Ca^{2+} on the outer bacterial membrane, thereby disrupting membrane permeability. Moreover, aminoglycosides can also be bacteriostatic, as they impair growth of bacterial cells by binding to the 30S subunit of the bacterial ribosome, thereby inhibiting protein synthesis. Susceptible bacteria are primarily aerobic Gram-negative bacteria, such as *Klebsiella* spp. and *Pseudomonas aeruginosa*, whereas most Gram-positive bacteria and anaerobes are not susceptible to this antibiotic class (35). Representative members of the aminoglycoside group include gentamicin, kanamycin, neomycin, and streptomycin. This group of antibiotics is primarily used for treating infections on the skin surface and in the respiratory system and is normally combined with other types of antibiotics for clinical therapy.

Quinolones

Quinolones are also bactericidal, as they inhibit DNA synthesis in bacterial cells by binding to DNA gyrase and DNA topoisomerase, both of which are involved in DNA replication, transcription, and repair systems (36–38). Quinolones are effective against many types of Gramnegative bacteria, including *Klebsiella* spp., *E. coli*, *Salmonella* spp., and *Shigella* spp. (39). Ciprofloxacin, gemifloxacin, moxifloxacin, and trovafloxacin are examples of quinolone antibiotics.

Bacteriostatic Antibiotics

Bacteriostatic antibiotics inhibit the growth of bacteria rather than killing them. Intracellular nucleotide or protein synthesis is most commonly targeted by this type of antibiotic, and a broad spectrum of bacterial species are affected. For example, sulfonamides are competitive inhibitors of dihydropteroate synthase (40–42), an enzyme that plays a key role in folic acid synthesis. The lack of folic acid eventually leads to the breakdown of nucleotide synthesis and interruption of cell proliferation. Four groups of sulfonamides have been defined based on their modes of absorption and excretion. These include shortto medium-acting sulfonamides, long-acting sulfonamides, GI tract sulfonamides, and topical sulfonamides. Commonly used sulfonamides include sulfamethoxazole, sulfadoxine, sulfasalazine, and silver sulfadiazine Tetracyclines are different from sulfonamides in that they inhibit bacterial growth by impairing protein synthesis. This type of antibiotic blocks aminoacyl-tRNA by binding to the 30S subunit of bacterial ribosomes (43, 44).

Spectrum of inhibition

Tetracyclines can inhibit a broad spectrum of bacteria, including most Gram-positive bacteria and Gram-negative bacteria, aerobes, and anaerobes. Tetracyclines are divided into three groups depending on their pharmacokinetic characteristics. These include short-acting tetracyclines (oxytetracycline and tetracycline), intermediate-acting tetracyclines (demeclocycline), and long-acting tetracyclines (doxycycline and minocycline).

The macrolides and ketolides are another type of bacteriostatic antibiotic and are capable of binding to subunits of the 50S ribosome, thereby inhibiting transpeptidation and translocation during protein synthesis. Macrolides have a relatively broad antibacterial spectrum, especially against Gram-positive bacteria. Members of the macrolide family include erythromycin,

clarithromycin, dirithromycin, and telithromycin, and they are often used to treat infections of the respiratory tract.

Mechanisms of AR in Bacteria

Besides biofilms, bacteria can become resistant to specific types or groups of antibiotics in many different ways, which can be summarized as (i) innate resistance; (ii) reduction of the intracellular concentration of corresponding antibiotics; (iii) inactivation of corresponding antibiotics; (iv) mutations at binding sites of corresponding antibiotics; and (v) formation of additional metabolic pathways that compensate for the loss of key enzymes or substrates (caused by corresponding antibiotics) (45, 46).

Bacterial cells with innate resistance to a particular antibiotic naturally lack the molecule or pathway targeted by the drug; therefore, the drug has no effect on the survival of the microbes. Bacteria can also become resistant to antibiotics by reducing the intracellular concentration of the antibiotic, which can be achieved with a reduction of membrane permeability (decreased import) and adoption of efflux pumps (increased export). Gram-negative bacteria generally have better control over importing molecules, because their outer membranes have a smaller size exclusion limit than the peptidoglycan layer surrounding Gram-positive bacteria. Hydrophilic solutes diffuse into Gram-negative bacteria primarily through porins on the outer membrane. Bacterial mutants that lack porins may present a broad spectrum of resistance. Decreased permeability to chloramphenicol is regarded as a cause of resistance in *Haemophilus influenzae* and *Pseudomonas cepacia* (47, 48). In Gram-positive *Staphylococcus epidermidis*, glycopeptide resistance may result from overproduction of glycopeptide binding sites within the cell wall peptidoglycan (49). *Bacillus subtilis* mutants resistant to the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) have altered membranes with reduced amounts of C_{16} fatty acids and increased ratios of iso- to anteiso-branches. The CCCP-

resistant mutants are crossresistant to other inhibitors, including 2,4-dinitrophenol, tributyltin, and neomycin (50).

Energy-dependent efflux pumps are another mechanism for reducing intracellular antibiotics in ART bacteria. The efflux pumps can be specific to single antibiotics, or they can export multiple antibiotics. For example, both multidrug-resistant pumps and tetracycline-specific pumps were discovered in tetracycline-resistant bacteria. The coordination of reduced membrane permeability and the presence of efflux pumps likely can contribute to high-level resistance to antibiotics. For example, it was determined that active efflux pumps, as well as a reduction in the amount of major porins, contributed to increased resistance to chloramphenicol in some *Enterobacter* strains (51). Antibiotic inactivation can be achieved by degradation or modification. An example of antibiotic inactivation is that brought about by β-lactamase, which is carried by many bacterial species. β-Lactamase inactivates the antimicrobial activity of β-lactam antibiotics by hydrolyzing the β-lactam ring in these antibiotics. Currently four types (classes A, B, C, and D) of β-lactamases are recognized, and each type possesses its own characteristic nucleotide and amino acid sequences. Some lactamases confer resistance to various β-lactam antibiotics, whereas others are responsible for resistance to a single type of β-lactam antibiotic. Many types of β-lactamases are also resistant to a variety of β-lactamase inhibitors. In some situations, antibiotics are not degraded but are modified by resistant bacteria. For example, aminoglycosides can be modified by bacterial phosphotransferase and acetyltransferase, thereby losing their capability to bind the ribosome subunit (52, 53). Chloramphenicol is inactivated by acetylation, which is catalyzed by the chloramphenicol acetyltransferase of resistant bacteria (54).

Resistant bacteria can modify aminoglycosides by a variety of enzymes, including acetyltransferases, nucleotidyltransferases, and phosphotransferases (53). Another strategy for gaining resistance is to modify (mutate) antibiotic recognition sites on the intracellular structure or enzymes of bacterial cells, so that the corresponding

antibiotics cannot recognize them. This mechanism is involved in the development of resistance to many types of antibiotics, such as quinolones (mutations on target sites in DNA gyrase), tetracycline, and macrolides (mutations on corresponding ribosome subunits) (46, 54). The formation of alternative metabolic pathways is responsible for resistance to some antibiotics that target critical metabolic activities of bacterial cells. For example, sulfonamides are effective against bacteria by competing with *para*-aminobenzoic acid, a substrate involved in folic acid synthesis, which is critical for nucleotide production. Bacteria can inhibit sulfonamide activity by producing additional *para*-aminobenzoic acid, which has been observed in many sulfonamide-resistant bacteria (55).

In summary, various mechanisms have been adopted by bacteria to provide resistance to different types of antibiotics. Depending on the mechanism, resistance to antibiotics can be low, medium, or high level. Bacteria can be resistant to a single antibiotic or become multidrug resistant via one or multiple AR mechanisms. Liu and Pop (56) created the Antibiotic Resistance Genes Database by compiling publicly available information. The database includes annotation of each AR gene and resistance type, with information on AR profile, mechanism of action, ontology, and external links to sequence and protein databases. The database also supports sequence similarity searches and has a tool for characterizing common mutations conferring AR. The site provides useful information for AR prevalence studies and for identification and characterization of resistance mechanisms.

Resistance Due to Point Mutation and AR Genes

Spontaneous mutations occur at different rates in different organisms, regardless of the presence of antibiotics. The reported frequency of mutation ranges from 10^{-6} to 10^{-10} per bacterial generation (57). Usually, it takes a long time to accumulate enough mutations for new AR genes to evolve. Once genes or gene clusters encoding specific AR mechanism(s) emerge, however, they can be

spread quickly through horizontal gene transfer (HGT). However, single mutations in certain genes can lead to resistance to certain antibiotics. For instance, certain point mutations in the *gyrA* gene can lead to fluoroquinolone resistance (58).

Generally, without the selective pressure from antibiotic usage, the mutants would account for only a very small portion of the total bacterial population and generally would not be detected. However, antibiotic usage selects for ART populations. Some data also indicate that certain ART bacteria have a survival advantage, which may become dominant even without the corresponding selective pressure, such as the fluoroquinolone-resistant isolates of *Campylobacter* with a point mutation in the *gyrA* gene (58). Data also suggest that constant exposure to inhibitory levels of antibiotics may increase the mutation rate in bacteria and lead to multidrug-resistant offspring (59).

Assessing AR Determinants and the Antibiotic Resistome

The molecular mechanisms of AR involve many different facets of bacterial cell function. Identification and assessment of AR determinants in bacteria exhibiting phenotypic resistance to a specific antibiotic(s) are important for fundamental and clinical research. The rapid development of PCR and sequencing techniques facilitates high-throughput analysis of AR due to genetic changes. Identification and quantification of a limited number of known AR genes can be conducted via conventional PCR and real-time quantitative PCR. However, several different AR genes may cause the same AR phenotype, and it is not uncommon for an ART bacterial strain to exhibit several AR phenotypes. The comprehensive information concerning all the AR genes and their precursors is called the antibiotic resistome.

The analysis of the antibiotic resistome in the ART bacterial genome or in environmental or host samples requires high-throughput sequencing, data processing, and AR gene database-dependent

analysis. The Antibiotic Resistance Genes Database and the Comprehensive Antibiotic Resistance Database are among the earliest AR gene databases (56, 60). Newly developed databases such as Structured Antibiotic Resistance Genes and DeepARG-DB are usually based on the former database but with improved accuracy and/or comprehensiveness (61, 62). AR genes located in gene cassettes are expected to be highly mobile. One advantage of the RAC database is that it provides information on integron-related AR genes (63). It is important to note that the detection of AR genes by molecular methods does not translate directly to an antibioticresistant phenotype, which can be validated only by using live cultures. Some AR determinants may share significant homology with structure genes, while other AR-encoding genes may be silent or may have lost activity during evolution. Therefore, false positivity by molecular detection is not unusual. On the other hand, the databases include only genes with confirmed AR function. They may not include newly evolved genes conferring an AR phenotype, so false-negative results are not unusual either. Functional genomics has been adopted to tackle both challenges of false positives and false negatives (64).

Transmission of AR in Bacteria

Resistance to antimicrobials can be due to genetic and structural changes or temporary adjustment of metabolic machinery. AR due to genetic changes can be transmitted to progeny by vertical transmission and to other susceptible bacteria by HGT. Resistance due to a particular mode of living or stress response is transient and is not transferable.

Vertical Gene Transmission

Both mutated genes (resulting from mutations in genes encoding the antibiotic recognition site, alternative metabolic pathways, membrane porin/efflux pump, or antibiotic degradation systems, etc.) and acquired AR genes can be transferred by vertical transmission to offspring during bacterial growth.

HGT

HGT mediates the spread of resistance determinants (AR genes) to susceptible bacterial cells, most likely by mobile gene elements, such as plasmids, transposons, and integrons (65). The transfer can occur via conjugation, transformation (uptake of free DNA), and transduction. In contrast to the time-consuming mutation accumulation process, resistant units can be disseminated rapidly among microorganisms, within or across species and genera, via HGT events; therefore, HGT mechanisms play a key role in the rapid emergence of AR in microbial ecosystems. It is worth noting that the immunity gene(s) in antibiotic-producing microbes that protects the organisms from the antimicrobial compound (if applicable) can potentially serve as an AR determinant(s) if acquired by others.

Three important mobile gene elements (plasmids, transposons, and integrons) are involved in HGT more commonly than others. A plasmid is a type of circular DNA that self-replicates independently of chromosomal DNA. Plasmids may carry beneficial genetic traits, such as additional genes for metabolic activities, and can be readily transferred to another host bacterial cell via conjugation (66, 67). Various AR gene-carrying, evolutionarily related plasmids have been isolated from clinical, environmental, food, and human microfloras, indicating that plasmids are an important shuttle for disseminating AR in nature. A transposon is a type of mobile DNA element that can “cut/copy and paste” itself using a transposase enzyme. Once separated from its original position, a transposon can integrate into a specific or random site on genomic or plasmid DNA (68). Transposons often contain an AR gene, thus contributing to AR dissemination. An integron is a mobile DNA element with a site-specific DNA recombination system that is capable of recognizing and capturing mobile gene cassettes (69, 70). Depending on the content of the gene cassettes, integrons can be divided into AR integrons, which carry mainly AR genes and can reside on plasmid and genome DNA, and superintegrons (large genomic DNA

elements containing an array of genes that encode a variety of adaptive functions, such as virulence and metabolic activities). Resistance integrons are an important contributor to the dissemination of AR throughout the food chain. More than 70 AR genes have been detected in AR integrons, and a single integron may contain multiple AR genes. Compared with integrons, insertion sequences are small and compact DNA elements that primarily code for mobility (71). Once an insertion sequence contains an AR gene, it becomes a mobile vessel capable of disseminating the resistance genes within and between bacterial populations.

Common Natural HGT Mechanisms

Conjugation is the transfer of genetic material from donors to recipients through direct contact between the cells. Susceptible recipient cells may become resistant to corresponding antibiotics by acquiring an AR-encoding genetic element (plasmid or transposon) from the donor cell. The frequency of conjugal transfer of functional genetic elements is affected by both the genetic compatibility of the donor-and-recipient pair and the cell concentration.

Apart from conjugation, it is also possible to transform susceptible bacterial cells with cell-free DNA segments containing AR genes, leading to acquired resistance in these cells to the corresponding antibiotics. A bacterial cell able to absorb naked DNA and integrate it into the genome is called a competent cell, and the process is called transformation. The frequency of transformation is commonly low. However, given the high concentration of free DNA in certain natural and host environments, the intake of DNA by bacteria via transformation may be a critical route in modifying the microbial ecosystem in such cases.

Transduction is another form of HGT mediated by bacterial phages, during which DNA from the donor cell can be packaged into the phage by mistake and then transmitted to the recipient cells in the subsequent round of infection and eventually integrated into the host (recipient) genome or plasmid. Besides facilitating AR

dissemination, transduction also plays an important role in bacterial evolution. For example, DNA sequence data suggest that a bacterial phage likely was involved in the evolution of *E. coli* O157:H7, during which Shiga toxin-encoding genes were initially transmitted from another toxin-producing bacterium to an *E. coli* strain via bacterial phage infection (72). Due to the extensive involvement of transduction in the evolution of microbes, including pathogens, it is important to reevaluate the long-term safety impacts of phage-based intervention strategies intended to control foodborne pathogens.

AR IN THE FOOD CHAIN

Antibiotic Usage in Food Animal and Agriculture Production

The usage of antibiotics extends beyond human medicinal purposes. Antibiotics are used in food and aquaculture production to treat infected animals, prevent infection of animals potentially exposed to pathogens, and promote growth. To a lesser extent, antibiotics are also used in plant agriculture production to prevent diseases and improve productivity. Estimates of the combined total use of antibiotics, including human and animal use, vary widely. According to a recent report from the FDA, the total domestic sales and distribution amount of antimicrobial drugs approved for use in food animal production in 2015 was close to 35 million pounds (73). Thus, on a mass basis, agricultural practices, including therapeutic treatment, contribute a significant portion of the antibiotics introduced into the biosphere. The growth-promoting function of antibiotics is only partly understood and is the subject of heated debate (74–76). It is generally believed that the increasing prevalence of AR is directly related to increased antibiotic uses, both prudent and frivolous, in clinical and agricultural settings. Many argue that the widespread use of antibiotics at sub-therapeutic levels exacerbates the selective pressure that enriches microbial

populations for ART bacteria and suggest that growth promotion and disease prevention could be achieved through nonantibiotic alternatives and novel management practices (77, 78).

As a result of the debate, many European countries have discontinued the administration of antibiotics for growth promotion. Denmark essentially banned the use of antibiotics for growth promotion in food animal production in an effort to decrease the prevalence of antibiotic-resistant human pathogens. While the total use of antibiotics in animals in Denmark decreased by >30% from 1996 (before the ban) to 2001, there has been a 96% increase in the use of antibiotics for therapeutic purposes during the same period (79, 80). This makes it difficult to assess the impact of this mitigation strategy. A 2002 meeting of the World Health Organization assessing the impact of the Danish ban on growth-promoting antibiotics revealed that ART enterococci decreased. For example, resistance of *Enterococcus faecium* to virginiamycin declined from approximately 60% to 10% among isolates found on broiler meat from 1997 (the peak of virginiamycin use) to 1999 (when there was no virginiamycin use). However, the incidences of *Salmonella*, *Campylobacter*, and *Yersinia* infections in humans were not affected (79).

In the United States, organic farming is increasing in response to consumer demand. A small three-state study compared the rates of pathogen isolation in conventional swine production (indoor rearing) versus antimicrobialfree farms (outdoor rearing) (81). The prevalence of both *Salmonella* and *Toxoplasma* was statistically higher in the antimicrobial-free production systems, although it is not clear how long the antimicrobial-free farms were in operation or if they had always been antimicrobial free. In 2007, Cox et al. proposed a model that estimated the increased load of *Campylobacter* on chickens when animals were reared without antibiotics (82). Hence, the risks and benefits of antibiotic use in animal agriculture need to be carefully weighed. Regardless, FDA released a new rule in January 2017 to phase out the use of antibiotics to promote growth in food animal production.

Agricultural antibiotic usage extends beyond animal use but presumably occurs in other settings to a much lesser extent. In the United States, fruit plants may be sprayed with oxytetracycline and streptomycin. Since there are no surveillance systems for ART bacteria on crops, it is difficult to estimate the number of ART bacteria on plant products. Although evidence for the direct transfer of AR determinants between plant pathogens and human pathogens under natural conditions is lacking, an impact of plant antibiotic usage on the environmental commensal flora and, consequently, pathogens is anticipated.

There is very limited use of antibiotics in U.S. aquaculture. Only sulfadimethoxine, ormetoprim, sulfamerazine, and oxytetracycline are approved for use, although sulfamerazine is no longer commercially available. The extent of antibiotic use in other countries that export seafood and produce is unknown and presumed to be much higher. The presence of antibiotic residues is a common reason for seizure of imported seafood. As indicated by Cabello (83), the use of prophylactic antibiotics in aquaculture compensates for unsanitary or otherwise poor fish production practices. Antibiotics administered through fish feed can be deposited in the aquaculture animals and the environment and may select for AR in environmental flora. These microbes, in turn, can be further involved in AR gene dissemination. Cabello's review suggested that the AR determinants in *Salmonella* DT104 originated from fish pathogens and resulted from HGT. Heuer et al. (84) also described the human health consequences of antimicrobial use in aquaculture, noting the likelihood of transmission of drug-resistant pathogenic bacteria to humans from the aquaculture reservoir and the evidence for exchange of AR genes between fish pathogens and human bacteria. Briefly addressing risk management options, the authors mentioned that the use of effective vaccine strategies by the Norwegian salmon industry reduced by 99% the use of antimicrobials in aquaculture between 1987 and 2007, despite an increase in production.

However, a study conducted in a U.S. aquaculture farm without a history of antibiotic usage identified a broad spectrum of ART bacteria with various AR genes across the farm environment, the fish feed, and the fish products (85). A new tetracycline resistance gene isolated from fish intestine was further characterized (86). Similar results were also reported from other food animal production systems and products and even human infants with no direct exposure to antibiotics (6, 87). The data indicated that direct exposure to antibiotics is no longer the sole risk factor for AR. AR ecology is complicated by multiple risk factors, and ART bacteria have been circulating among the sectors within the connected ecosystem.

Foodborne ART Bacteria

In the past few decades, a variety of ART foodborne pathogens have been isolated and characterized. While it is important to realize the significance of the contribution of foodborne pathogens to foodborne illness, as mentioned previously, pathogens account for only a small percentage of the microbes associated with foods, with the number of foodborne ART pathogens being even smaller. Furthermore, raw meat and poultry are subjected to cooking before consumption, which effectively kills bacteria. Thus, the prevalence of ART pathogens in retail meat and poultry did not justify treating foodborne AR, nor its impact on AR ecology, as a significant food safety challenge. Since 2004, a series of studies revealed not only the prevalence but also the abundance of a broad spectrum of ART commensal bacteria in retail ready- to eat foods. A very large AR gene pool was identified in ready-to-eat products consumed without further processing, and the foodborne AR genes led to acquired resistance in human pathogens via HGT events (4, 88). These data clearly demonstrated that conventional food intake serves as a critical avenue for spreading AR to the public.

ART Foodborne Pathogens

Extensive research has led to an in-depth understanding of microbe-specific mechanisms in AR emergence, dissemination, and persistence, although only for a small number of zoonotic pathogens. An enhanced understanding of the differences in AR prevalence among pathogen species and serovars within the same genus is still beneficial. Such information may enable a better assessment of the relative contribution of different animal hosts and environmental factors, including selective pressure, to AR development and persistence for a given pathogen population. Furthermore, a comprehensive understanding of the relative contribution of pathogens in AR development in complex microbial ecosystems along the food chain, from the natural environment to acquisition by animal hosts, is needed for conducting a proper risk assessment and developing a well-targeted mitigation strategy. Several important foodborne pathogens as well as nonpathogenic *E. coli* strains and their resistance to antibiotics have been well documented.

Salmonella

Considering the large number of *Salmonella* serotypes (more than 2,500) and the differences in AR among different *Salmonella* serotypes, it is difficult to make generalizations regarding AR in *Salmonella*. This is compounded by differences that occur among *Salmonella* serotypes in the incidence of the human illnesses they cause. For example, the incidence of foodborne illness caused by *Salmonella enterica* serovar Typhimurium decreased by 40% between 1996–1998 and 2004, whereas *Salmonella* serovar Newport-related foodborne illnesses increased by 40% during the same period (89). Changes in serotype prevalence should be considered when trends in AR are examined, so that the potential impact of AR on human health can be evaluated in the context of pathogen prevalence. Despite the variation that occurs in AR, pathogenicity, and incidence of human illness associated with different serotypes of *Salmonella*, there are some general relationships that occur among *Salmonella* species and AR. AR rates

vary depending on the product (e.g., chicken versus turkey or layers versus broilers), country, year, and the type of antimicrobial. Gyles (90) proposed that differences in AR profiles observed among *Salmonella* serotypes in different countries or regions could be the result of dissemination of dominant *Salmonella* clones specific to these locations. However, in general, resistance to tetracycline and streptomycin in *Salmonella* is high, whereas ciprofloxacin resistance is usually low, if it exists at all. Resistance to extended-spectrum cephalosporins in *Salmonella* is of particular concern, because this class of antibiotics is commonly used to treat salmonellosis. Extended-spectrum-cephalosporin resistance is mediated by genes encoding β-lactamases. One gene, bla_{CMY2}, has been found in plasmids carried by *Salmonella* isolates worldwide. β-Lactamase-mediated resistance in *Salmonella* is encoded mainly by transposons and transmissible plasmids (91). Over the past several years, *Salmonella* serovar Newport MDR-AmpC, which is resistant to at least four additional antibiotics, has received much attention due to its epidemic spread. The bla_{CMY2} gene was identified in resistant isolates examined and was successfully transferred from a representative *Salmonella* isolate to *E. coli* by conjugation (92–94).

Campylobacter

In 2015, FoodNet identified 6,289 laboratory-confirmed infections by *Campylobacter*, corresponding to a rate of 12.82 per 100,000 persons in the United States (95). While sporadic cases are typically associated with poultry, outbreaks are most often associated with consumption of raw milk, unchlorinated water, and produce (96). Occasionally, a *Campylobacter* infection must be treated with antibiotics; erythromycin or a fluoroquinolone is most commonly prescribed, followed to a lesser extent by tetracycline and gentamicin. Fluoroquinolones are also used in animal agriculture in different countries. In the United States, fluoroquinolones had been used to control disease in poultry. However, the FDA withdrew in 2005 the approval of the fluoroquinolone enrofloxacin, marketed as Baytril, for use in poultry because the agency judged that

fluoroquinolone use in poultry increased the prevalence of fluoroquinolone-resistant *Campylobacter*, which potentially compromised the treatment of human campylobacteriosis (97). Resistance to fluoroquinolones in *Campylobacter* is due to a single chromosomal mutation in *gyrA* (98), unlike most other pathogens, which require multiple mutations for clinically relevant resistance. There appears to be no fitness cost associated with fluoroquinolone resistance in *Campylobacter*, and in fact, the mutation in GyrA confers a fitness advantage, increasing colonization of the pathogen in chickens (58). Resistance to fluoroquinolones can be detected shortly after exposure to the antibiotic. This is in contrast to resistance to macrolides, which requires repeated exposure to the antibiotic (98).

The macrolide erythromycin is also used to treat human campylobacteriosis. In a study using chickens colonized with the macrolide-sensitive species *Campylobacter coli* and *Campylobacter jejuni*, the effect of erythromycin dose on developing resistance was examined (99). One group of chickens was given a subtherapeutic dose of tylosin (a macrolide), and the other was given a therapeutic dose. Approximately 63% of the *Campylobacter* isolates were resistant to erythromycin after the subtherapeutic dose was administered, whereas only 11.4% were resistant among the isolates from the group treated with the therapeutic dose (99). This finding was consistent with the results of a laboratory study in which longterm use of subtherapeutic doses of tylosin, but not therapeutic treatments, selected for macrolide-resistant *Campylobacter* (100). It appears that *C. coli* has higher frequencies of macrolide resistance than *C. jejuni*; however, the reasons for this difference are unknown (98). Macrolide resistance in *Campylobacter* most commonly results from point mutations in 23S rRNA, which results in modifications to the antibiotic. Active efflux mediated by CmeABC is another mechanism that works synergistically with antibiotic modification in conferring macrolide resistance (98). Compared to other antibiotics, tetracycline resistance in *Campylobacter* is relatively high and is mediated by the *tet*(O) gene. The *tet*(O) gene

in *Campylobacter* is most often carried on a plasmid but occasionally is chromosomal. The protein product of this gene binds to the ribosome, protecting it from the inhibitory action of tetracycline (98, 101).

Shigella

When considering antibiotic-resistant *Shigella*, it is important to understand that humans, not food animals, are the host for this pathogen. Hence, human cases of shigellosis are not due to carriage of the pathogen in food-producing animals or to contamination of crops with manure from food animals. Human fecal contamination of food, either on the farm or during food preparation, is the root source of shigellosis. The infection is classified as a foodborne illness because food is often the vehicle (not the source) for transmitting *Shigella*. The NARMS has maintained a database of antimicrobialresistant enteric bacterial isolates from humans, food, animals, and fowl, including human *Shigella* isolates, since 1996. In 2010, most (81.8% of 407 isolates) of the *Shigella* species reported were *Shigella sonnei* (102), and most (91.2%) of the NARMS *Shigella* isolates were resistant to streptomycin; 40.8% were resistant to ampicillin; 48.2% were resistant to trimethoprim-sulfamethoxazole; 30.2% were resistant to sulfamethoxazole/sulfisoxazole; 31.7% were resistant to tetracycline; 10.1% were resistant to chloramphenicol; 4.4% were resistant to nalidixic acid; 1.7% were resistant to ciprofloxacin; less than 1% were resistant to gentamicin, ceftiofur, ceftriaxone, and ceftriaxone; and 0% were resistant to amoxicillin-clavulanic acid and kanamycin.

The emergence of cefotaximase-producing *Shigella* isolates in the United States has become a concern, because cefotaximase-type β-lactamases are among the extended-spectrum β-lactamases that confer resistance to extended-spectrum cephalosporins and significantly compromise shigellosis treatment options (102). A study by Wong et al. (103) on antimicrobial resistance trends among *Shigella* isolates from patients with shigellosis in New York City

between June 2006 and February 2009 revealed that 70% of 477 patients received an antibiotic for shigellosis, and 11% of these were treated with an antibiotic to which their *Shigella* infection was resistant. High levels of resistance against amoxicillin-clavulanate, ampicillin, and trimethoprim-sulfamethoxazole were found among these isolates, and resistance at a lower level to ciprofloxacin was also reported.

Listeria monocytogenes

Infection with *L. monocytogenes* is typically treated with a combination of ampicillin and aminoglycoside. Rates of AR in this pathogen have not been studied to the same extent as in other pathogens, such as *Salmonella* and *Campylobacter*. Because *L. monocytogenes* is considered widely distributed in the environment, it is not strictly a zoonotic organism and has not been considered problematic in terms of AR. Hence, it is not included in any of the NARMS surveys. Prazak et al. (104) reported that 95% of the *Listeria* isolates from cabbage and environmental or water samples in Texas were resistant to two or more antibiotics, with 85% of those being resistant to penicillin. More recent studies (105–109) have revealed that *L. monocytogenes* isolates from raw meat and a variety of retail foods in several countries outside the United States are ART. A study of food products in China reported multidrug resistance (i.e., resistance to ≥2 antibiotics) in 63.9% of 72 *Listeria* isolates and resistance to only one antibiotic in 19% of isolates (106). A study by Pesavento et al. (108) revealed that eight *Listeria* isolates were resistant to ampicillin and nine were resistant to methicillin. This finding is of note because ampicillin is used for treatment of listeriosis and *Listeria* can potentially transfer the methicillin resistance gene to *Enterococcus* spp., thereby compromising the treatment for *Enterococcus* infections. However, more likely the dominant commensal bacteria have played key roles in disseminating the AR genes. Yücel et al. (105) also reported *Listeria* resistance to ampicillin (five isolates), nalidixic acid (nine isolates), cephalothin (nine isolates), trimethoprim-

sulfamethoxazole (six isolates), and kanamycin (one isolate) among isolates from meat products in Turkey. Lyon et al. (110) examined the antimicrobial resistance profiles of 14 *L. monocytogenes actA* types (n = 157) isolated from a poultry processing plant and found that the prevalence of antimicrobial resistance among all *actA* types and the diversity within resistotypes were both low. All isolates were sensitive to the antibiotics used to treat listeriosis, and except for tetracycline, resistance was not related to type or lineage. In the same study (110), it was also observed that persistence of *L. monocytogenes* in a poultry processing plant was not related to antimicrobial resistance and that there was little difference in antimicrobial resistance variability between clinical and environmental isolates.

Escherichia coli

Pathogenic strains of *E. coli* may be among the more publicly recognizable foodborne pathogens, although only certain pathogens, such as enterohemorrhagic *E. coli* (e.g., *E. coli* O157:H7), cause illness. Human infections caused by enterohemorrhagic *E. coli* are not typically treated with antibiotics. This makes AR in these strains less practically relevant from a public health standpoint. However, when animal and human clinical isolates of *E. coli* O157:H7 have been tested for antibiotic susceptibility, resistance against tetracycline, streptomycin, and sulfamethoxazole is most commonly found. While the subject of innate stress resistance is outside the scope of this chapter, it is a noteworthy characteristic of *E. coli* O157:H7. A major outbreak of *E. coli* O157:H7 illness was associated with unpasteurized apple juice, an acidic product in which conventional knowledge regarding *E. coli* deemed that this pathogen would not survive. However, the enhanced ability of *E. coli* O157:H7 to survive acidic conditions by acid stress response mechanisms is an innate quality and has been confirmed in multiple studies (111–114). The vast majority of *E. coli* strains are nonpathogenic and are part of the natural microbiota of many animals, including humans.

E. coli organisms make up about 1% of the colonic microflora (115–117).

A European Food Safety Authority (EFSA) Community Summary Report (2010) on antimicrobial resistance in zoonotic and indicator bacteria from animals and food in the European Union from 2004 to 2007 indicated that resistance to antimicrobials was common among indicator (commensal) *E. coli* organisms, with resistance being greater among isolates from fowl and pigs than cattle and varying among fowl and pig isolates from the 20 member states providing information (118). In North America, Alexander et al. (119) investigated the effect of subtherapeutic use of chlortetracycline plus sulfamethazine, chlortetracycline, virginiamycin, monensin, and tylosin on the development of AR in *E. coli* in fecal samples from feedlot steers (n = 300) in Canada. They found that cattle not receiving antimicrobials prior to arrival at feedlots were carrying *E. coli* strains that were resistant to tetracycline and ampicillin and that clonal dissemination of ampicillin-resistant *E. coli* appeared to readily occur between animals within the same pen. They also found that administering subtherapeutic levels of chlortetracycline in combination with sulfamethazine increased the prevalence and cell numbers of tetracycline- and ampicillin-resistant *E. coli*, and tetracycline-resistant *E. coli* populations were higher in animals fed a grain-based diet than in those fed a silage-based diet. The investigators concluded that the GI and environmental factors influencing the development and dissemination of antimicrobial-resistant *E. coli* in feedlot cattle are complex and that other factors in addition to the use of antimicrobials need to be considered when assessing the acquisition of AR by bacteria, such as relationships between environmental stressors and AR genes.

Morley et al. (120) compared differences in AR between non-type-specific *E. coli* (NTSEC) isolates and *S. enterica* isolates recovered from pen floors of feedlot cattle in Colorado raised without exposure to antimicrobial drugs (40 pens and 4,557 animals) and those reared using conventional practices (44 pens and 4,913

animals). They determined that overall, the prevalence of resistance among all NTSEC isolates was very low (<1% for 9 of 15 drugs, 1 to 5% for more than 2 drugs, 6 to 15% for 2 drugs, and >15% for only 1 drug, including antibiotics of interest in zoonotic transmission to humans, e.g., potentiated penicillins, cephalosporins, quinolones, and aminoglycosides) and that conventional feedlot production methods predictably or uniformly increased the prevalence of antimicrobial resistance among fecal NTSEC isolates compared with rearing methods that restrict exposure to antimicrobial drugs. The investigators suggested that it is possible that the impacts of different antimicrobial drug exposures were overwhelmed by environmental exposures. Marshall et al. (115) concluded that although there is strong evidence for direct transfer of AR traits from animal- to human-associated microorganisms, there is still little direct evidence of transfer of resistance genes from animal-associated *E. coli*, *Bacteroides*, or *Enterococcus* spp. into comparable human microflora members, which subsequently resulted in ART infections.

The EFSA Community Summary Report indicated that *E. coli* is thought to be a reservoir of resistance genes that may be transferred to pathogens and that resistance in *E. coli* over time may serve as an "early warning system" for resistance in potentially pathogenic bacteria (118). However, recent studies have revealed that although nonpathogenic *E. coli* strains are commensals of the GI tracts of animals and humans, they are not the major and earliest carriers of AR genes in various ecosystems from foods to the human gut microflora (4, 87, 121). Hence, proper selection of an AR indicator should be based on a comprehensive understanding of the AR ecology, which is described below.

Staphylococcus aureus

Toxin production by large cell numbers of *S. aureus* causes foodborne illness; ingestion of the bacterium itself does not. There has been much publicity in the medical community and among the general public regarding MRSA. MRSA usually causes health care-

related infection rather than foodborne illness, although recent reports suggest potential zoonotic (or foodborne) transmission due to the finding of common genotypes in both animal and human MRSA isolates (122). However, since there are no reports of increased toxin production by MRSA, while this pathogen is of great clinical significance, its AR has no effect on staphylococcal food poisoning.

Foodborne Commensal Bacteria and AR

Although the idea that commensal members of the microflora might play an important role in the development of resistance in bacterial pathogens was raised at least 40 years ago, this concept did not receive serious attention and there was lack of strong scientific evidence supporting this hypothesis. From a regulatory agency's perspective, the immediate threat of AR to human health is due to pathogens, not commensals; therefore, studies on ART commensal bacteria have received minimal attention compared to AR of pathogens. In addition, AR was not considered a major health problem when new antibiotics were developed on a regular basis. Moreover, a technical challenge for microbiologists is the lack of methods to culture all the bacterial species in complex samples and determine gene transmission by such members of the microbiota to enable the measurement of the overall impact of unidentified commensal bacteria on AR dissemination. It has been much more direct to study mechanisms of AR in designated bacterial species, such as particular pathogens or opportunistic pathogens, including *Salmonella*, *Campylobacter*, *E. coli*, enterococci, etc., than in commensal bacteria. Therefore, for a long time, the influence of commensal bacterial populations on AR dissemination, and particularly the size and breadth of the resistant population and the spectrum of bacteria involved, has remained unknown.

Roles of Commensal Bacteria in AR Dissemination

Andremont (3) reported that horizontal transmission of AR genes between commensal and pathogenic microorganisms in ecosystems

is a much more likely event than direct gene dissemination from one pathogen to another. AR gene reservoirs in commensal microbes in various environmental and host ecosystems have been identified (123–128). Nandi et al. (124) determined that Grampositive bacteria, not Gram-negative members of the *Enterobacteriaceae* such as *E. coli*, served as a major reservoir of class 1 AR integrons in poultry litter. More specifically, Luo et al. (129) determined that an inherited, clumpingassociated high-frequency conjugation system, previously considered beneficial for disseminating fermentation-related traits in *Lactococcus lactis* (a beneficial bacterium known for its application in dairy fermentation), can enhance the transmission of a broad-host-range drug-resistant plasmid, pAM β1, more than 10,000 times in laboratory settings. Since similar clumping-associated high-frequency conjugation systems were previously detected in *Enterococcus*, *Bacillus*, and *Lactobacillus*, this finding likely has broad biological implications. This study revealed for the first time that commensal bacteria could serve as not only a reservoir of AR genes but also a facilitator of their dissemination. Furthermore, it indicates that beneficial bacteria, including fermentation starter cultures and probiotic microbes, are not exempt from AR gene dissemination.

Magnitude and Spectrum of the Commensal ART Bacteria

Data from the past decade have revealed that ART bacteria are prevalent in commercial cheese and other retail food products, including seafood, meat, poultry, produce, and delicatessen and restaurant foods (4, 88, 121). A survey conducted during 2004 to 2005 with samples from grocery chain stores in Columbus, OH, involving multiple types and brands of commercial cheese products revealed that most of the cheeses contained large numbers of ART bacterial cells, with *tet* and *erm* genes being detected in about 10% of the isolates assayed (4). As many as 10^7 copies of AR genes/g of food were detected in retail cheeses before 2007, and AR gene

carriers included a broad spectrum of commensal bacteria, including *Streptococcus thermophilus*, *L. lactis*, *Enterococcus* spp., and *Pseudomonas* spp., with some being used as fermentation starter cultures (4, 121, 130). Due to limitations of the cultivation conditions that were used, the reported data were likely an underestimate of the actual AR status of the commensal bacteria in the samples. Kastner et al. (131) also reported that starter culture and probiotic strains used in fermented dairy and meat products contained AR-encoding genes. A study by Durán and Marshall (132) revealed the existence of a broad spectrum of ART commensals and pathogenic bacteria in ready-to-eat shrimp products. Because many of these products were directly consumed without further processing, the data suggest that members of the public who consumed these products were often exposed to high levels of ART bacteria, including AR genes, even without being exposed to clinical antibiotic treatments. The use of new methods in lieu of the standard selective enrichment-based approach played a key role in more accurately assessing the extent of AR in the commensal bacterial population. For instance, instead of testing for antibiotic susceptibility of individual isolates, Durán and Marshall (132) applied an antibiotic disk diffusion method directly to food samples and successfully isolated a large number of ART bacteria associated with shrimp products. The resistant bacteria were further identified by gas chromatography fatty acid methyl ester and API tests, revealing a broad spectrum of ART commensal and pathogenic bacteria. Using a direct-plating method supplemented with DNA profiling techniques, such as 16S rRNA gene sequence analysis and denaturing gel gradient gel electrophoresis coupled with 16S rRNA gene fragment sequence analysis, the Wang research group isolated a wide variety of ART commensal bacteria from dairy, seafood, meat, produce, and delicatessen and restaurant foods (4, 88, 121). These new methods are also applicable to detecting ART bacteria in a variety of hosts and environmental ecosystems.

AR Amplification, Dissemination, Evolution, and Persistence in Microbial Ecosystems

Animal and human fecal wastes are by far the most significant sources of ART bacteria (124, 133, 134). However, contrary to common belief, the initial and rapid development of the ART bacterial populations in the host GI tract, at least in the case of Tet^r bacteria, likely no longer results from exposure to antibiotic treatment as well as food intake (135). Zhang et al. (87) reported that Tetr bacteria and a *tetM* gene pool developed rapidly within days following natural birth in the GI tracts of infants without their being exposed to antibiotic treatment. Bacteria resistant to additional antibiotics have also been found to be prevalent in infant fecal samples. The investigators concluded that the oral-nasal exposure to mother's vaginal and skin microbiota through natural birth and breastfeeding may be routes for seeding resistant bacteria in the GI tract. Furthermore, since the inoculated resistant bacteria were much less numerous than those excreted through feces, the resistant bacteria must have been significantly enriched in the gut, even without exposure to antibiotics (87). Stanton et al. (136) reported the abundance of chlortetracycline-resistant anaerobic bacteria in fecal samples from organically raised swine, but the number was significantly lower in fecal samples from feral swine.

ART bacteria were also found in poultry without the corresponding antibiotic treatment (137). These data illustrated the prevalence and abundance of ART bacteria in the gastrointestinal tracts of animals lacking direct exposure to antibiotics, as well as the potential impact of human activities, environmental factors, and also possibly host specificity on AR development. In an early study involving home-bred hamsters, not only were Tet^r bacteria isolated from both parents and their offspring, none of which had been treated with antibiotics, but the Tet^r gene patterns from Tet^r isolates from the baby hamsters were similar to those of the mother but different from those of the father (138). These data suggest that the ART bacteria initially found in the infant GI tract were acquired from the

mother, through either the birth canal or other contact that eventually resulted in oral exposure of the infants, independent of therapeutic antibiotic exposure and regular food intake. This conclusion is in agreement with recent data on the development of the microbiota in animal and human GI tracts, suggesting that transmission of both specific pathogens such as *Salmonella* spp. and the general microbial population is largely from mother to infant (139–141). This is also in agreement with findings from a study of a remote Bolivian community in which ART bacteria were isolated from 73% of the population's fecal samples (142), although antibiotics were not used in agriculture and were rarely used by humans in the community. ART bacteria colonizing the host digestive system can further serve as an "immobilized" source of AR genes for potential HGT events, regardless of the presence of antibiotics. A constant supply of large populations of AR gene-containing bacteria acquired through daily food consumption, coupled with occasional colonization and HGT events, will inevitably have an impact on the AR status of the host gut microflora (4).

Jacobsen et al. observed that, after inoculating a tetracycline-resistant *Lactobacillus plantarum* strain and a nonresistant *Enterococcus faecalis* strain into the digestive tracts of germ-free rats, HGT occurred even in the absence of selective pressure and that the administration of tetracycline caused only a slight increase in transmission of AR (143). Evidence also suggested AR gene transmission among *Bacteroides* spp. and bacteria of other genera in the human colon, indicating that the colon likely provides a suitable environment for HGT events to occur (144). Based on quantitative analysis, the cell populations of ART bacteria in fecal samples were much greater than those in foods consumed during daily food intake, suggesting that ART bacteria were enriched in the host GI tract, even in the absence of antibiotic exposure (5, 87, 145). Once released into the environment, these ART bacteria can then spread to other human and animal hosts through contact with water, food, and the environment (146–149). Hence, proper waste

treatment is a critical control point for targeted mitigation of ART bacteria (5, 145).

Many human pathogens and their ART derivatives do not cause disease in animal hosts, such as *L. monocytogenes*, *Salmonella*, and *E. coli* O157:H7, but can cause disease in humans when consumed via raw, smoked, fermented, or undercooked foods. Marshall et al. (117) reported that there was strong evidence for direct transmission of AR traits from animal- to human-associated microorganisms, such as *Salmonella*, *Vibrio*, *Campylobacter*, *Yersinia*, and *Listeria*. Kieke et al. (150) observed that exposure to poultry was associated with acquisition of a quinupristin-dalfopristin resistance gene and inducible quinupristin-dalfopristin resistance in human fecal *E. faecium*. Johnson et al. (151) determined that many human-source drug-resistant fecal *E. coli* isolates in the U.S. population were more likely to have originated from poultry than from humans, whereas drugresistant poultry isolates likely were derived from drug-susceptible poultry isolates. Stanton and Humphrey (152) isolated Tet^r *Megasphaera elsdenii* strains having a novel mosaic gene containing hybrids of *tetO* and *tetW* from swine fed with or without chlortetracycline. Their results revealed a likely role of commensal bacteria not only in the preservation and dissemination of AR in the intestinal tract but also in the evolution of resistance.

A significant portion of AR genes from resistant bacteria are quite stable in the absence of antibiotic selective pressure (88). Some AR genes have been integrated into the host genome, and others may reside on plasmids with special stabilization mechanisms (5). For example, a pRE25-like plasmid with the toxin-antitoxin (TA) stabilization system has been found to be widely distributed among *Enterococcus* spp. and other bacteria (153–157). The TA system was also found to be responsible for persistence of the *vanA*-bearing plasmid in vancomycinresistant enterococci isolated from the clinical and farm environments (153, 157). In addition, a TA-independent plasmid stabilization mechanism is likely responsible

for the persistence of plasmid pM7M2, although the plasmid is closely related to pRE25 (158).

ANTIMICROBIAL USES IN THE FOOD CHAIN AND RESISTANCE

Besides antibiotics, microorganisms often encounter many antimicrobial hurdles during the journey from farm to fork. Sanitizers and disinfectants such as quaternary ammonia compounds (QACs), chlorine-containing agents, and peroxyacids are often used in the food system, from production to processing, and in household environments. During food processing, raw meats may be decontaminated with a variety of physical processes or acidic rinses. Fruits and vegetables may be washed in chlorine rinses and/or packed in modified-atmosphere environments. While antibiotics are not permitted in food products, whether as residues from production or as a means to inhibit the growth of pathogens and spoilage organisms in the final products, various food antimicrobials are added to food to control the growth of both pathogens and spoilage microbes. Many natural food antimicrobials, such as nisin, lysozyme, lactoferrin, essential oils, and organic acids, not classified as antibiotics, have been used in food products (159). Triclosans are used in food processing plants and are now being introduced into many consumer products. Some of these antimicrobial compounds may be removed by washing or inactivated by consumers before consumption; others may be consumed along with the foods.

Microbial Resistance to Food Antimicrobials, Sanitizers, and Disinfectants

Overall, microbial resistance to food antimicrobials, sanitizers, and disinfectants is less well understood and more difficult to characterize and quantify than resistance to antibiotics, and standardized methods have not been developed, nor are surveillance

systems in place. Active export mechanisms contribute in part to bacterial resistance to many agents, including antibiotics, but their potential involvement in bacteriocin resistance has not been reported. However, strains of nisin-producing *L. lactis* contain immunity mechanisms encoded by *nisI* and *nisFEG*, which are located on a transposon (160, 161). Acquisition of the immunity gene(s) can lead to acquired nisin resistance in recipient cells (162). The intracellular accumulation of protons from organic acids acidifies the cytoplasm, inhibiting many metabolic processes. The cell tries to maintain pH homeostasis by pumping these protons out of the cytoplasm using ATP as an energy source. The resultant energy depletion is a major factor in the inhibition caused by organic acids (163). Cells have numerous mechanisms, some of which are inducible, to survive in acidic environments, including proton pumping, changes in the cell envelope, and the production of acid shock proteins and chaperones, as well as the generation of alkali in the immediate external environment (164, 165). In addition, degradation of preservatives by enzymes, either specialized or general, but different from the enzymes that inactivate antibiotics, may also contribute to resistance to antimicrobials. For example, some bacteria metabolize citric acid, rendering it ineffective in their presence. Many proteases inactivate bacteriocins in a nonspecific fashion.

Heir et al. (166) investigated the molecular epidemiology and disinfectant susceptibility of *L. monocytogenes* isolates from food processing plants and human infections. The prevalence of QAC-resistant *L. monocytogenes* was much higher in isolates from food processing facilities than in clinical isolates. While QAC resistance could increase the prevalence of *L. monocytogenes* in the processing plant, these strains or serotypes may not be the dominant causes of human listeriosis. No correlation between resistance and pulsed-field gel elec Heir et al. (166) investigated the molecular epidemiology and disinfectant susceptibility of *L. monocytogenes* isolates from food processing plants and human infections. The prevalence of QAC-resistant *L. monocytogenes* was much higher in

isolates from food processing facilities than in clinical isolates. While QAC resistance could increase the prevalence of *L. monocytogenes* in the processing plant, these strains or serotypes may not be the dominant causes of human listeriosis. No correlation between resistance and pulsed-field gel electrophoresis profile or between resistance and persistence was observed. Isolates of *L. monocytogenes* resistant to the bacteriocin nisin have been reported and characterized. While the genetic determinant of the resistance is currently unknown, these strains typically have a more rigid cell membrane, which may physically impair attachment or insertion of nisin molecules (167, 168). Nisin is approved for use in some foods to prevent clostridial outgrowth but not to control *L. monocytogenes*. It is not known if nisin resistance results in increased survival of *L. monocytogenes* in foods treated with nisin and potentially increased foodborne illness. Some strains of *S. aureus* are resistant to QACs, due to either plasmid acquisition or chromosomal alteration that results in efflux of the sanitizer (169–171). While this may help the pathogen persist in the food processing environment, most cases of foodborne illness related to *S. aureus* are related to postprocessing contamination, making the industrial significance of QAC resistance questionable. While some *L. monocytogenes* and *S. aureus* strains were described as "resistant" to QACs, the levels of QACs at which "resistance" was observed are severalfold lower than what is used industrially (172). While these organisms may be classified as resistant in a laboratory, they are likely still susceptible to QACs at the levels normally used in food processing plants.

Adaptation to Stressors at Sublethal Levels

Microbes may be innately resistant to certain food antimicrobials, but sensitive organisms do not typically mutate or acquire resistance to food antimicrobials. However, exposure to subinhibitory levels of antimicrobials may cause temporary adaptation, so that subsequent exposure to levels that are normally lethal is less effective. "Bacterial adaptation" is the term used to

describe temporary phenotypic changes in response to stress. Adaptation is more common in response to food antimicrobial agents than antibiotics. New genetic material is not required for bacteria to adapt; instead, the stressor quickly activates certain existing pathways and mechanisms to produce a physiological response that helps the organism withstand the stressor. In contrast to the antibiotic selecting for resistance, in the case of adaptation, the antimicrobial causes the observed resistance.

Some mechanisms of adaptation are known. The synthesis of stress response proteins, including heat shock proteins, is triggered by low levels of the stressor, and alternative sigma factors are involved in the process. These stress response proteins protect the cells from subsequent related or unrelated stressors. In many cases, these proteins have a general protective function. In addition, the alternative sigma factors are involved in regulating additional metabolic pathways, likely with universal impact. For example, exposure to low levels of acid may activate the stress response system, protecting cells from subsequent heat exposure as well as acid exposure (173). Quorum sensing allows cells to communicate with each other and exchange information in a nongenetic fashion. Signal compounds secreted by stressed cells may also be sensed by surrounding cells, including the nonstressed progenies, and impact the corresponding gene expression, until the signal becomes sufficiently diluted.

Cross-Resistance and Coselection

The ability of a bacterial strain to display resistance to more than one antimicrobial can also be due to cross-resistance or coselection. In cross-resistance, the multidrug resistance may be due to a single resistance mechanism that is effective against multiple antibiotics. For example, an alteration in a cellular target may make a cell resistant to allantibiotics that act on that target; acquiring a general drug efflux may increase the resistance to several antibiotics. In cases of coselection, the microbe may be resistant to a number of different antibiotics, each with a distinct mechanism of action. The

different resistance determinants are generally genetically linked. This also applies to resistance against antibiotics, heavy metals, and other biocides (174–177). Further, as mentioned previously, exposure to subtherapeutic drug levels may increase the mutation rate and lead to multidrug resistance (59). In a farm study, Alexander et al. (119) evaluated the effect of subtherapeutic administration of antibiotics on the prevalence of antibiotic-resistant *E. coli* in feedlot cattle, finding that subtherapeutic administration of tetracycline in combination with sulfamethazine increased the prevalence of tetracycline- and ampicillin-resistant *E. coli*. However, because this resistance may also be related to additional factors, such as diet and additional oral exposure through environmental contacts, it is important to characterize the corresponding molecular mechanism(s) and confirm the correlation by means of well-controlled laboratory studies. The multiple-AR (*mar*) operon, which controls the expression of chromosomal genes involved in intrinsic multidrug susceptibility and resistance in *Enterobacteriaceae*, is of special interest in food microbiology. This global regulator (*marRAB*) can be expressed by a number of pathogens, including *E. coli* O157:H7 and *Salmonella* serovar Enteritidis. Activation of the operon in *Salmonella* serovar Enteritidis by chlorine, sodium nitrite, sodium benzoate, or acetic acid, all of which are used by the food industry, causes increased resistance to antibiotics such as tetracycline and ciprofloxacin (178). Some sanitation agents and disinfectants may also cause coresistance to other antimicrobial compounds, such as antibiotics.

A review by Hegstad et al. (179) cites several studies that examine the relationship between bacterial resistance to QACs and clinically important antibiotics. The cross-resistance can be attributed to nonspecific resistance mechanisms, such as altered membrane composition and efflux pumps. An EFSA panel on biological hazards considered the possible effects of chlorine dioxide, acidified sodium chlorite, trisodium phosphate, and peroxyacids as used for poultry carcass decontamination. The EFSA scientific opinion is that there are currently no published data supporting the conclusion that

application of these antimicrobials under the proposed conditions of use will lead to acquired reduced susceptibility to these substances or lead to resistance to therapeutic antimicrobials (180).

A few studies have examined the response of ART bacteria to food processing treatments, such as heat. ART *Salmonella* and *L. monocytogenes* are as sensitive to heat as their drug-susceptible counterparts (173, 181). However, there is a relationship between AR and heat resistance: activation of the AR confers increased protection against heat (173). This can be explained by the AR mechanism.

The induction of stress response proteins (due to exposure to acid) results in increased heat resistance, in which stress response proteins also play a role. This exemplifies the need to examine situations of cross-resistance on a case-by-case basis; broad assumptions about cross-resistance cannot be made without an understanding of the mechanism(s).

One study has linked nisin resistance with AR (182). *S. aureus* nisin-resistant isolates were resistant to antibiotics. The MICs of the antibiotics increased 4-fold in the nisin-resistant strains (183). However, similar studies with *L. monocytogenes* revealed no significant increase in resistance to antibiotics (184, 185). Nisin-resistant *L. monocytogenes* and *Clostridium botulinum* isolates are more sensitive to food preservatives, such as low pH, salt, sodium nitrite, and potassium sorbate, than nisin-susceptible isolates (168). Their increased acid sensitivity is caused by increased ATPase activity, which decreases intracellular energy stores (163, 186, 187). Alonso-Hernando et al. (188) also examined cross-resistance between decontaminants and antibiotics in strains of *L. monocytogenes* and *Salmonella* serovars Enteritidis and Typhimurium. Although standard deviations were not provided, the data appear to reveal little or no increase in the MICs of the pathogens to peroxyacetic acid or trisodium phosphate. However, for acidified sodium chlorite (ASC), the MIC increased between 1.88- and 2.71-fold. Exposure to sublethal concentrations of ASC

was related to cephalothin resistance in one strain of *L. monocytogenes* and one strain of *Salmonella*. For both strains of *Listeria* but neither of the *Salmonella* strains, ASC exposure was associated with increased resistance to chloramphenicol. For both *Salmonella* serovars, ASC exposure was associated with increased streptomycin resistance. The authors cited previously identified mechanistic relationships but did not speculate on mechanisms that may cause the cross-resistance observed in this study.

COMPREHENSIVE AND TARGETED MITIGATION OF FOODBORNE AR AND GUT MICROBIOTA DYSBIOSIS

After decades of problematic practices, AR has become a broad and complicated ecological issue impacting many segments of human society. The applications of antibiotics in food animal, crop, and aquaculture production, especially oral drug administration in animals, contribute to AR evolution as well as its enrichment, dissemination, and persistence in the ecosystem; conventional food intake serves as a key avenue connecting the host and environmental microbiota, and AR-rich feces have prominent impact on the environmental AR gene pool. Thus, targeted control of foodborne AR becomes critically important for effective mitigation of AR in the ecosystem. However, simply stopping the growth-promoting uses of antibiotics is not sufficient to get rid of the AR genes and ART bacteria that have already emerged and continue to persist in animals, plants, and the environment. Antibiotic applications in agriculture essential for infection prevention and treatment, particularly administered through feed and water in food animal production, still contribute to the increasing AR gene pool in feces and the ecosystem.

As illustrated in Fig. 34.1, the identification of critical control points throughout the food chain is essential for targeted AR mitigation. In food animal production, it is important to (i) enhance food

processing treatment at the manufacturer and consumer levels to inactivate ART bacteria and pathogens, minimizing the direct introduction of ART bacteria into the host GI tract; (ii) reduce the need for antibiotic applications, such as by prompt infection detection and improving facility, sanitation, and production management to reduce infections and the spread of diseases; (iii) change the antibiotic administration route and select drugs with proper excretion routes to avoid unnecessary exposure of the gut microbiota to antibiotics; (iv) establish effective waste management and processing practices to inactivate ART bacteria and pathogens; and (v) regulate the safety of biocontrol approaches. A significant percentage of the probiotics marketed for food animal applications still contain AR genes. The application of these probiotics as growth promoters facilitates the dissemination of AR genes in the agricultural and aquaculture production system, rather than reducing it.

The new approaches have already led to impressive outcomes. Following the knowledge breakthrough on the potential involvement of lactic acid bacteria and even probiotics in AR ecology (4, 129), an effective collaboration between academia and industry led to the rapid identification of the risk factors in cheese fermentation and removal of the problematic cultures from the market. Data from a follow-up study illustrated that effective mitigation of the largest AR gene pool in the food chain was achieved in just 4 years (2006 to 2010) (121). The European Union has already established safety screening standards for bacterial strains intended for beneficial applications, and China is in the process of adopting such standards. The same strategy needs to be adopted by the United States and should cover probiotics for agricultural and aquaculture production as well. The use of bacterial phages is another proposed strategy to eliminate pathogens in the food chain. However, bacterial phages have been extensively implicated in bacterial (including pathogen) evolution through HGT. Therefore, extreme caution should be used regarding their

application, to avoid another round of unforeseen detrimental consequences, as happened with antibiotics.

Although eliminating ART bacteria from the food production environment is impractical, it is possible to interrupt the transmission of foodborne ART bacteria through conventional food intake by proper food processing. The reduced prevalence of ART bacteria in both retail meat and poultry as well as fresh produce in recent years is in fact an encouragement for improving microbial inactivation at the food processing level in processing plants. Proper education to further minimize foodborne ART bacteria ingestion through proper cooking and other processing by consumers is another essential strategy.

In summary, the misuses of antibiotics in human medicine and food animal production have a paramount impact on AR ecology. Systematic investigation for a comprehensive understanding of risk factors of AR and the development of targeted controls are essential for effective AR mitigation in the food chain as well as in medicine. Proper detection approaches and a predictive surveillance system for early warning are critical components for accurate risk forecast, assessment, and reporting. Innovative strategies are further needed to mitigate persistent AR in the hosts and the environment, as well as gut microbiota dysbiosis and modern diseases. Food microbiologists are well positioned for the mission to improve food safety and public health.

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35
Genomics of Foodborne Microorganisms

The genomics and postgenomics era has transformed microbiological and molecular biology research, and genomic analysis is emerging as an important tool in food microbiology and safety. Microbiologists investigating foodborne pathogens, probiotics, starter cultures, and recently the intricate microbiota of a food system are relying more and more on DNA sequencing technologies, genomic analysis, and metagenomic techniques. Foodborne illness represents an important economic and public health burden worldwide, and food contamination can occur at any step along the food supply chain. It is critical to be able to detect microbes with high sensitivity and reliability for surveillance monitoring and to prevent further outbreaks. Traditional methods based on culturing or biochemical tests are often laborious and time-consuming and have limited sensitivity. Testing procedures based on DNA sequencing methods have accelerated pathogen detection, improved consistency, and allowed accurate species identification during outbreaks. Indeed, as outlined in this chapter, genomics-based methods are currently being implemented for foodborne-organism surveillance and tracing. In the early days of DNA sequencing, efforts primarily focused on pathogen detection and on the content of pathogen genomes. More recently, however, analyzing the genomes of probiotic microbes for their potential functional food development has also gained increasing interest. Additionally, advances in high-throughput DNA sequencing technologies, and particularly bioinformatics capabilities, have impacted the available resources for the investigation of the

microbiota in food processing environments or within a complex food matrix.

In an earlier edition of this book, the genomic and proteomic advances in the area of food microbiology were described. Since then, there have been significant developments in high-throughput technologies, metagenomics, and computational capabilities. These are reflected in this updated chapter. We also outline the large-scale use of sequencing technologies for outbreak surveillance, and we look specifically at documented examples of how genomics has expanded our knowledge of foodborne microorganisms and its impact on the food industry and food microbiological research in recent years.

ADVANCES IN DNA SEQUENCING

There have been many changes in DNA sequencing technologies over the last 20 years, the most dramatic being a movement from the sequencing of single stretches of DNA at a time to the in-parallel sequencing of millions of reads, with a large reduction in sequencing costs. These have impacted all areas of microbial genomics, including whole-genome sequencing (WGS), 16S rRNA compositional and metagenomics analysis, and gene expression studies, with the development of direct sequencing of RNA (RNA-Seq) and transcriptomic technologies, all discussed below. Over the years, improvements in sequencing output, accuracy, and speed have generally been defined as first-, second-, and third-generation technologies, the basics of which are briefly outlined below. More extensive overviews of DNA sequencing technology platforms can be found elsewhere (1, 2).

First-Generation (Sanger) Sequencing

First-generation sequencing was based primarily on the Sanger approach, which relies on the incorporation of chain-terminating dideoxynucleotides during the DNA replication process (3).

Automated Sanger sequencing was later introduced by Applied Biosystems through fluorescent base labeling. The first completed bacterial genome sequenced using this method was the 1.83-Mb genome of *Haemophilus influenzae* in 1995 (4). This was a time-consuming and labor-intensive approach, as the genomic DNA of the bacterium was isolated, sheared, and subsequently cloned into a plasmid vector for sequencing. Despite this, however, hundreds more bacterial genomes were sequenced by this method in the following years, including those of *Mycobacterium tuberculosis*, *Escherichia coli*, *Bacillus subtilis*, and the first foodborne pathogen, *Campylobacter jejuni*, in 1999 (5).

Second-Generation Sequencing

Second- or next-generation sequencing (NGS) technologies are capable of massively parallel sequencing, producing millions of reads in one run, in contrast to Sanger sequencing, which can produce just 96 reads per run. NGS technologies differ from conventional (first-generation) sequencing in the basic chemistry. Additionally, they generate a much larger base output and have a reduced DNA concentration requirement. The first NGS sequencer was launched by 454 Life Sciences in 2005 and was based on a pyrosequencing "sequencing by synthesis" technology. This technology is based on a series of enzymes to detect the nucleic acid bases incorporated during strand synthesis. Following the initial launch of the 454 GS20 pyrosequencing platform in 2005, there were many technology advancements over the years, including machine upgrades (GS FLX and GS Junior). This approach was used for many applications, including WGS (*de novo* sequencing and resequencing), metagenomic sequencing, and transcriptomics. Indeed, 454 pyrosequencing was central to the completion of the human microbiome project (6). Despite this, however, the 454 technology could not compete with other NGS technologies that subsequently emerged on the market over the last 10 years and that could produce greater outputs at a reduced cost.

Currently, the market-leading NGS technologies include those offered by Illumina Technologies. Since their launch of their first sequencer in 2008, Illumina sequencing output has grown over 1,000-fold, from less than 1 Gb to 6 Tb per run, while the number of reads has increased from millions to billions. Initial read lengths were typically between 35 and 70 nucleotides, but these have been extended in recent years, with 2 □□300 bp paired-end sequencing available with the MiSeq V3 sequencing kit (https://www.illumina.com/systems/sequencing-platforms/miseq.html). Illumina's sequencing platform utilizes ligation of adapters to fragmented single-stranded DNA, but the fragments are then attached in isolated spots on a solid surface that contains primers complementary to the adapters. After PCR, the solid surface contains millions of isolated colonies of amplified single-stranded DNA (7). Illumina currently offers a range of machines within their benchtop MiniSeq, MiSeq, and NextSeq series and the large-scale HiSeq and NovaSeq series (www.illumina.com). The repertoire of machines provides a range of potential applications, including small-scale WGS on the MiniSeq and MiSeq machines, exome sequencing on the NextSeq instruments, and metagenomics studies on the MiSeq and NextSeq series, while the HiSeq and NovaSeq machines are generally used for large (nonmicrobial) genomic projects, as well as RNA-Seq and other projects (8).

There are also a number of sequencers offered by Thermo Fisher Scientific based on ion semiconductor sequencing (9) (www.thermofisher.com/ie/en/home/brands/ion-torrent.html). Ion torrent technology is based on the principle of detection of released hydrogen ions during the incorporation of new nucleotides into a growing DNA template. Depending on the chip used, the Ion PGM system can run up to 5.5 million reads, with an output of 2 Gb and a read length of 400 bases. The PGM platform is therefore suited for sequencing small genomes or gene sets. The available benchtop machine, the Ion Proton, produces 15 Gb of sequence data with read lengths of 200 bases, which makes the machine suitable for

transcriptomics and exome sequencing. The new Ion GeneStudio platform combines many of the attributes of both of these systems.

Third-Generation (Single-Molecule) Sequencing

Recently, methods that do not require an amplification step have emerged, enabling sequencing of a single molecule and eliminating issues caused by amplification or variability within a strain. These so-called third-generation technologies involve direct sequencing of single DNA molecules and have resulted in much longer sequencing reads than was previously possible. The first singlemolecule sequencer, the HeliScope, developed by Helicos Biosciences, utilized deoxynucleoside triphosphates attached to very bright fluorophores and incorporated a sequencing-by-synthesis method similar to that of the Illumina platform without amplification. Pacific Biosciences delivers its single-molecule real-time technology based on a nanophotonic structure known as a zeromode waveguide. This company developed a method to sequence fragments in zeptoliter wells with fluorophoreassociated deoxynucleoside triphosphates, resulting in sequence reads up to several kilobase pairs in length (10). Oxford Nanopore Technologies, launched in 2014, also has a number of sequencers (MinION, GridION, and PromethION) currently on the market and the promised SmidgION, the smallest device based on nanopore-sensing technology. These third-generation sequencers deliver much longer reads than previously achievable, and although they still have a sizable documented error rate, these technologies are being used in more and more sequencing projects.

GENOMIC ADVANCES IN FOOD MICROBIOLOGY

Pathogen Genomes

With the number of completely sequenced and draft bacterial genomes increasing daily, one powerful approach to defining unique

or conserved gene content and understanding how these microorganisms evolved is comparative genomics, via *in silico* analysis. This was demonstrated in the early years of bacterial genomics, when sequencing of the food pathogen *Listeria monocytogenes* and the nonpathogenic species *Listeria innocua* revealed extensive collinearity (11). It was found that 270 genes (9.5%) were specific to the *L. monocytogenes* genome, whereas only 149 (5%) were specific to the *L. innocua* genome. Many of the *L. monocytogenes*-specific genes were key to survival in the mammalian gut, including those coding for bile salt hydrolases, acid tolerance via glutamic acid decarboxylases, and a virulence locus responsible for infection and motility during pathogenesis. Genomics also revealed that pathogenic *L. monocytogenes* and nonpathogenic *L. innocua* were likely derived from a common ancestor, with the latter subsequently losing most of the genes directing pathogenesis. Therefore, *in silico* comparative analysis of complete genomes can reveal important clues regarding an organism's genetic content, capabilities, and relationships to other microorganisms.

Additionally, other genomic approaches have been used in the identification and characterization of genes of foodborne organisms that confer resistance to environmental stresses. These genes are of interest because they are pivotal to understanding how both foodborne pathogens, such as *E. coli* O157:H7, and potentially beneficial microbes, such as *Lactobacillus* spp. and *Bifidobacterium* spp., survive the dynamic environment of the intestinal tract. Bacteria can adapt and survive in these environments through different mechanisms, including the induction of an acid tolerance response. The best-characterized acid resistance mechanism is the Gad system of *E. coli* (12), first described before the sequence of *E. coli* was published in 1997 (13). The ability to withstand the effects of bile in the intestinal tract is also central to a microbe's capacity to cause and maintain an infection, as bile acts as a detergent that can dissolve the phospholipid cytoplasmic membranes of bacteria, leading to their death (14). *L. monocytogenes* is able to withstand

the presence of high concentrations of bile. Bile salts exist primarily as steroid rings attached through an amide bond to an amino acid group, either glycine or taurine. While the mechanisms of bile tolerance are not fully understood, gene loci such as those for bile salt hydrolases are the most studied, and indeed, disruption of the *bsh* gene in *L. monocytogenes* leads to greater sensitivity to bile (15, 16).

Genomic analysis of *L. monocytogenes* also illustrated the genetic regulation controlling stress resistance in the pathogen. Bioinformatics analysis of the *bsh* promoter region revealed that it is under the control of the transcriptional regulator PrfA, which controls expression of other known virulence genes in *L. monocytogenes* (17). Further analysis revealed that PrfA is regulated by the alternative sigma factor, sigma B, an analogue of RpoS, which is found in Gram-negative bacteria such as *E. coli*. Sigma B is the general stress response sigma factor in Gram-positive bacteria and has a pivotal role in stress resistance and adaptation to the intestinal tract (18, 19). Transcriptomic studies deciphered the sigma B regulon among *L. monocytogenes* isolates (18, 20), identifying many of the genetic determinants responsible for acid, bile, and salt tolerance. Additional transcriptomic analysis by Toledo-Arana et al. elucidated further regulatory complexity within *L. monocytogenes* stress resistance strategies by identifying a diversity of RNAs, including 50 small RNAs and antisense RNAs involved in global transcriptional regulation of virulence (19). RNA-Seq analysis (described below) also was used to identify the genome-wide transcriptome of *L. monocytogenes* following internalization by murine macrophages, also indicating the importance of antisense RNAs to the intracellular growth of this pathogen (21).

Starter Cultures

In recent years, there has also been a surge in the analysis of the genomes of starter cultures of *Lactobacillus* and *Lactococcus* spp. to identify the adaptation of the isolates to a food environment and to

allow the determination of the metabolic capabilities of starters (reviewed in reference 22). Additionally, genome sequence information has provided key insights into the evolution of microorganisms and their adaptation to specific foods (23). *Streptococcus thermophilus* is a lactic acid bacterium used worldwide for the manufacture of yogurt and some hard cheeses. Sequencing the genome of two yogurt strains of *S. thermophilus* and comparison with other streptococci revealed that the organism had undergone extensive rounds of genome decay, with over 10% of the genes being characterized as nonfunctional genes, or "pseudogenes." Notably, with the exception of lactose metabolism, many of the other gene regions associated with carbohydrate transport and metabolism were also degraded. Genes commonly associated with pathogenic streptococci (e.g., *Streptococcus agalactiae* and *Streptococcus pneumoniae*) were either lost or inactivated due to mutations or transposon insertions. Bolotin et al. concluded that *S. thermophilus* has evolved specifically to grow in milk and that, during the adaptation process, much of its genomic complement that is not required for growth in this environment was eliminated or degraded (24).

Probiotic Genomes

Although we have been consuming probiotics for many years, it is only since the advent of DNA sequencing technologies that we have begun to more comprehensively understand the potential mechanisms that confer their benefit to human health. One of the earliest probiotic genome sequences available was that of *Lactobacillus johnsonii* NCC533 (25). Genomic analysis of this organism showed a number of loci important to survival in the gastrointestinal tract, including three for bile salt hydrolases and two for bile acid transporters. Additionally, the genome sequence shows that this commensal lacks loci for the biosynthesis of amino acids but compensates via a plethora of transporters, peptidases, and proteases, suggesting that it relies heavily on its surrounding host for nutrients and metabolism (25). Further genome

sequencing of porcine isolates of *L. johnsonii* indicated large genomic rearrangements and evidence of horizontal gene transfer, suggesting host-specific genetic divergence (26).

In the years following this analysis, numerous complete and draft genomes of strains belonging to species of *Lactobacillus* and *Bifidobacterium* were made available on GenBank to advance the field of probiogenomics (27), and more recently, a "newer generation" of potential probiotics, including strains of *Akkermansia muciniphila* and *Faecalibacterium prausnitzii*, have become widely available for genomic analysis. Indeed, genome-scale metabolic reconstructions have allowed the evaluation of metabolic cross-talk between the gut inhabitants *Bifidobacterium adolescentis* L2-32 and *F. prausnitzii* A2-165 and provided insight into the demand and supply of acetate and butyrate in the gut, which have an important role in cancer prevention and the microbial balance in the intestinal tract (28). Results also suggest a beneficial role for *A. muciniphila* in maintaining the integrity of the mucus layer in the intestine, contributing to the control of glucose homeostasis and insulin resistance (29). Recent whole-genome comparative analysis of 40 genomes of *A. muciniphila* traced the identity of three species-level phylogroups and examined their correlation to body mass index and antidiabetes-drug usage (30).

Overall, the accumulating genomic data that are becoming available for a multitude of potential probiotic species enables analysis of how the strain has adapted to the intestinal tract, its metabolic profile, its technological robustness, and its potential safety for use in foods. In fact, in Europe, any probiotic with potential commercial application is now required by the European Food Safety Authority to undergo full genomic safety assessments before use. A probiotic strain has to be genome sequenced so that it can be analyzed for overall genome stability, the presence of transferable antibiotic resistance loci, potential virulence genes, or genes for adverse metabolites that may compromise the safe use of the strain in food (reviewed in reference 1).

USE OF WGS IN FOODBORNE SURVEILLANCE

The advancements in NGS technologies and the rapid reduction in per-base cost of sequencing have led to the genome sequencing of microbes becoming routine and have allowed even the smallest lab groups to obtain the genome sequence of their organism of interest. WGS is being used more and more in public health laboratories for foodborne-pathogen surveillance and tracing the source of foodborne-illness outbreaks. Potentially, WGS allows the replacement of multiple “wet-lab” procedures with a full genetic blueprint of an organism for full genomic analysis. A number of government-run programs and participating public health laboratories are implementing WGS in surveillance and source tracking for foodborne microorganisms. Some of these initiatives are outlined below.

PulseNet

PulseNet International is an initiative based on the dedicated global laboratory surveillance of foodborne microbes (reviewed in reference 31). At present, it is dedicated to disease surveillance in 86 countries in total, including those in Africa, Asia, Europe, and the Americas. This project is complementary to the Food and Drug Administration (FDA)-driven GenomeTrakr food safety initiative described below. The overall mission is to establish standard genotyping methods to identify and track outbreak strains, and PulseNet currently prevents ~270,000 cases of annual foodborne illnesses due to organisms such as *E. coli* and *L. monocytogenes* in addition to easing the associated financial burden of foodborne illness. Since its conception more than 20 years ago, the main methods of strain detection and identification used by PulseNet have been traditional molecular biology-based methods, primarily pulsed-field gel electrophoresis and multilocus variable tandem repeat analysis on chosen strains where required (32). Although the traditional methods used have indeed been effective in organism

surveillance, sequencing has become a focus of the PulseNet initiative due to lowering DNA sequencing costs in addition to the high level of detail and specificity that can be achieved with WGS. Standardized typing in the form of large-scale WGS and more specifically whole-genome multilocus sequence typing (WG-MLST) is now the vision of the PulseNet program (31).

WG-MLST is a gene-by-gene typing approach allowing MLST on a genomewide scale. Traditional MLST has generally been based on approximately seven housekeeping genes within a genome for which each unique sequence is given an allele number assignment. The drawback of this is that because of the high conservation among housekeeping genes, the technique is suitable only for very closely related organisms and is not useful among species with very low diversity. Additionally, with just seven alleles, it gives only a glimpse of the genetics of a genome and does not take into account the extent of genetic transfer. A genome-wide approach allows analysis of all the loci within an isolate, which can then be compared to the equivalent loci in other isolates; therefore, this method has very large discriminatory power. To achieve this successfully and allow implementation across PulseNet International laboratories, many factors are taken into consideration, including the sequence read set, the storage of data, the deposition of data in public repositories, read assembly, the bioinformatics capabilities required, and the standard strain and allelic nomenclature to be used (31). Overall, the intent of PulseNet International is to standardize a typing method using the discriminatory power of WGS of foodborne organisms to allow dedicated surveillance of foodborne-illness outbreaks globally.

GenomeTrakr

GenomeTrakr is an FDA food safety and traceability initiative consisting of a distributed network of laboratories (university and public health) that collect and share whole-genome sequences for food pathogen identification (https://www.fda.gov/Food/FoodScienceResearch/WholeGenomeS

equencingProgramWGS/ucm363134.htm). Originally a U.S.-focused sequencing initiative dedicated to food microorganisms, GenomeTrakr has expanded internationally in recent years. All sequenced genomes are deposited in public databases at the NCBI (https://www.ncbi.nlm.nih.gov/genbank/) and are fully accessible for analysis of outbreak investigations, with the aim to reduce foodborne illnesses and deaths. The first GenomeTrakr genome was uploaded in February 2013, and currently, almost 5,000 genomes are being added monthly (http://www.fda.gov/Food/FoodScienceResearch/WholeGenomeSequencingProgramWGS/default.htm). At the time of writing, the Genome Trakr network has over 183,000 isolates sequenced, with over 175 closed genomes. Initial efforts by GenomeTrakr member labs focused primarily on pathogenic *Salmonella* and *Listeria* isolates; however, other pathogens are now being added to the network, including strains of *E. coli, Campylobacter, Vibrio*, and *Cronobacter*, as well as parasites and viruses. As the number of genomes increases, the ability to gain insight into outbreak etiology will strengthen. WGS has been specifically applied to the surveillance of *Salmonella enterica* serovar Enteritidis, and it was shown by den Bakker et al. that whole-genome cluster analysis of *Salmonella* isolates largely improved the detection and resolution of outbreak strains compared to the more traditional pulsed-field gel electrophoresis method (33).

The *Listeria* Initiative

In recent years, *Listeria* sequencing has focused on outbreak strains, typing, and traceability. Due to the importance of *Listeria* as a foodborne pathogen, the Centers for Disease Control and Prevention (CDC) launched the *Listeria* Initiative (https://www.cdc.gov/listeria/surveillance/listeria-initiative.html), which is a network dedicated to identifying isolates responsible for outbreaks of listeriosis in the United States. Traditionally, data collected were based on clinical, laboratory, and epidemiologic data, with more recent analyses focusing on genomic data. More

specifically, the focus was initially on a questionnaire for patients and on DNA "fingerprinting" in the form of pulsed-field gel electrophoresis, but since 2013, WGS has been used to type strains (https://www.cdc.gov/listeria/surveillance/listeria-initiative.html). A recent review (34) described how WGS has now been used to trace listeriosis in foods not generally associated with *L. monocytogenes* outbreaks (celery, stone fruits, etc.). As WGS provides the genetic information for the entire genome and as the cost has decreased and the speed of sequencing has increased, it can potentially be used in routine epidemiological investigations.

FOOD METAGENOMICS

Metagenomics, also known as environmental genomics, is the study of genetic material recovered directly from a given environment. This approach allows the gene content of the entire microbial load of an environment or sample to be analyzed without the need for culturing, removing the need for lengthy incubation periods and eliminating the bias towards the small proportion of microorganisms that have been successfully cultured under lab conditions.

While genomic sequencing aims to completely catalog the genetic information within a single food isolate, the aim of metagenomics is to evaluate the composition or function of the entire community of microorganisms and is often used to compare the communities present in different environments or in the same environment under different conditions (Table 35.1). This is achieved by extracting microbial DNA from a sample before sequencing using one of the sequencing technologies described above. The method used to extract, prepare, and sequence this DNA will depend on the goal of the study and the sequencing technologies available. The main factor to consider is whether the composition or the potential function of the microbial community is being examined. The different metagenomics techniques available are outlined in Fig. 35.1.

Compositional Metagenomics

Compositional metagenomics uses a specific marker gene to allow the identification of the individual members of a community and infer their relative abundance within the population. The chosen marker must be present in all microorganisms under consideration, variable enough to provide maximal phylogenetic information, and stable enough to be reliably identified across a broad taxonomic range. The markers most commonly used are the 16S rRNA gene for bacteria and archaea and either the 18S rRNA gene or the internal transcribed spacer sequence for eukaryotes. These marker genes have become the gold standard for their respective domains as, in the years since the development of compositional metagenomics, large databases of marker gene sequences have been assembled against which the sequences generated can be compared. Once the microbial DNA has been extracted, PCR amplicons are generated for the chosen marker before sequencing. The generated reads are then clustered into groups of closely related sequences known as operational taxonomic units and assigned taxonomy based on similarity to sequences in a curated database of marker genes. There are many bioinformatics tools available for analyzing compositional metagenomic data, but two of the most commonly used are mothur (35) and QIIME (36); both are open-source pipelines which connect a large number of bioinformatics programs by transforming their inputs and outputs so that they can be used together. The QIIME pipeline also contains programs for visualizing and interpreting compositional data using methods adapted from classical community ecology, such as diversity indices and multidimensional scaling.

Table 35.1. Summary of the strengths and limitations of genomic technologies

Technology	Description	Strengths	Limitations
Compositional metagenomics	Uses well-characterized marker genes to identify the microorganisms present in a community	Low cost Well-characterized databases	Limited accuracy below genus rank Amplification bias towards specific taxa Marker gene copy number variation between species Compositional information only
Shotgun metagenomics	Uses all DNA present in a sample to identify the microorganisms present and their functional capacity	Resolution to species (and sometimes strain) level Characterizes functional capability Not limited to a single domain	High cost Contamination by host DNA Difficult to identify unknown or poorly characterized species Complex interpretation of results High requirement for data storage and analysis

Metatranscriptomics	Uses all RNA present in a sample to identify metabolically active microorganisms and their functional activity at a specific moment	Functional snapshot (gene expression)	High cost Contamination by host DNA Complex interpretation of results High requirement for data storage and analysis

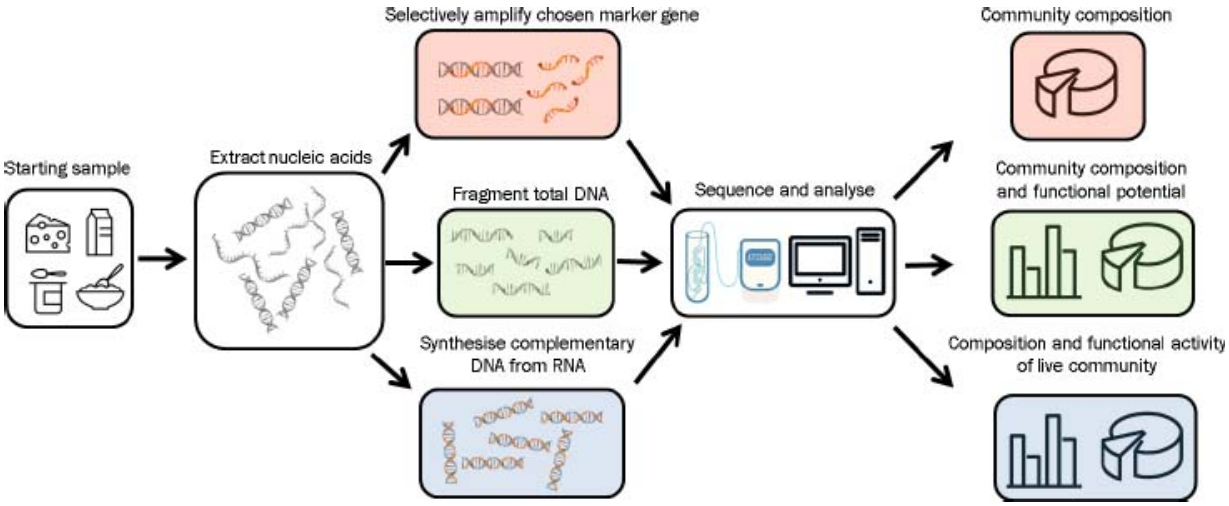


Figure 35.1. Sequencing pipeline for metagenomic and metatranscriptomic analysis from a complex food matrix.

Functional Metagenomics

Functional metagenomics does not rely on marker genes; instead, the total DNA from a sample is randomly fragmented before sequencing, resulting in thousands of short reads. This untargeted method of library preparation led to the approach becoming known as shotgun sequencing. These reads are compared against a database of genes with known functions to gain insight into the functional capacity of the community. The unrestricted approach of

shotgun sequencing means that all microorganisms in a sample are considered, not just those possessing the chosen biomarker gene. Shotgun reads can also be used for compositional profiling of the community and, as they are more heterogeneous than the marker genes typically used, contain greater potential phylogenetic information, enabling classification to lower taxonomic ranks. The HUMAnN pipeline (37) is perhaps the most widely used tool for shotgun data, as it performs both functional and taxonomic profiling. SUPER-FOCUS is another well-known program for functional profiling (38), while KEGG (39), MetaCyc (40), EggNOG (41), and Pfam (42) are all databases that can be used to functionally annotate metagenomic reads. For taxonomic classification, software such as MetaPhlAn (43), Kaiju (44), and Kraken (45) is commonly used.

Use of Metagenomics in Food Microbiology

An example of both comparative and functional metagenomics being utilized in a food setting was in the identification of *Thermus thermophilus* as an agent responsible for the pink discoloration defect in cheese (46). Compositional metagenomics, utilizing both 16S rRNA and shotgun approaches, revealed high proportions of the genus *Thermus* in cheeses with a pink defect but no *Thermus* organisms in unaffected cheeses. A subsequent functional metagenomics approach then noted that genes responsible for the production of carotenoids were identified in defective cheeses only. This led to the isolation of *T. thermophilus* from defective cheeses and the dairy processing environment as well as re-creation of the pinking effect by inoculation of the species (46).

Shotgun sequencing, combined with recently developed bioinformatics tools, shows potential in the future of food safety assessments. For example, an examination of nunu, a traditional Ghanaian drink produced by the spontaneous fermentation of raw cow's milk, highlighted frequent contamination by bacteria associated with the bovine gut and potentially pathogenic strains of *E. coli* and *Klebsiella pneumoniae* (47). Shotgun metagenomics has

also been used to detect foodborne pathogens such as *S. enterica*, *L. monocytogenes*, and *Campylobacter* spp. in the microbiome of the beef production chain (48).

Shotgun metagenomics can also play a preventive role in food safety if employed appropriately. Viral sources of foodborne disease are notoriously difficult to detect and pose a considerable public health risk, particularly relating to seafood and imported fresh food products. Shotgun sequencing has been proven to be an effective method to detect viral DNA from field-grown lettuce (49), and the bioinformatics tool PanPhlAn has proven capable of detecting and characterizing the strain responsible for the German 2011 *E. coli* outbreak from metagenomic data (50).

RNA SEQUENCING

Historically, investigation of the bacterial transcriptome was performed using microarrays, a collection of DNA spots attached to a solid surface which fluoresce upon hybridization to a cDNA strand. This cDNA is reverse transcribed from bacterial RNA, and the level of fluorescence is indicative of RNA abundance and, therefore, expression of the corresponding gene. Microarrays greatly increased the amount of information known about bacterial transcriptomes but are limited to use with known sequences and present technical challenges arising from cross-hybridization and background levels, requiring complex normalization procedures.

RNA-Seq provides many advantages over microarrays, such as removing the need for prior sequence knowledge, eliminating cross-hybridization effects, and allowing better quantification of genes expressed at low and high levels. Similar to shotgun sequencing, these RNA transcripts can be used to identify the community composition in addition to function. The sequences provided by RNA-Seq enable additional information to be obtained in experiments directed at gene expression profiling. For example, while microarrays may be used to discover single nucleotide

polymorphisms (SNPs) through specialized arrays in experiments directed at discovering SNPs, RNA-Seq provides SNP information in all experiments by providing the sequence of each transcript (51). Knowledge of SNPs has been useful for molecular subtyping of *L. monocytogenes*, which may be applied in foodborne-disease investigations (52).

It is important to note that the mRNA transcripts that are most often of interest during transcriptional profiling account for only about 5% of the total RNA. Most of the RNA pool consists of rRNAs and tRNAs, which need to be depleted before sequencing; otherwise, most reads obtained will be housekeeping RNAs rather than the mRNAs or small noncoding RNAs that are of interest. In eukaryotes, mRNA enrichment is easily accomplished by selection for the 3′-polyadenylated tail that is present only on mRNAs. The absence of this tail on prokaryotic mRNA complicates the process required to obtain useful information from RNA-Seq experiments, but several rRNA and tRNA depletion strategies have emerged for prokaryotic RNA-Seq (53).

The aim of many RNA-Seq studies is detection of differentially expressed (DE) genes under different environmental conditions. One of the most widely used tools for detecting these DE genes is DESeq2 (54), a package for the R statistical environment as part of the Bioconductor project (55). This approach uses count data, derived from mRNA transcript numbers, to detect DE genes and quantify the change in expression while compensating for outliers and other problematic features of transcriptomic data. This is just one approach to modeling RNA-Seq data, however; other widely used alternatives are EdgeR (56), BBSeq (57), DSS (58), baySeq (59), and ShrinkBayes (60).

Comparison of the transcriptomes of persistent and nonpersistent *L. monocytogenes* strains from a food processing environment highlighted the role of increased peptidoglycan biosynthesis in benzethonium chlorideresistant strains (61). A subsequent study by the same group also reported that the peptidoglycan biosynthesis

gene was, among other genes, differentially expressed when *L. monocytogenes* was exposed to sublethal concentrations of benzethonium chloride (62). As mentioned above, transcriptomics and RNA-Seq have been used extensively to identify the global transcriptome of *L. monocytogenes*. Additionally, RNA-Seq has also been used to characterize the transcriptome of important food fermentation microbes such as *Aspergillus oryzae*, a mould involved in many oriental food fermentations (63), and investigate the regulatory response of the brewer/baker's yeast *Saccharomyces cerevisiae* to acetic acid (64).

In metatranscriptomic studies, RNA-Seq is applied to microbial communities to measure differences in gene expression between samples under different environmental conditions or over time. For example, a longitudinal RNA-Seq analysis of the lactic acid bacteria present during kimchi fermentation revealed that the microbiome was dominated by six species whose relative abundance changed throughout the fermentation (65). Many genes related to carbohydrate transport and hydrolysis and lactate fermentation were active over the time course. Of particular note to the authors was a high level of *Leuconostoc mesenteroides*-associated mannitol dehydrogenase genes at the early stages of the fermentation, highlighting the species' role in flavor development.

RNA-Seq analysis revealed strong correlations between the abundance of nonstarter lactic acid bacteria and the levels of expression of genes involved in amino acid and fatty acid catabolism in a traditional Italian Caciocavallo Silano cheese, suggesting a role for these bacteria in maturation (66). Similarly, RNA-Seq revealed that genes associated with proteolysis and lipolysis in *Geotrichum candidum* were highly expressed in a surfaceripened cheese, indicating that the fungus is important for flavor development (67). The authors also identified a subset of differentially expressed genes across ripening stages, which suggests that the ripeness of the cheese could be assessed by measuring the expression level of those genes.

CONCLUSIONS

The rise of genomic technologies has led to an explosion of knowledge on both the composition and the functionality of the microorganisms associated with our foods. With this information, it is now possible to understand the complete genetic complement of pathogenic, spoilage, and bioprocessing organisms and determine how environmental conditions affect their survival and the expression of key genetic traits. As outlined in this chapter, genomic sequences are being used to track foodborne pathogens in food and have led to international initiatives that enable outbreak surveillance. Additionally, the availability of the whole-genome sequences of pathogens, starter bacteria, and probiotic strains means that they can be compared for their genetic content and that their pathogenic or potentially beneficial capabilities can be reliably predicted. The advent of metagenomics and transcriptomics has also allowed further understanding of the role of microorganisms in our foods and how they interact in a complex food matrix. There is little doubt that the future of food microbiology will rely heavily on genomics and other "-omics" technologies in the efforts to trace, understand, and control microorganisms associated with our foods.

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36
Metagenomics of Meat and Poultry

Meat and poultry products, the animals that produce them, and the environments they are processed in are biologically diverse and contain many (thousands) species of bacteria, viruses, and fungi arranged in complex living environments. These organisms work together and share resources (such as metabolites), much like a community. Within this community, each organism has its own genome, yet some genes are ubiquitous across the community, while others are more specialized, present in only a few or one single species in the community. Together, the genomes and genes within this community are known as the pangenome (1) (Fig. 36.1). By looking at samples through the context of community-wide interactions, instead of a few selected organisms, we can obtain a more holistic model of what is occurring in a given microbial community. This ecological way of thinking can be incorporated into any biological research, including that related to meat and poultry production.

However, even with advancements in microbiological culture techniques, many organisms within and around meat and livestock products will likely never be cultured in a laboratory. This issue is known as the plate count anomaly, which leads to estimates that from 76% (2) to upward of 99.9% (3) of cells within samples cannot be found with culture plating or enumeration procedures alone. While the vast majority of cells will never be cultured in a laboratory setting, these unculturable bacteria still interact within biological communities, contribute to meat safety and quality, and

affect the dissemination of genes of concern to public health, such as virulence factors. To overcome culture-dependent methods, researchers have started integrating culture-free approaches into traditional studies with techniques such as metagenomics, transcriptomics, proteomics, metabolomics, and whole-genome sequencing (WGS).

This chapter specifically addresses the science of metagenomics and how it is used in meat and poultry production to advance food safety and quality.

OVERVIEW OF METAGENOMICS

Metagenomics is defined as the direct genetic analysis of genomes contained within an environment (such as a processing facility, animal model, clinical samples, etc.) (4); such studies can be sequence or function based. The term “metagenomics” was first coined in 1998 by Handelsman et al. (5) in reference to the functional analysis of all of the genomes within a soil sample. Sequence-based studies deal with the direct genetic analysis of genomes contained within environmental samples without a culture step (4). Function-based studies, on the other hand, are conducted by cloning large portions of screened DNA into a host (6). Here, we focus on sequence-based metagenomics.

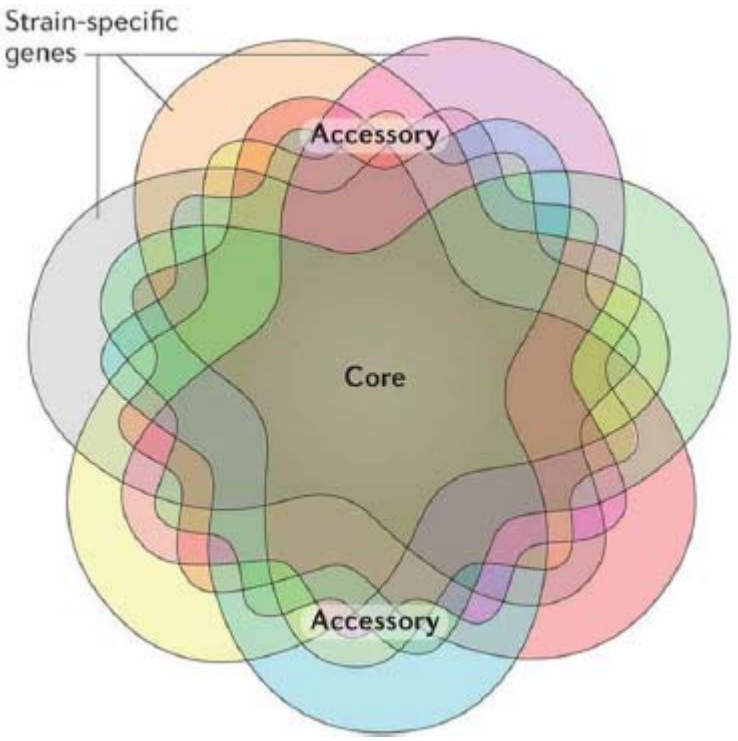

Strain-specific genes
Accessory
Core
Accessory

Figure 36.1. The pangenome and core genome, based on the Edwards-Venn cogwheel and designed by O. Zhaxybayeva, Dartmouth College. The pangenome of a group refers to the sum of all the genes that are present in members of the group. Pangenomes comprise the core genome, which comprises the genes found in all members of a group of interest, and the accessory genome, genes that are present in only one or a few members of the group. The concept of a pangenome has led to the idea that steps in metabolic pathways may be distributed over several individuals within a community. The Black Queen hypothesis suggests that the combination of leaky functions—genes that produce a product that is shared with others in the community—with a selection for small genomes will lead to a situation in which leaky functions are encoded in the genomes of only a fraction of community members that produce this function as a common good. The pangenomes of many taxa seem to be open (that is, of an unlimited size), although the combination of limited population size and limited time of divergence from a common ancestor certainly limits the numbers of genes actually present in a given taxon. Estimated pangenome sizes taking population size and divergence time into consideration can be large; for example, the *Prochlorococcus* pangenome has been estimated to contain approximately 58,000 genes, whereas the individual genomes of the members of this genus contain only about 2,000 genes each. Reprinted from reference 1.

History and Process

Modern whole-community DNA analysis using sequencing was preceded by cloning environmental DNA into recipient cells (7). This method was eventually replaced by methods that directly isolate nucleic acids from the environment and sequence them; the first such method was Sanger sequencing, published in 1977 (8). Then, in the mid-2000s, next-generation sequencing (NGS) was introduced, with the adoption of pyrosequencing technologies. NGS uses a variety of methods for sequencing, including a popular method called sequencing by synthesis, which allows parallel

sequencing reactions to occur simultaneously, increasing sequencing ability from 96 reactions at a time to several hundred thousand (9). The introduction of NGS brought sequencing costs down by a factor of 10,000 and greatly reduced the labor requirements, which were intensive for Sanger-based sequencing (Fig. 36.2). This technology was first commercialized by 454 Life Science and improved upon by a method known as bridge sequencing, first marketed by Solexa, which was then acquired by Illumina (10). As a result, DNA sequencing became a tool that could be implemented in many laboratories to address more applied topics, including those associated with agricultural production and processing (11). While NGS was initially used for sequencing of single genomes, it eventually became feasible to sequence entire communities of microbial DNA without any need for culture- or PCR-based isolation.

Sequencing a nonenriched sample from an environment of interest (whether that be a processed meat product, feces from a food-producing animal, a sample from a production facility that packages ready-to-eat products, human clinical samples from a foodborne outbreak, or a sample from any other environment of interest to a food microbiologist) provides insights into the structure, composition, and function of the microbial community, which, together, can be referred to as the microbial ecology of the sample. The microbiome is defined as the entire population, and the makeup of the community, of organisms in a given sample or animal. In order to investigate the microbial ecology (the interactions of the microbiome with the environment and host) of a given environment, many steps need to be performed, from study design to molecular biology to bioinformatics to statistical analysis. Metagenomic workflows (i.e., the set of steps conducted to complete a study from study design to publication) are somewhat similar to single-genome workflows; an example of such workflows is presented in Fig. 36.3.

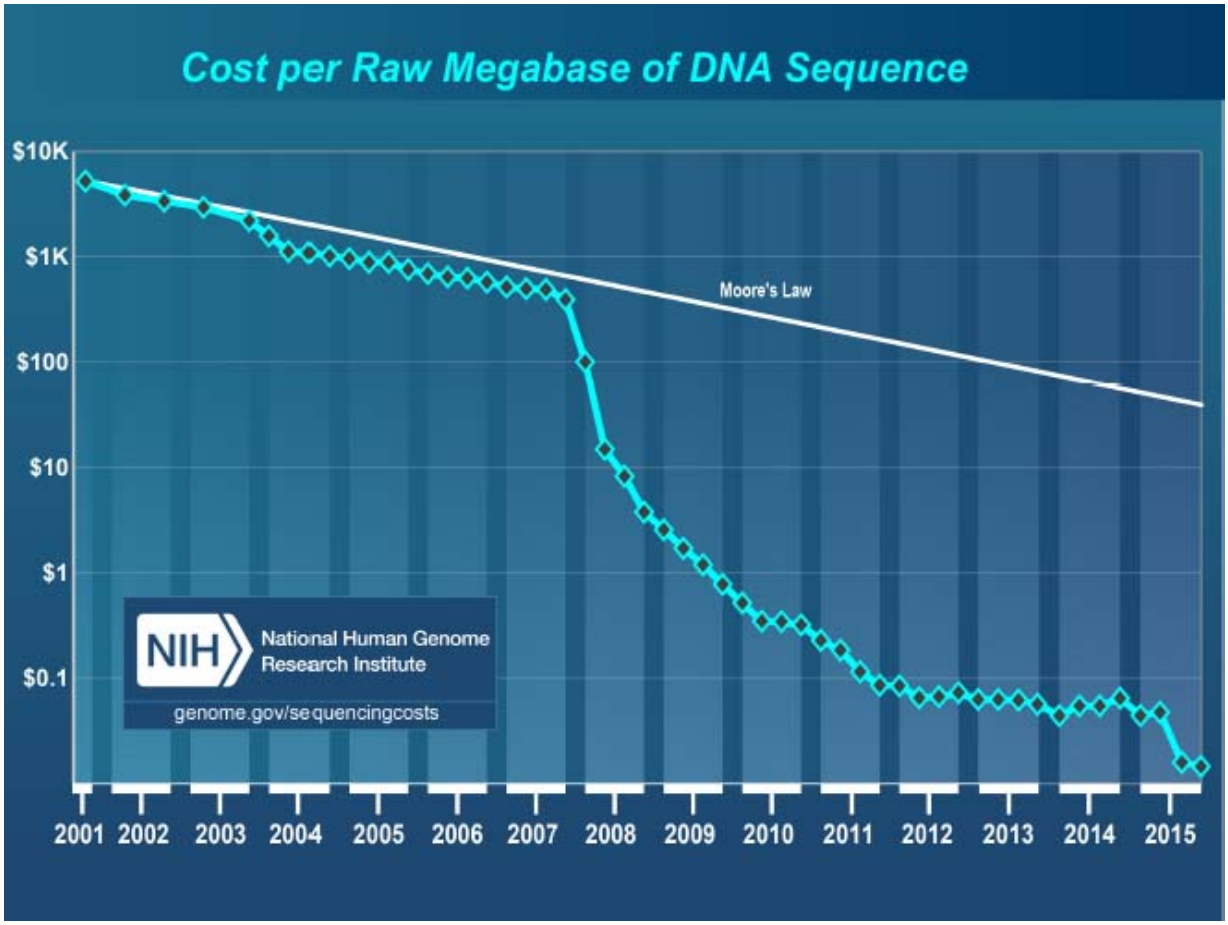


Figure 36.2. The cost of sequencing per megabase pair from 2001 to 2015. Reprinted from reference 87.

Before sampling is conducted, an experimental design is constructed to meet research objectives. While many metagenomics studies currently conducted in meat and poultry science are more explanatory in nature (i.e., determination of what organisms or genes are present in an environment and in what quantities), a shift to hypothesis-driven research is beginning to occur. After the experiment is structured, sampling occurs as aseptically as possible, with special attention paid to not introducing foreign DNA into the sample. From there, DNA is extracted and prepared for sequencing through PCR amplification, “cleanup” steps, and ligation of adapters onto the DNA so that the sequencers can recognize the reads (known as library preparation). Fragmentation, breaking DNA into smaller pieces, is also conducted during library preparation, and

input fragment length is a key difference between short-read sequencing, where this occurs, and long-read sequencing, where it does not.

The manner in which samples are handled (including the specific kits and protocols used to extract DNA and conduct library preparation) can introduce bias into both sequencing and downstream analysis and must be considered carefully in the study design and planning stages (12). A major breakthrough in the preparation of libraries for sequencing was the ability to multiplex samples through the use of bar coding. Bar coding involves the addition of a known, sample-specific nucleotide sequence onto each fragment of DNA, followed by pooling of DNA from multiple samples. This technique greatly reduces sequencing costs by enabling simultaneous sequencing of multiple samples, while maintaining the ability to match DNA sequences back to their originating sample during postsequencing analysis.

It is important to note that one of the main differences between a whole-genome and a metagenomic pipeline is the absence of an enrichment step in the metagenomic pipeline. While single-genome workflows enrich for specific bacteria (through selective culturing and broths), a main objective of metagenomics is to look at a nonenriched view of the entire community. Thus, no steps are taken to differentially increase abundance of specific species, which would not allow accurate relative-abundance estimates. Sample-processing steps must be as unbiased as possible so as not to artificially perturb the composition of the microbial community (12).

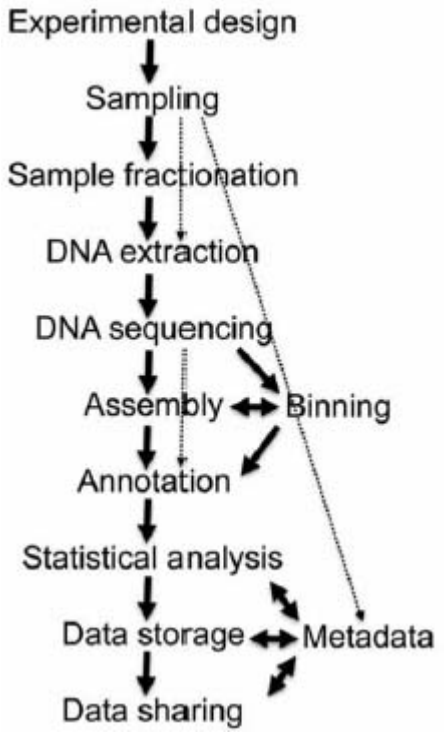


Figure 36.3. Flow diagram of a typical metagenome project. Dashed arrows indicate steps that can be omitted. Reprinted from reference 4.

Once DNA has been extracted, fragmented, and made into a library, DNA is sequenced either through shortread (e.g., Illumina platform) or long-read (e.g., Pacific Biosciences [PacBio]) sequencing. To date, metagenomic studies have mostly utilized short-read sequencing due to lower costs, greater general accuracy, and higher output compared to long-read sequencing technologies. The primary disadvantage of short-read technology is the inability to assign what genes or features of interest belong to what organism due to the shearing of DNA for library preparation.

The process of sequencing DNA produces FASTQ files that contain the DNA sequence of each fragment of DNA, as well as the sequencing quality score for each nucleotide. These short fragments are referred to as “reads,” and each read within the FASTQ files has a unique identifier. With these raw sequence reads as a starting point, bioinformatic analyses can begin. The first step of the bioinformatics pipeline is typically quality control of the sequence

data, which involves removal of library adapter sequences, removal of low-quality nucleotides on the ends of reads, and removal of reads that have overall low quality or are too short. For analyses in which bacterial community is of main interest (such as a study on the shelf life of meat), reads that belong to the host (e.g., *Bos taurus* or *Gallus gallus* DNA) can be removed as part of the quality control process. After quality control is complete, assembly and/or alignment of the sequence reads can begin. Assembly involves stringing the short reads back together into longer sequences called "contigs," typically using de Bruijn graph-based or overlap-layout-consensus algorithms (13). Due to the nature of metagenomic DNA, assemblies tend to be highly fragmented; i.e., they have numerous short contigs, especially compared to assembly of single-genome DNA sequence data. Once contigs are assembled, they typically are compared to existing databases using matching algorithms such as BLAST (14) in order to identify sequences of interest.

Alignment, on the other hand, allows bypassing of assembly and attempts to match unassembled sequence reads directly to existing databases, typically using algorithms such as the Burrows-Wheeler aligner (15). Whether assembled or unassembled data are used, the process of matching DNA sequences to existing databases allows the identification and quantification of genes and/or organisms of interest within the metagenomic data (note that, in the case of 16S sequencing [discussed below], only phylogenetic classification can occur, while shotgun metagenomic analysis provides a broader picture of genomes present in the sample as well as functional and gene differentiation). Once genes and/or organisms of interest have been identified and counted, descriptive and formal statistical tests can be conducted in conjunction with previously collected metadata (i.e., descriptive data associated with the samples, such as location, sample matrix, environmental characteristics, etc.). The specific comparisons made during statistical analysis depend largely on study design, although basic descriptions, such as number of reads, read quality, and level of host contamination, are typically reported for all metagenomic studies. After formal comparisons have been

conducted, raw data and critical output files must be stored on a secure server or backed up in another manner, such as in the "cloud." As with other sequencing studies, metagenomic datasets are often deposited as raw sequence data onto public repositories such as the National Center for Biotechnology Information Sequence Read Archive (16), as this is frequently a requirement for publication in highquality scientific journals.

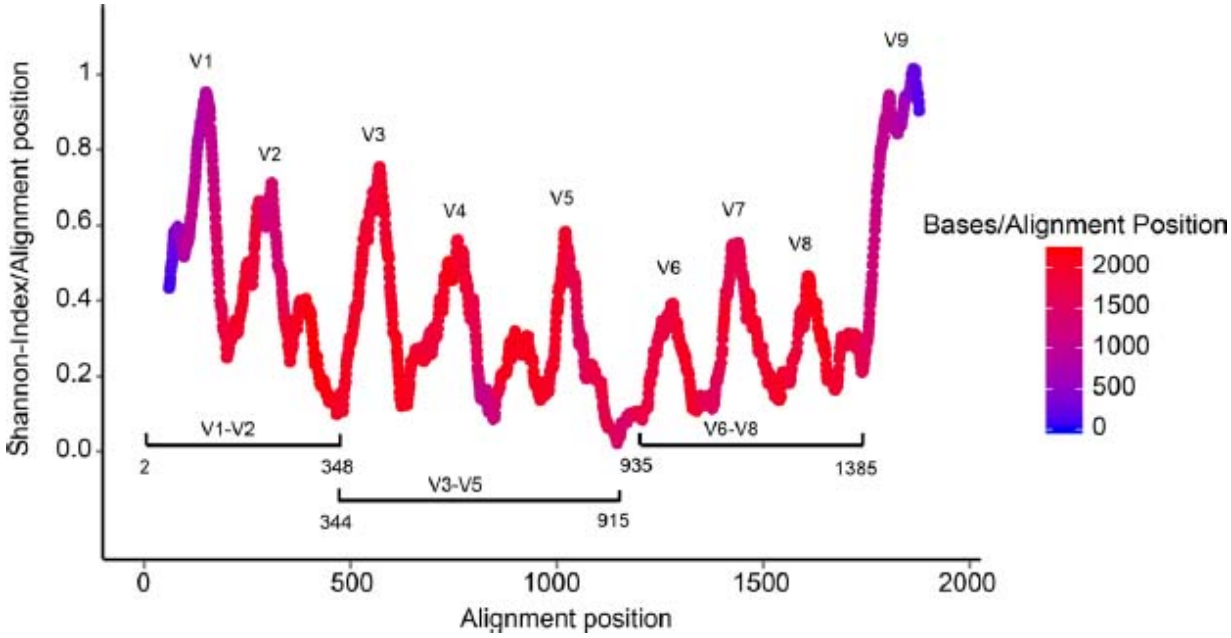


Figure 36.4. Coverage of the archaeal 16S rRNA gene by sequences included in RIM-DB. Sequence variability is expressed as a Shannon index for each alignment position using a 50-bp moving average. Sequence coverage per base is indicated by the heat map and was calculated using a 50-bp moving average. Approximate positions of frequently targeted regions (V1-V2, V3-V5, and V6-V8) for amplicon sequencing are shown for orientation and nucleotide numbering and correspond to positions in the *E. coli* 16S rRNA gene. Reprinted from reference 88.

Terminology used in discussions of metagenomic studies currently is in flux, and 16S microbiome studies and/or extensive sequencing of a single species are often referred to as "metagenomics." It is important to note that, from a purist point of view, the term "metagenomic" means "genome of genomes" and thus refers to

whole-community, unrestricted sequencing of all the genomes in a sample (known as shotgun metagenomics). Alternatively, 16S sequencing studies involve PCR amplification and sequencing of the 16S rRNA gene as a marker to investigate the taxonomic bacterial composition of a sample (17). Because shotgun sequencing is unspecific with regard to what piece of the bacterial genome is amplified and sequenced, it can be used to look at microbial community composition as well as other features of interest, such as specific genes and detailed metabolic and functional profiles (18). On the other hand, 16S rRNA sequencing uses primers that anneal to conserved genomic regions that contain segments of hypervariability (known as variable regions V1 to V9) (Fig. 36.4). The 16S rRNA gene itself is specific to bacteria and archaea and is highly conserved between species, making it a useful target for capturing most of the bacteria within a sample; the hypervariable regions within the 16S rRNA gene, on the other hand, tend to differ between bacterial genera and/or species and thus can be used to reconstruct the taxonomy of the bacteria and archaea within the sample.

While it is much less expensive than shotgun metagenomics, there are some drawbacks to 16S rRNA analysis that are caused by amplifying only a portion of the genome (as opposed to unrestricted sampling of the entire genome, as in shotgun studies). Namely, 16S rRNA sequencing reads cannot be assigned to taxa at high resolution, such as at the species or strain level (17). Additionally, because only the 16S rRNA gene is targeted and sequenced, use of 16S rRNA sequencing does not allow direct functional analysis of the communities being studied. Some pipelines attempt to perform correlative analysis between the taxonomic composition of a sample and its potential functional capacity (e.g., potential metabolic pathways). Some of these pipelines use curated databases of both genomes and so-called marker genes, the latter of which have been confirmed to correlate to both specific bacterial taxa and specific bacterial functions. It is important to note that the accuracy of such

correlative analyses is under debate within the scientific literature (19).

Bioinformatics

A unique component of analyzing large biological data sets is reducing gigabytes (or more) of data down to manageable, useful information. Bioinformatics is defined as the application of computational tools to the capture and interpretation of biological data (20). Bioinformatics is multidisciplinary, as it combines the expertise of fields such as computer science, statistics, and biology to understand and interpret the information contained in large data sets.

In shotgun metagenomics, there are many tasks that must be automated through shell scripts and algorithms in order to make them efficient. These tasks can range from simple to complex, but all of the tasks must be well understood in order to verify that the bioinformatics tools are being used appropriately. An example of a simple, but essential, tool is read trimming and filtering (i.e., quality control; see above). After raw sequences are produced by the sequencer, removal of sequencing adapters and low-quality nucleotide reads is an essential task. While this would be easy to do manually on one read, a typical study has billions to trillions of reads to process. Tools like Trimmomatic (21) provide free, open-source scripts for public use. Published tools can be focused on a specific task, such as trimming or alignment, or can be in the form of "pipelines" that wrap multiple tools into one bioinformatic workflow. Wrapping multiple tools into one computational workflow helps not only with speed but also with data reproducibility. Galaxy (22) is an example of a reproducible research system, which is an environment for executing and tracking computational analyses (23). The advantage of these types of systems is that they not only keep the analysis organized but also can be published alongside research findings for reproducibility.

On-Target Reads

When one is preparing to conduct an analysis, evaluating whether there are enough sequences in relation to the sample type and diversity is a precursor to a statistical analysis. One way to assess this is to perform rarefaction of the sequence data to produce a rarefaction curve (Fig. 36.5). A rarefaction curve traditionally displays sampling depth on the *x* axis and features (species, genes, etc.) or a measure of richness on the *y* axis. If sampling depth (i.e., the number of sequences attributed to each sample) is appropriate, the curve constructed for the sample (or group of samples) should level out when an appropriate sampling depth is reached. The depth of sequencing that needs to occur differs by bacterial community type as a function of diversity (i.e., the more diverse the matrix, the more reads are needed). While deep sequencing (i.e., sequencing a high number of samples) would be ideal for research, the cost makes "unlimited" sequencing impossible. As a result, prestudy work with a smaller subset of the sample type of interest, or relying on previously published work, needs to be considered before a sequencing depth is targeted.

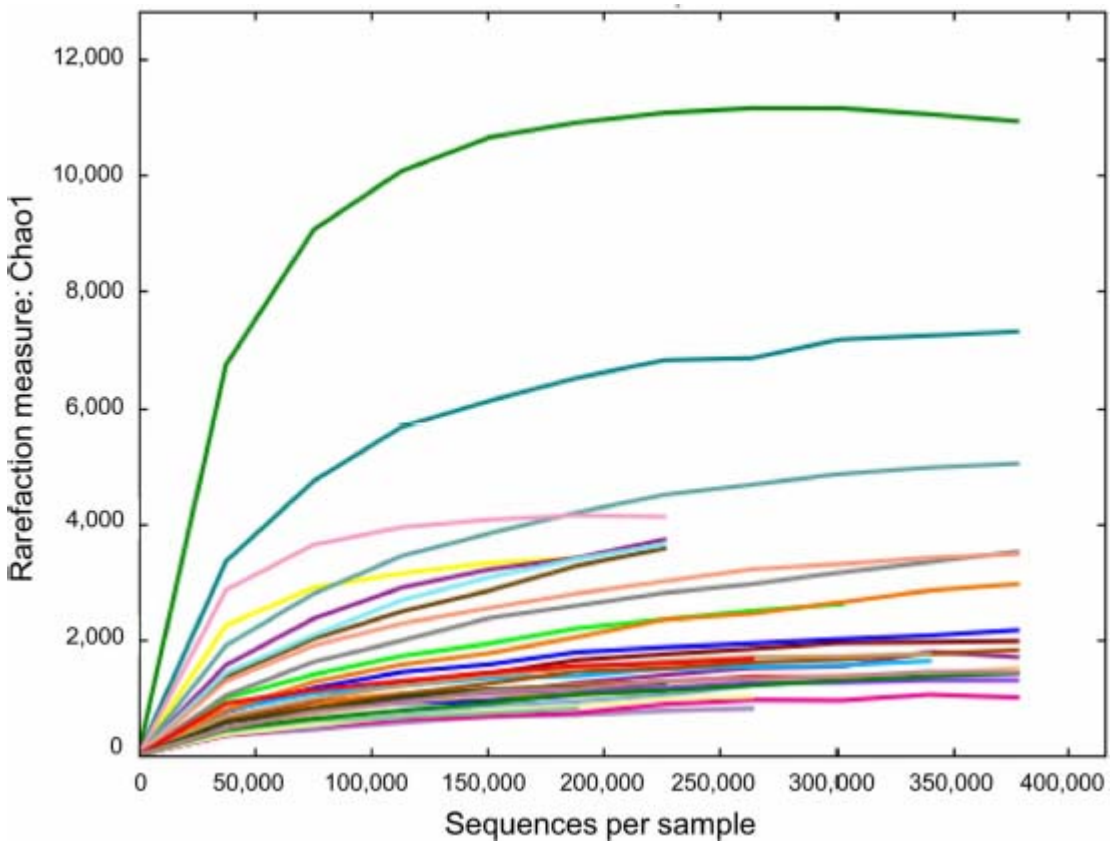


Figure 36.5. Rarefaction plot of individual liver sample from Chao1 measurements. The leveling off in all samples indicates that an appropriate sampling depth was reached to estimate the diversity of the community. Reprinted with permission from reference 72.

While appropriate sequencing depth is a concern, the number of reads that map to a feature of interest (known as a hit) must also be considered. For example, in a study conducted on beef cattle production (24) that sampled both feces and meat, only 1.5% of total raw reads aligned to the bovine host genome and were removed from the sequence data, while 99.6% of sequences in the meat were associated with the bovine genome. This study was conducted to evaluate antibiotic resistance genes in the samples, yet across all samples, only 0.04% of all of the raw sequences were attributed to one of these genes. As a result, 99.6% of the sequences in this study did not contribute to the research objectives and could be considered off-target. This is not an uncommon occurrence in metagenomic studies in which the objective is to investigate a

subset of genes within a wider population. There has been some work to combat this issue, including use of both functional metagenomics (not discussed in this chapter) and targeted sequencing. Targeted sequencing involves specifying features of interest and designing "baits" for these targets (25). Once baits have been designed, they can be used to enrich for specific sequences within the metagenomic DNA via magnetic streptavidin beads, resulting in more on-target hits than a sample without targeted sequencing.

Dealing with Different Library Sizes

There are many decisions that must be made to minimize bias when a metagenomic study is carried out. These decisions start with experimental design and carry through to data presentation. One decision that must be made is how to "normalize" the sequence data. Weiss et al. describe normalizing as "the process of transforming the data in order to enable accurate comparison of statistics from different measurements by eliminating artifactual biases in the original measurements" (26). While library preparation and sequencing technologies have improved dramatically in short periods of time, there still can be an issue with uneven sequencing of DNA from samples. This results in sequence read data sets with differing sizes. For example, equal volumes of DNA extracted from two samples can be sequenced on the same sequencer at the same time, but one may generate 20 million reads while the other may generate 40 million reads.

Table 36.1. Comparison of number of reads in a 16S rRNA sequencing study and a shotgun metagenomics study

	No. of reads		
Study type	Minimum	Maximum	Median
Shotgun metagenomics (24)	12.0 million	93.4 million	46.3 million
16S rRNA sequencing (72)	190,551	996,910	350,716

Such sequencing bias is common with NGS; Table 36.1 provides an example of a shotgun and a 16S rRNA study's minimum, maximum, and mean numbers of reads per sample. While differences in the number of sequences obtained from different samples are common, they are rarely a reflection of true biological differences, but more of sequencing inconsistency. A few factors that affect read number include raw DNA allocation during pooling of DNA for sequencing, sequencing protocol and sample matrix, and sequencer efficiency—such as variation in output from lane to lane on the same sequencer (27). Because it is currently impossible to ensure completely uniform sequencing depth, postsequencing tactics must be used to normalize for the uneven numbers of reads generated per sample.

Two common methods of normalizing are rarefying and scaling (Fig. 36.6); both provide advantages and disadvantages. Rarefying is the process of randomly sampling without replacement from each sample up to a common count (26). For example, if one sample had 1,000 reads and another had 2,000 reads, and the rarefying depth was set at 1,000 reads, the smaller of the two samples would be completely represented because all1,000 reads would be sampled. From the 2,000-read sample, 1,000 reads would randomly be drawn without replacement and the rest discarded. This would result in an equal number of sequences for each of the two samples (i.e., 1,000), and these reads would be carried forward into statistical analyses. The minimum number for rarefying could be selected as the lowest number of reads per sample within the data set; alternatively, a higher number could be chosen, resulting in exclusion of samples with a lower number of reads. Scaling, on the other hand, is multiplication of count by a number or proportion. While total sum scaling divides counts by total number of reads, a more sophisticated technique known as cumulative-sum scaling builds on this method such that counts are divided by the cumulative sum of counts up to a percentile determined by the data (28). Another approach to dealing with count data is a logarithmic-ratio transformation. However, because a log transformation can be used only with positive numbers, pseudocounts must be added to zeros

(i.e., usually adding a 1 to each observation), an issue that can result in bias (29).

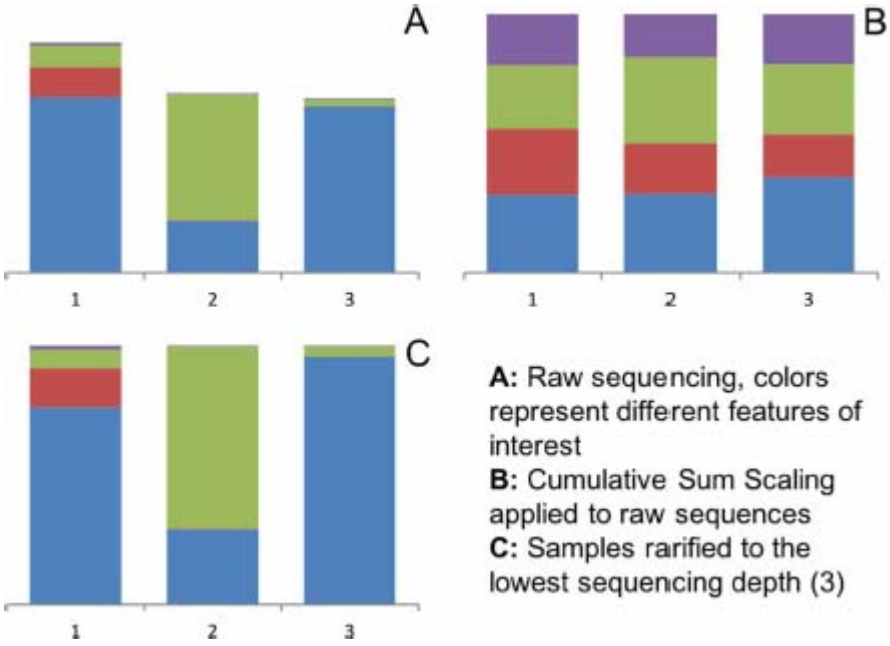


Figure 36.6. Demonstration of cumulative sum scaling (B) and rarefying (C) of raw sequences (A).

Rarefying effectively mitigates varied sample read count size and is especially versatile in handling small and uneven library sizes (26). Disadvantages to rarefying include "throwing away data" (i.e., using only a small proportion of total reads) and adding an additional uncertainty to the data (30). In contrast, cumulative-sum scaling and other scaling methods utilize all counts in the sequencing data by adjusting them by a proportion. However, scaling also has problems associated with it. It can over- or underestimate rare features in samples (26). These issues can be illustrated by looking at two library sizes, one of 10,000 counts and another of 100,000 counts. In the small library, overestimation of abundance can occur. For example, if a feature is present in both samples at a minimal number, the scaling factor applied to the smaller library may overestimate a feature that is, in fact, very rare in the population.

Normalizing is currently a necessary tool due to vastly different sequencing library sizes among samples. Unfortunately, there is no

perfect method that allows all data to be evaluated while avoiding over- or underestimations of features of interest. However, tools are being developed that incorporate more robust models to handle these types of variably sized data sets (31). This is an area that has seen recent growth and will continue to evolve as bioinformaticians and statisticians develop models that more correctly handle count data.

Data Presentation

Metagenomic data are multivariate, and studies routinely generate gigabytes and even terabytes of data. While bioinformatic tools reduce these large data sets to more manageable sizes, the way in which metagenomic results are reported and visualized often differs from that of traditional culture-based reporting methods. While, depending on the objective(s), there are many hypothesis-driven tests that can be performed, Fig. 36.7 displays examples of various descriptive graphical representations of analyses common to metagenomic studies. A taxonomy plot, or 100% stacked bar graphs, can represent individual sample diversity or the taxonomy of groups of samples that share common phenotypic characteristics. Ordination plots (including principal component analysis and nonmetric multidimensional scaling plots) are used to visualize the calculated distances between samples based on some characteristic, typically the composition of the microbiome. This allows visualization of “like” and “unlike” samples, i.e., clustering. Heat maps can be used to illustrate feature abundances (such as a species of interest or specific genes or classes of genes) across samples or groups of samples, again with the goal of visualizing clusters of like samples. Phylogenetic relationships are represented as “trees” built on how similar or dissimilar species or communities are. Uses for these trees can include illustrating the full phylogenetic diversity of a sample and comparing a subsection of data, such as the diversity of species within a genus. Network analysis is the visualization of associations between genes or other features of interest. Co-occurrence of these features is something that can be demonstrated

with a network analysis, as well as associations. While these methods of visualization are a starting point for data interpretation, there are numerous, more robust hypothesis-testing methods, such as ZIG models (28), PERMANOVA (32), and Bayesian network analysis (33), that are used in metagenomics but that are outside the scope of this chapter.

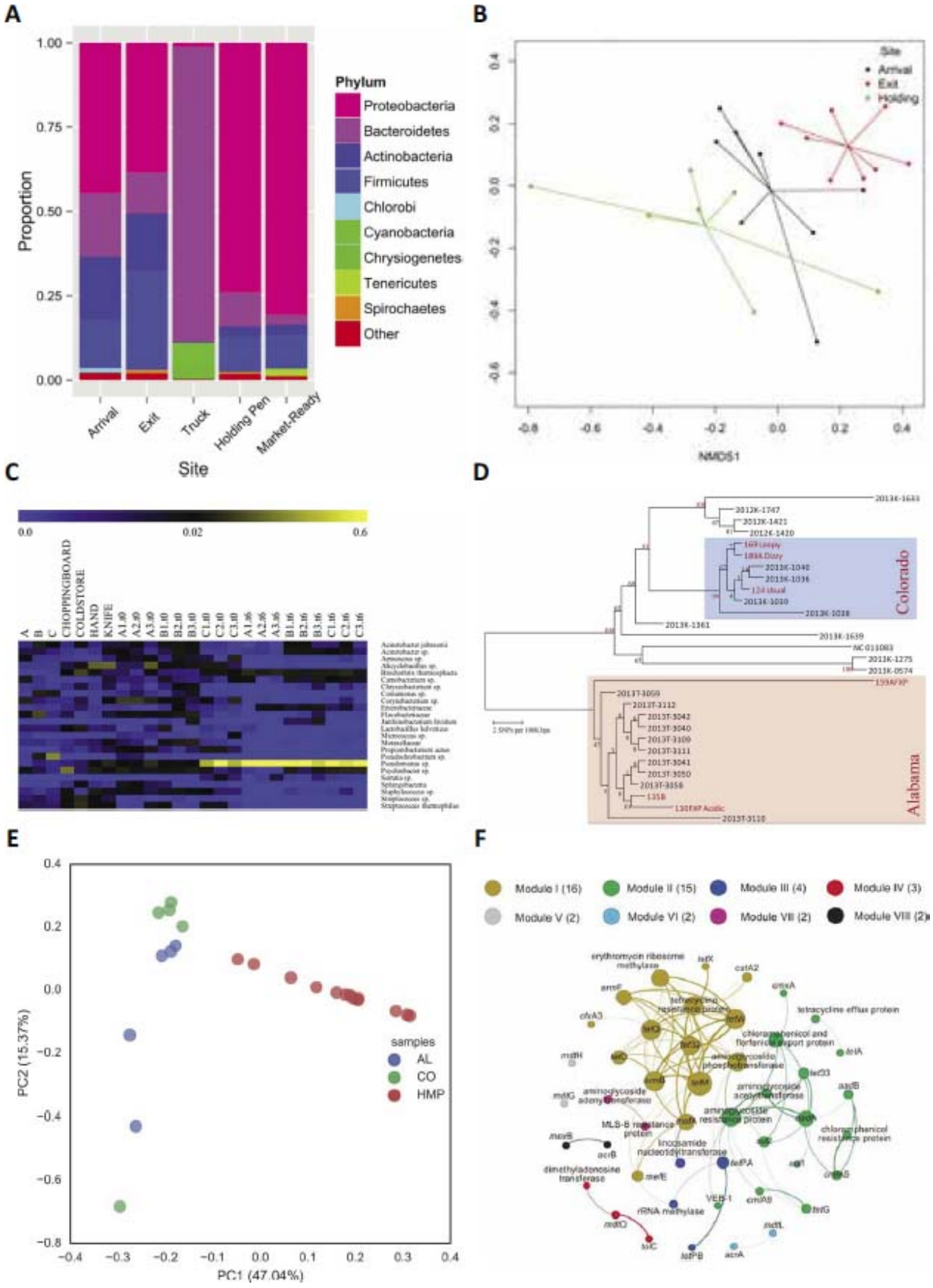

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Actinobacteria
Firmicutes
Chlorobi
Cyanobacteria
Chrysiogenetes
Tenericutes
Spirochaetes
Other
B
Site
Arrival
Exit
Holding
NMDS1
C
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A
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CHOPPINGBOARD
COLDSTORE
HAND
KNIFE
D
Colorado
Alabama
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PC2 (15.37%)
PC1 (47.04%)
samples
AL
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HMP
F
Module I (16)
Module II (15)
Module III (4)
Module IV (3)
Module V (2)
Module VI (2)
Module VII (2)
Module VIII (2)
erythromycin ribosome methylase
tetracycline efflux protein
chloramphenicol and florfenicol export protein
aminoglycoside phosphotransferase
aminoglycoside acetyltransferase
aminoglycoside adenyltransferase
MLS-B resistance protein
chloramphenicol resistance protein
lincosamide nucleosidyltransferase
dimethyladenosine transferase
rRNA methylase

Figure 36.7. Examples of common ways metagenomic data are presented. (A) Taxonomy plot (reprinted from reference 47); (B) nonmetric multidimensional scaling ordination plots (reprinted from reference 47); (C) heat maps (reprinted from reference 75); (D) phylogenic relationship trees (reprinted from reference 89); (E) principal coordinate analysis (reprinted from reference 89); (F) network analysis (reprinted from reference 90).

Advantages of Metagenomics

In addition to the relative reduction in cost in the past decade and the ability to study uncultured organisms, metagenomic methods have other advantages. These include the ability to assess community ecology and to study unknown pathogens of interest. The most touted advantage of metagenomics is the ability to look at an entire community of bacteria. As discussed in chapter 3, culture-based techniques often use indicator organisms, such as generic *Escherichia coli* or aerobic plate counts, as markers for pathogenic bacteria. While indicator organisms are used to indicate pathogens as a fraction of a larger population, some studies have shown only limited direct relationships between indicators and pathogens (34). Instead, in some cases, the use of indicator organisms rests on the assumption that if a reduction is induced or occurs in the larger microbial population, the pathogenic subpopulation also will decline in magnitude accordingly (35). Metagenomics, on the other hand, can be used to directly assess the presence and abundance of organisms in a microbial population, as well as the presence of specific genes of interest. While metagenomics has primarily been used to develop inferences concerning bacteria, the technique also allows indepth investigation of the virome (all the viruses in a given environment), the mycobiome (all the fungi), and the plasmidome (all the plasmids). This is of special interest in food microbiology, where processes like shelf life and aging are also heavily influenced by fungal growth.

In addition to identifying and quantifying the organisms within a given microbial community, interactions between organisms can be

studied. To assess relationships between features of interest (genes, genomes, or sets of genomes) in a bacterial population, co-occurrence and coexclusion relationships can be studied in the context of network analysis. Co-occurrence and coexclusion relationships look at whether a specific feature of interest occurs at a higher rate alongside another feature; for example, two genes that are on the same plasmid would have a higher co-occurrence than two chromosomal genes from different species of bacteria. Network analysis can provide insight into intertaxon associations to understand symbioses between community members; this allows more complex pattern discovery than traditional diversity metrics (36). Understanding associations between different microorganisms within a bacterial community can guide researchers towards specific targets for alteration or manipulation of the community—a systems approach to food safety and quality control.

Disadvantages of Metagenomics

While NGS has substantially lowered sequencing costs, there are still financial barriers. These barriers are not limited to generation of sequence data but extend to computational capabilities, data storage, and, in some cases, the need to hire a bioinformatician and/or laboratory personnel familiar with NGS. As sequencing costs are dropping rapidly, costs associated with data analysis and storage are forecast to occupy a larger proportion of total experimental costs (Fig. 36.8). After the financial cost associated with the technique has been overcome, there are hurdles related to metagenomics being a relatively new technique; namely, shortcomings associated with public databases and the availability and usability of analytical tools.

With respect to public databases used in metagenomic analysis, there are several difficulties that researchers may encounter, including incomplete databases, misannotation, and inconsistent nomenclature. While the rise in NGS has helped fuel the expansion of many databases, there are still features and organisms that have yet to be entered into a database. To combat this issue, one method

of *de novo* discovery of novel genes or organisms, called *de novo* assembly, has been developed (37). The issue of unannotated sequence data is especially prevalent for fungi; while public databases exist, they are generally a few years behind those established for bacteria (38). This is especially challenging to food researchers studying shelf life and fermentation, where fungi play an important role in bacterial communities. The second issue surrounding databases is the possibility of misannotation (i.e., that a gene or species of interest is erroneously attributed to an incorrect species due to an error in the database). When misannotation occurs, the only remedy is careful curation of the database, which is timeand labor-intensive. Finally, there is the concern over inconsistent nomenclature and taxonomic grouping "rules." This can be addressed by instituting standards for the naming of features or organisms. An example of this is found in antibiotic resistance gene naming, where a gene needs at least a 2% difference in nucleotide makeup from its closest relative for it to be designated as a "new" gene (39).

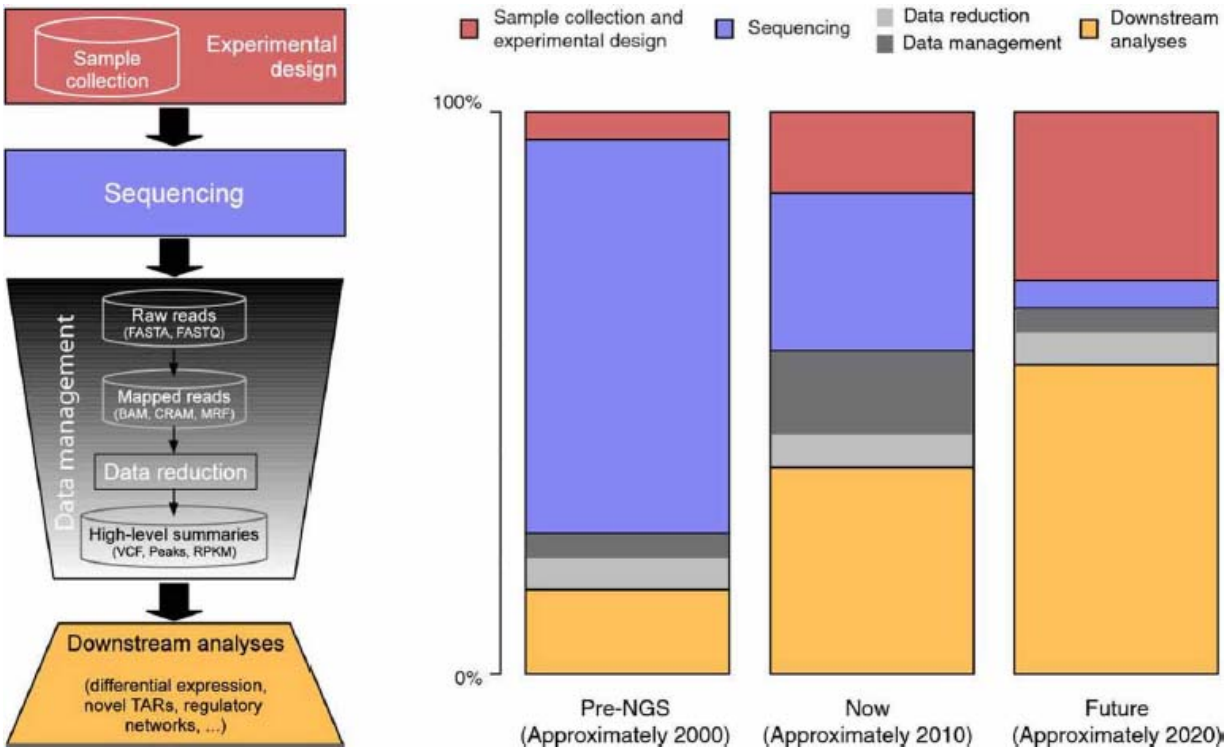


Figure 36.8. Contribution of different factors to the overall cost of a sequencing project across time. (Left) The four-step process: (i) experimental design and sample collection, (ii) sequencing, (iii) data reduction and management, and (iv) downstream analysis. (Right) Changes over time of the relative impact of these four components of a sequencing experiment. BAM, binary sequence alignment/map; CRAM, compression algorithm; MRF, mapped read format; RPKM, reads per kilobase million; TAR, transcriptionally active region; VCF, variant call format. Reprinted from reference 91.

Metagenomic studies require the merging of computational and biological knowledge. Historically, these disciplines (i.e., computer science and the life sciences) have been largely separate. As life science researchers increasingly utilize bioinformatic software, the userfriendliness of these tools continues to improve (38). 16S rRNA sequencing, which has a longer track record than more costly shotgun metagenomic sequencing, can be analyzed using two widely available platforms (mothur [40] and QIIME [41]), both of which have graphical user interfaces for improved accessibility for life science researchers. For shotgun metagenomics, such tools have not

yet been established, and many analyses can still be performed only via the command line (i.e., interacting with a computer only via coded commands, with no mouse capacities or graphics to guide the user).

METAGENOMICS OF MEAT AND POULTRY

Limitations associated with use of culture-dependent methods have been known in food microbiology for many years. Before NGS became common, food microbiologists attempted to look at unculturable bacteria through other avenues. For example, 16S rRNA gene PCR and denaturing gradient gel electrophoresis fingerprinting were tools used as a precursor to the modern 16S rRNA sequencing protocol. These tools separate equal-length DNA fragments based on sequence-specific melting in a polyacrylamide gel with a gradient of a denaturant chemical (42). The difference between this method and more modern analysis of 16S rRNA is that, after extraction and PCR, fragment lengths are separated on a gel as opposed to being prepared for library sequencing. While widely employed as a precursor to modern-day microbial ecology studies, this method does have drawbacks. In addition to less-developed whole-community DNA extractions, other problems include formation of chimeric sequences (a new artificial gene created when two or more biological genes overlap and combine during PCR) affecting the band distribution, limited DNA fragment length (500 bp), comigration of DNA fragments (resulting in different fragments having identical melting behavior), and a single species with multiple rRNA copies, resulting in multiple bands and diversity overestimates (43).

As a result of these issues, shotgun metagenomic techniques are starting to be used to answer specific food safety and quality questions regarding meat and poultry. There are many areas within food microbiology in which metagenomics can aid further research. While targeting pathogenic bacteria is related to food safety and assessment of specific spoilage bacteria is more closely associated

with meat quality, both safety and quality studies are beginning to look at the entire community of organisms in an environment. In the past, metagenomics in meat and poultry production was primarily used for descriptive purposes; e.g., finding the true polymicrobial diversity of a specific food product or environment (38). As more background understanding is obtained, hypothesis testing is now beginning to emerge, using both 16S rRNA sequencing and shotgun metagenomics in randomized, controlled studies (44).

Food Safety

Mitigation of specific culturable pathogen strains or indicator organisms has been the standard in meat and poultry production as a result of the sensitivity and affordability of these methods. However, some culture methods take up to a few days to confirm a positive presence of pathogens of interest (45). While rapid diagnostic methods, such as culturing or BAX PCR, allow fast screening of potential pathogenic bacteria, results are only a presumptive positive or negative and still require a cultural confirmation through biochemical, serological, or genetic tests (46). While not currently deployable within the framework of on going food safety programs, a future goal of metagenomics is to provide confirmatory rapid pathogen detection in complex sample matrices. Currently, metagenomic research with respect to pathogenic organisms can be broken down into detection and reduction of organisms, as well as more downstream public health application within a greater microbial community.

Pathogenic Bacteria

Detection of Pathogenic Bacteria with Metagenomics

While whole-genome sequences for many pathogens are available in the public domain, there has been limited use of metagenomics as a tool in true regulatory pathogen identification. An example of limitations regarding pathogen detection in metagenomic samples

was demonstrated in a metagenomic study of a feedlot beef production system. This study highlighted both the challenges associated with genetic similarity between many bacterial species and the downfalls of short-read analysis. Because shotgun metagenomics has a nonbiased enrichment PCR step in library preparation, there is randomness with regard to what portion of any bacterial genome will be sequenced. For example, in the beef study, a 100-bp read assigned to the *Salmonella enterica* serovar Newport reference genome could have come from this serotype, but *S. enterica* serovar Typhimurium and other members of the *Enterobacteriaceae*, such as *E. coli*, also shared the same conserved genetic region (Fig. 36.9) (47). As a result of these homologous regions between bacteria of different strains, and even different species, short-read sequencing cannot offer a definitive answer regarding what strain a specific 150- to 250-bp fragment belongs to. In situations where strain-level differentiation is the difference between an adulterant in a meat product and an organism that is considered part of the natural microflora, metagenomic tools are not capable of these distinctions due to intraspecies genetic homology and limitations of short-read sequencing.

As a result, pathogen detection within unenriched samples (i.e., within metagenomic data) is still a future goal and not a current reality. In contrast, WGS is already being used in outbreak investigations, as this technique enables highly sensitive differentiation of extremely similar serotypes (even down to a single nucleotide polymorphism) (48). The reason that WGS is currently better suited to such applications is its ability to create full-length genomic assemblies from the sequence data, as opposed to metagenomic data, which typically produce very fragmented, low-quality assemblies.

With metagenomic limitations in mind, there has been some work to solve issues associated with pathogen detection in metagenomic data. The creators of SeqSero, a Web-based tool for determining *Salmonella* serotypes using high-throughput genome sequencing data from more than 2,300 serotypes, have attempted to identify

Salmonella in shotgun metagenomic data (49). While the tool is aimed primarily at WGS assembly of *Salmonella*, it does provide the option for pathogen detection within shotgun metagenomic data. The SeqSero developers tested the method by infecting mice with *Salmonella* Typhimurium and then using metagenomic sequencing of DNA extracted from the feces from the mice; they were able to identify *Salmonella* Typhimurium (Table 36.2). As a follow-up, fecal samples from patients known to harbor *E. coli* O104:H4 also were evaluated using the same tool to test for false positives; no reads from this sample group mapped to *Salmonella* spp. (49). While this work provides a framework for identification of pathogens within metagenomic data, the *Salmonella* in the mice was at an elevated level due to an active illness. In contrast, detection of food-associated pathogens would need to detect specific strains at a lower relative abundance than those present in clinical infection doses.

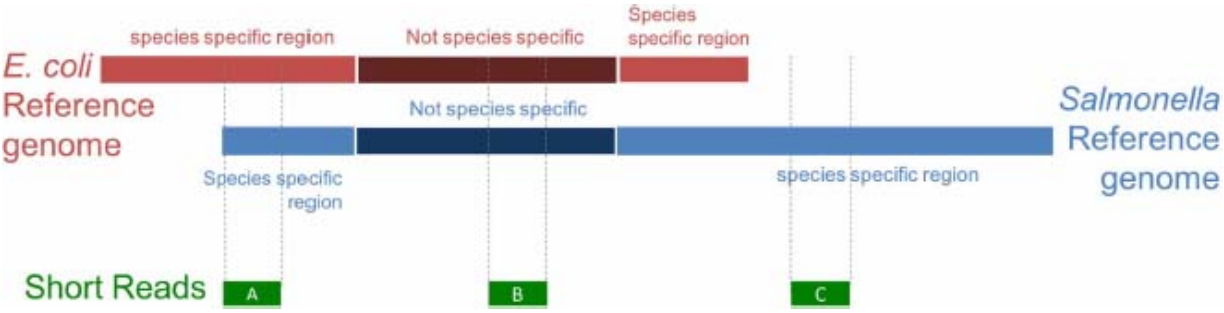


Figure 36.9. Because metagenomics is primarily associated with short-read sequences, species-specific differentiation is difficult due to different species containing the same homologous sections of nucleotides. Therefore, the section of the genome sequenced may be in a species-specific region of the genome (A or C) or may be shared by both bacteria (B) and would therefore not allow differentiation.

Table 36.2. *Salmonella* serotype determination utilizing high-throughput genome sequencing data[a] No. of reads mapped to individual antigen alleles

		No. of reads mapped to individual antigen alleles		
Sampling time[b]	Sample accession no.	*wzx/wzy*	*fliC*	*fljB*
Day 1	SRR916930	273	2	2
Day 3	SRR916932	521	10	11
Day 6	SRR916933	519	12	10
Day 14	SRR916931	1,572	21	21

[a] Reprinted from reference 49.

[b] Time after oral inoculation of *Salmonella*.

Detection of Pathogen Reduction Using Metagenomics

While metagenomics was not used during the 2011 European *E. coli* O104:H4 outbreak, retrospective use helped characterize the event. After the outbreak, 45 archived fecal samples from patients were evaluated using shotgun metagenomics. Using this retrospective analysis, researchers were able to create a draft sequence of the outbreak *E. coli* strain, but they were successful at detecting the strain in only 67% of the cases that were confirmed via culture (50). While not effective at overt pathogen detection, this study did provide evidence of the ability to create draft genomes from metagenomic samples. This demonstrated the usefulness of metagenomics as an approach to characterize outbreak strains and find co-occurrences of outbreaks. Metagenomics also can be paired with other NGS techniques to investigate reductions in pathogen prevalence or abundance. While not directly related to detection of pathogens, a study conducted to identify metabolic pathways of enterohemorrhagic *E. coli* demonstrated that the pairing of techniques was effective by first using 16S rRNA sequencing to

understand the bacterial community of ground beef and then looking into metabolic pathways (51).

Metagenomics in a Public Health Setting

While there are clear limitations to the clinical application of shotgun metagenomic assessments to detect pathogenic bacteria, it has been used in some instances. For example, in a clinical setting (52), a patient that presented with symptoms consistent with a foodborne illness was tested for the presence of several bacterial and viral pathogens within the feces through traditional culture methods; however, no pathogen was detected, and therefore the illness could not be diagnosed. After the patient recovered from the illness, another stool sample was obtained and the metagenomic DNA was compared to DNA extracted from the original sample collected while the patient was ill. Reads associated with *Campylobacter jejuni* were detected, which then was confirmed by PCR of the sample taken during illness but not the sample taken after recovery (52). This resulted in the ability to confirm the causative agent of a foodborne illness that was not possible through traditional culture methods. This case demonstrated the utility of shotgun metagenomics in finding a causative pathogen when it is not clear which pathogen is being sought.

Commensal Bacteria

While identification and quantification of pathogenic bacteria have been the cornerstone of food safety for years, researchers are beginning to understand the importance of commensal bacteria in modulating health and disease of both individuals and environments, such as factories and meat and poultry products. Bystander bacteria, while not harmful on their own, can carry genes of interest that escalate risk of disease (Fig. 36.10). Metagenomics allows increased understanding of the relationships between commensal microbes and pathogens, because researchers can sequence whole-community DNA to obtain a “full-resolution picture” of the microbial community. Currently, identification and

quantification of the organisms in a sample are the cornerstone of metagenomic studies, but more advanced analyses are beginning to find their way into the literature, including those designed to answer questions such as what portion of the microbiome is transmissible both intra- and interspecies, what the mechanisms of this transmission are, and what—if any—organisms in the commensal microbial population should be considered more or less risky (53).

A specific example of the role that commensal bacteria can play in dissemination of genes of interest lies in the case study of antibiotic resistance genes. Some research has demonstrated that when an antibiotic is introduced into a bacterial community, the microbial community can become stressed, resulting in increased rates of horizontal gene transfer (54). Commensal bacteria can donate and accept genes via horizontal gene transfer, and as a result, both the commensal and pathogenic bacteria can acquire, carry, and disseminate these genes (55). A widely studied commensal bacterium is generic *E. coli*. While diarrheagenic *E. coli* and enterohemorrhagic *E. coli* (also called Shiga toxin-producing *E. coli* [STEC]) are the main public health concern, understanding harmless *E. coli* aids in the understanding of pathogenic *E. coli*. This is due to the fact that horizontal gene transfer occurs at a higher frequency in bacteria that are most closely related (1).

A specific community dynamic of importance to meat and poultry production is biofilm formation in processing facilities. Shotgun metagenomics has allowed study of multispecies biofilms and their complex microbial interactions, including genetic makeup, metabolite exchange, and quorum sensing, that may occur between microorganisms in biofilm communities (56). In biofilm communities, *E. coli* O157:H7 has been shown to possess the ability to cocolonize with commensal bacteria, and shotgun metagenomics assays have been used to study microbial competition for essential macronutrients (56).

Genes of Interest

Biological hazards in meat and poultry products have primarily been described in the context of a pathogen or toxin of interest (certainly there are exceptions to this, such as *E. coli* differentiation based on virulence). However, with new technologies, instead of considering the microorganism in its entirety, specific biomarkers can be evaluated (57). In this way, pathogenic bacteria can be further delineated based on specific genetic attributes. Two common genetic components of interest in foodborne pathogens are antibiotic resistance genes and virulence factor genes.

Antibiotic resistance

Antibiotic resistance in meat and poultry products is of concern due to use of antimicrobials in the raising of food-producing animals. The apprehension surrounding antibiotic use in livestock stems from concerns that consumption of products derived from animals administered antimicrobials could increase the risk of antibioticresistant bacterial infections and treatment failure in humans (58). In the past, antibiotic resistance has mainly been evaluated using cultural methods such as those of the National Antimicrobial Resistance Monitoring System (NARMS) (59). This protocol is intended to monitor trends in antimicrobial resistance among enteric bacteria from humans, retail meats, and animals (59). However, the current workflow calls for enrichment and culturing of specific pathogens of concern in relation to foodborne illness, followed by antimicrobial susceptibility testing of cultured isolates; enrichment changes the relative abundance of organisms in the community, and therefore, biases result. Furthermore, some of the tested isolates are sequenced as part of the NARMS WGS initiative, after which the sequences are deposited in GenomeTrakr, a public database of genomes (59). Currently, however, there is no exploration of commensal bacteria in these NARMS samples, and therefore no investigation into the potential for a “commensal” reservoir of antibiotic resistance genes (60). Previous research has demonstrated that many commensal bacteria harbor antibiotic resistance genes. Hence, accessing these commensal bacteria may

be critical in furthering our understanding of antimicrobial resistance and its transmission to humans (60). While antimicrobial resistance regulatory and surveillance programs continue to rely on testing of cultured indicator organisms, the research sector has produced studies that identify and quantify all known resistance gene sequences within metagenomic DNA (i.e., the resistome) (24). Such an approach allows detection of antimicrobial resistance genes within both pathogens and commensal bacteria and potentially allows detection of horizontal gene transfer events within the microbial population (61).

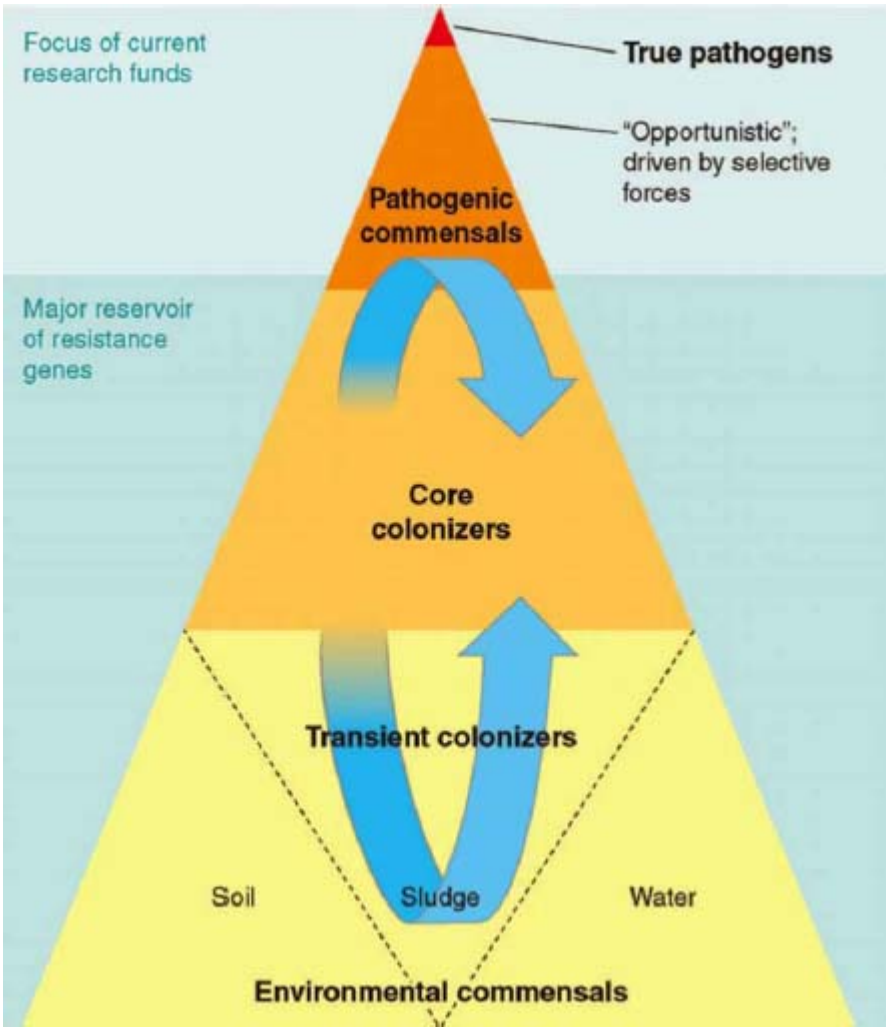

Focus of current research funds
True pathogens
"Opportunistic"; driven by selective forces
Pathogenic commensals
Major reservoir of resistance genes
Core colonizers
Transient colonizers
Soil
Sludge
Water
Environmental commensals

Figure 36.10. Hierarchy of commensals (not to scale). Pathogenic commensals make up only a tiny fraction of the total microbial environment. Greatly under rated and under studied are the multitudes of "core" and transient colonizers, i.e., commensals that constitute the major reservoirs of resistance genes. To some degree, commensals can be distinguished by their place in the environment and the relationships with their hosts. Some colonizers of the skin, oropharynx, and intestinal tract rarely if ever cause dis ease (e.g., the lactic acid bacteria). Yet another group is considered generally nonpathogenic, but when imbalances or shifts occur in the selective pressures on their microbial niches, these species can be propelled to the status of pathogens, made more problematic if they have acquired resistance or virulence genes from neigh boring commensals. These constitute the group of opportunistic or pathogenic commensals. *Staphylococcus aureus*, which regularly or transiently colonizes about 80% of humans, now frequently crosses this border. *Staphylococcus epidermidis* and other traditionally commensal coagulase-negative staphylococci only occasionally cause nosocomial infections and only under extreme selective pressures, such as those exerted by indwelling catheters and depressed immunologic states. Organisms that commonly harbor native or "constitutive" resistance in their chromosomes (e.g., *Pseudomonas, Stenotrophomonas, and Acinetobacter*) also may emerge under these conditions. Most commensals, however, exist as environmental residents of soil and water habitats, many of which may be come transient colonizers of humans and animals through the food chain and other routes of exposure. The accumulated evidence suggests wide spread gene exchange among these groups. Reprinted from reference 60.

Another challenge in the evaluation of antibiotic resistance genes in a metagenomic sample is the disuniform methods of analysis. From sampling to processing to data interpretation, there are no standard methods of assessing the risk of antibiotic-resistant genes in culture-free methods. While some metagenomic studies of antibiotic resistance evaluate the functional assays of resistance,

others infer risk based on the particular database being used (62). As a result of the varying analyses, it is a challenge to directly compare results from one study to the next.

Virulence factors

Virulence factors have previously been explored by food microbiologists, most notably as a means to differentiate *E. coli* strains, but primarily in the context of pathogen differentiation instead of community-wide characterization. Virulence factors are known to spread through bacterial communities using horizontal gene transfer avenues such as bacteriophages (63). While the presence of genes like *eae* and *stx* is used to classify *E. coli* O157:H7 as pathogenic, the ability to identify such genes in a shotgun metagenomic sample is challenging due to the short-read nature of the data and inability to create robust assemblies (47). Furthermore, linkage inferences between genes and bacteria are limited because library preparation and sequencing do not amplify evenly, resulting in uneven sequence depth. However, understanding what virulence factors are present in a bacterial community can allow researchers to evaluate how these genetic factors interact within the microbial community. An example of this approach was used to conduct a culture-free assessment of naturally versus conventionally raised beef cattle in North America (64).

Environment

In addition to looking at the meat and poultry products themselves, a very appropriate use of shotgun metagenomics is in characterization of processing facilities used to produce meat and poultry products. Analysis of the biogeography of all potential fomites is a discipline known as building ecology (65). In meat or food processing facilities, metagenomic analysis has been used to identify food spoilage bacteria and pathogenic bacteria on processing surfaces (66). Another valuable capability that metagenomics affords is the investigation of co-occurrence of organisms (Fig. 36.11). Such an analysis can be helpful in mitigating

pathogens and spoilage organisms because it enables identification of organisms whose abundance is either directly or inversely correlated with the pathogens or spoilage organisms of interest.

The ability to track a microbial community of a given environment is another example of how metagenomics can be used in environmental analysis. In addition to characterizing the microbes in a given environmental community, a longitudinal study allows researchers to observe changes in the composition of the microbial community over time, or to compare the characteristics/makeup of a community with those of a community from another environment. For example, in a study of cooked sausage, *Lactobacillus* spp. increased in relative abundance during production, while *Enterobacteriaceae* declined between the processing steps of meat receiving and final product (Fig. 36.12) (67).

Recent advancements in biostatistical capabilities (i.e., Bayesian modeling) also have enhanced source attribution to product or environmental contamination. For example, SourceTracker (68) takes 16S rRNA sequencing data collected from both the location of concern and potential sources of contamination and attempts to determine which potential source was the actual source of the contamination (for example, tables in a packing room for ready-to-eat products could be the location of interest and potential sources of contamination could be employee boots, packaging material receiving, and the cooler areas where meat is stored prior to packaging). While this could be a valuable tool for improving general understanding of contamination, the 16S rRNA sequencing input does not allow strain-level identification, thus limiting the tool's usefulness in identifying a pathogenic contaminant. The software MetaMLST is a more recently developed tool that allows microbial strain tracking and identification within complex shotgun metagenomic data obtained directly from an environmental sample (69). This tool works by reconstructing loci identified by multilocus sequence typing of pathogens (multiple areas of one genome) of interest from publicly available databases and comparing them to

microbial communities from shotgun metagenomic data (69). This tools holds promise for identifying pathogen contamination from different areas within a processing facility.

While not directly related to meat and poultry products, metagenomic data have also been used to better understand plant worker health. Understanding what microflora and contaminants workers are exposed to throughout their work shifts helps to develop mitigation strategies to prevent possible negative results of exposure. For example, assessment of airborne bacteria found in the air vents of a slaughterhouse could become a proxy for worker airborne exposures, including air quality (70).

Rearing of Animals

The use of metagenomics is not limited to end product food safety and abattoir sanitation. Many scientists have focused on the use of metagenomics to discover potential targets that can be used to influence the microbiome of food animals before slaughter. Objectives of such studies include reduction of pathogen levels within animals, alteration of growth status, mitigation of unwanted outcomes such as liver abscesses in cattle, and manipulation of the nutrient profile of the meat being produced. As methods have become more refined and we have accumulated knowledge of animal microbiomes, research goals have correspondingly evolved. Early studies focused more on characterization of animal microbiomes (71) as a way to understand what organisms were present in normal situations. From there, specific locations within the animal's body that impacted end product food safety were mapped using a microbiome approach (72). Metagenomics then were employed to better understand movement of pathogens within livestock populations. For example, metagenomics was used to evaluate STEC in feedlot cattle populations, resulting in the observation that STEC colonization was correlated with a lower diversity of gut microflora, which increased as cattle matured (73).

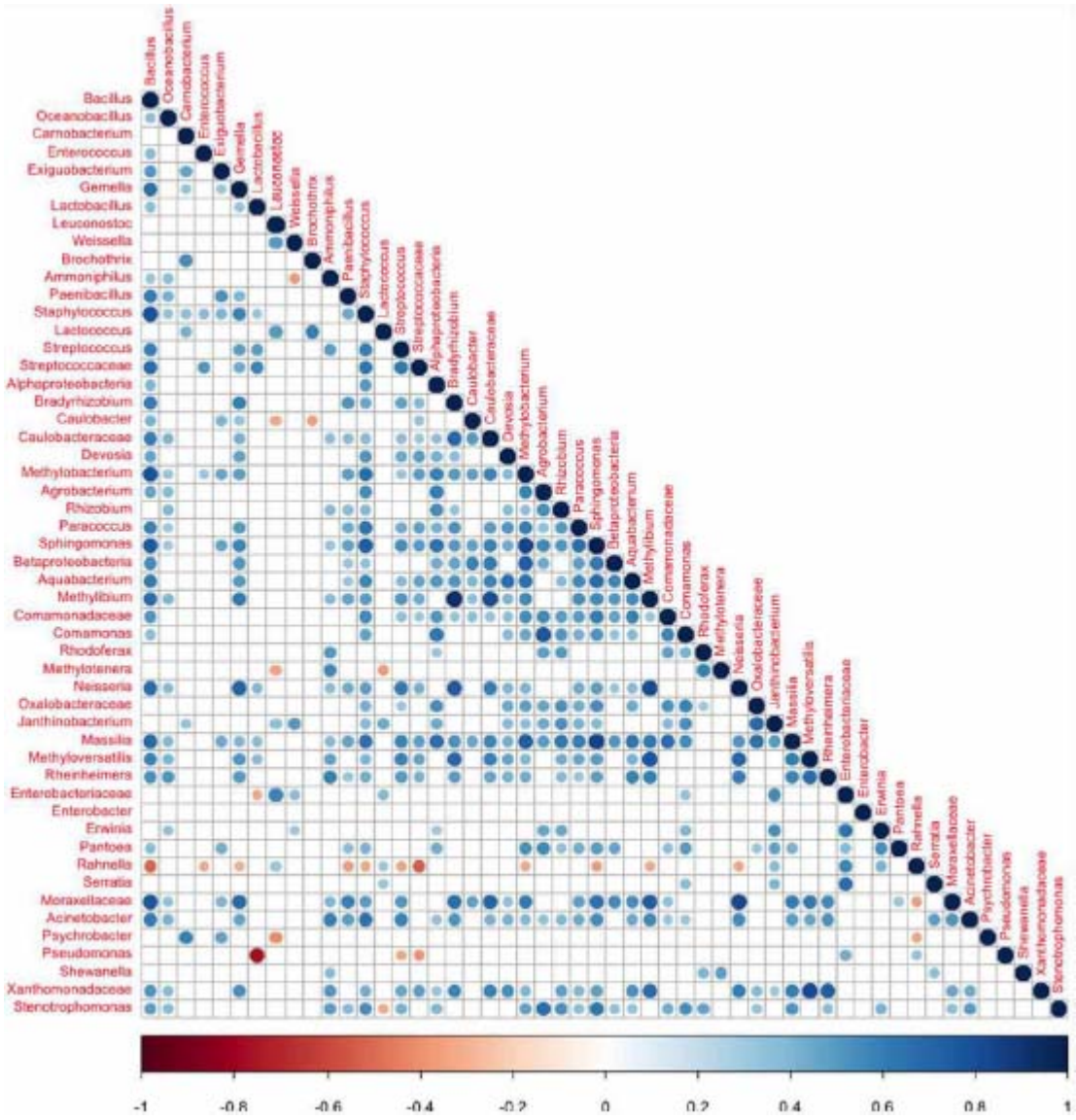

Bacillus
Oceanobacillus
Carnobacterium
Enterococcus
Exiguobacterium
Gemella
Lactobacillus
Leuconostoc
Weissella
Brochothrix
Ammoniphilus
Paenibacillus
Staphylococcus
Lactococcus
Streptococcus
Streptococcaceae
Alphaproteobacteria
Bradyrhizobium
Caulobacter
Caulobacteraceae
Devosia
Methylobacterium
Agrobacterium
Rhizobium
Paracoccus
Sphingomonas
Betaproteobacteria
Aquabacterium
Methylibium
Comamonadaceae
Comamonas
Rhodoferax
Methylotenera
Neisseria
Oxalobacteraceae
Janthinobacterium
Massilia
Methyloversatilis
Rheinheimera
Enterobacteriaceae
Enterobacter
Erwinia
Pantoea
Rahnella
Serratia
Moraxellaceae
Acinetobacter
Psychrobacter
Pseudomonas
Shewanella
Xanthomonadaceae
Stenotrophomonas
-1
-0.8
-0.6
-0.4
-0.2
0
0.2
0.4
0.6
0.8
1

Figure 36.11. Significant co-occurrence and coexclusion relationships between bacterial operational taxonomic units. Spearman's rank correlation matrix of operational taxonomic units with an abundance of ≥0.1% in at least five samples. Only phylotypes assigned to the phyla *Proteobacteria* and *Firmicutes* were considered. Strong correlations are indicated by large circles, whereas weak correlations are indicated by small circles. The colors of the scale bar denote the nature of the correlation, with 1 indicating a perfectly positive correlation (dark blue) and −1 indicating a perfectly negative correlation (dark red) between two phylotypes. Only significant correlations (false discovery rate < 0.05) are shown. Reprinted from reference 66.

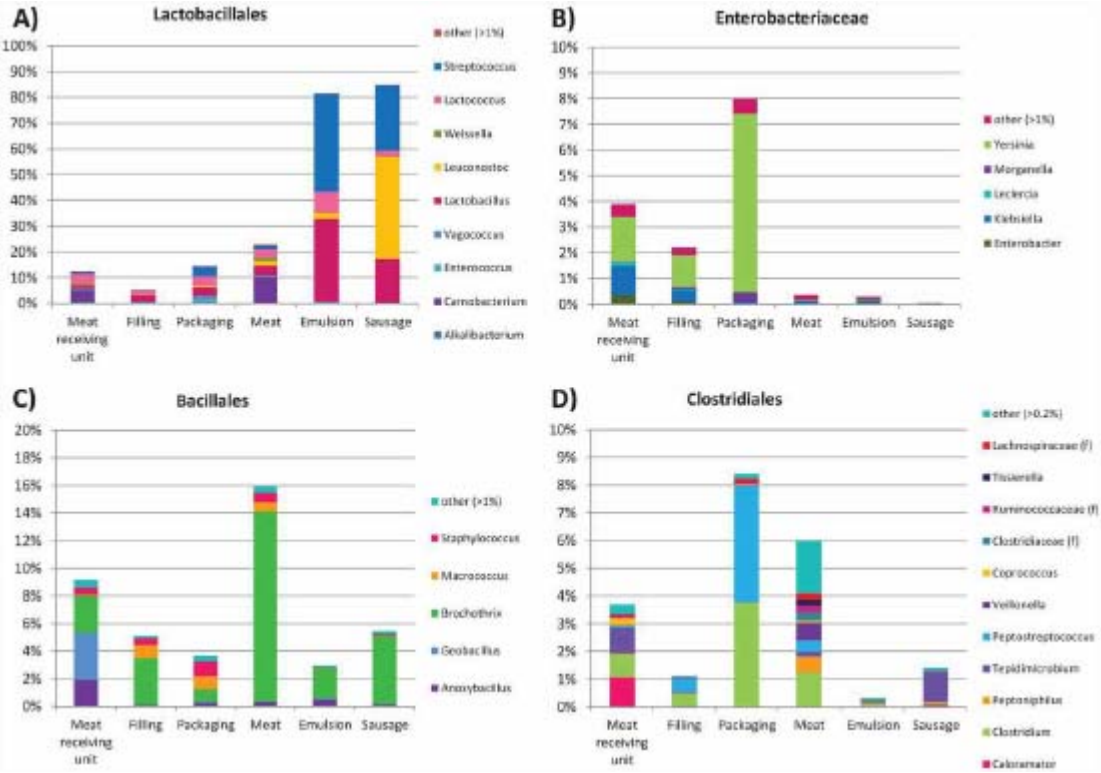


Figure 36.12. Relative abundances of sequence reads assigned as indicated. f, family-level classification. Reprinted from reference 67.

Food Quality

Metagenomics not only has increased our understanding of pathogenic and harmful bacteria but also has helped us to better

understand how all bacteria and organisms change over time and as a result of specific interventions. For instance, fermentation and aging of meat add value and longevity to the final products. While these processes have been characterized through culture-based assays in laboratories, metagenomics allows these processes to be viewed through an entirely different lens.

Shelf Life

Metagenomics has been used to understand how different processing procedures affect the bacterial community and known causative spoilage organisms. In shelf life studies, a metagenomics approach was used to characterize the impacts of different ingredients, packaging types, and processing variation on shelf life. For example, in marinated broiler meat, the addition of a marinade (which contained, among other things, oil and sugar) altered microbial communities such that spoilage-associated *Leuconostoc gasicomitatum*, *Leuconostoc gelidum*, and *Lactobacillus* spp. were favored (44).

Metagenomic investigation also has been used to understand the effects of using different types of packaging. For example, comparison of meat products packaged in mainly air, modified-atmosphere packaging (MAP), vacuum packaging, and active vacuum packaging revealed that *Brochothrix thermosphacta* made up a larger proportion of the microbiome of meat packaged in air and MAP during the early days of a 45-day shelf life study, while *Pseudomonas* spp. became more dominant in air over time (74). While many of the bacterial species present on the product had previously been associated with meat spoilage, bacteria that had not previously been associated with meat spoilage were also found (Fig. 36.13).

In addition to profiling microbes present in meat subjected to shelf life conditions, work was conducted to evaluate whether processing environments and other factors contributed to shelf life variation. One study of beef steak production investigated the microbiome of

steaks, carcass swabs, and environmental samples over time (75). The investigators discovered many different types of bacterial contaminants across the samples on day 0 of sampling, while samples from later time points showed that phyla associated with spoilage outcompeted skin-associated bacterial environmental contaminants (*Propionibacterium acnes*, *Staphylococcus equorum*, and *Staphylococcus* sp.) to become the most dominant phyla in the community. Other metagenomic studies have demonstrated that variation in production systems, including assessments of lot variation in beef that was packaged in modified atmosphere, can also have an effect on the abundance and composition of phyla present (76).

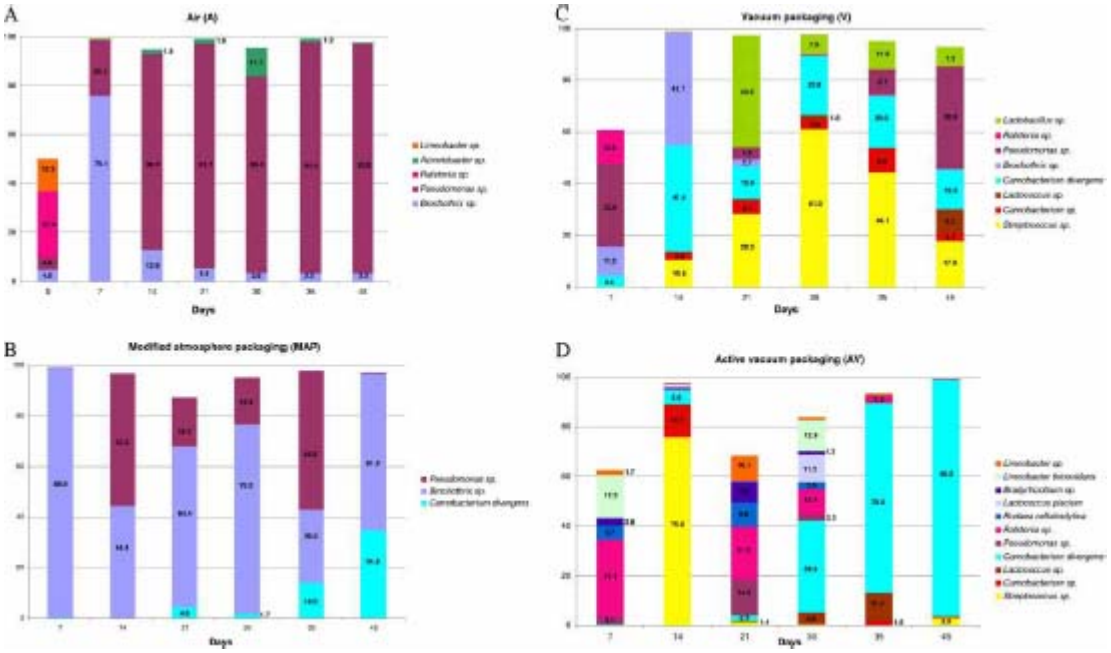


Figure 36.13. Incidence of operational taxonomic unit tracebacks based on bTEFAP (bacterial tag-encoded FLX amplicon pyrosequencing) analysis of allthe DNA samples directly extracted from meat at different times and under different storage conditions. Only taxa with an incidence above 9% in at least one sample are shown, and only percentages above 1% are displayed in the histograms. Shown are results for storage in air (A), MAP (B), vacuum packaging (C), and active vacuum packaging (D). Reprinted from reference 75.

Fermentation

Culture-based studies associated with fermented meats, such as salami, have been plentiful, but these studies have focused on a few organisms and were not reflective of an entire microbiome. A 16S rRNA sequencing study of Italian salami highlighted the complexity of the ripening process by documenting a diverse bacterial community with higher-than-expected numbers of *Lactobacillus* spp. and *Staphylococcus* spp., as well as the presence of previously undocumented rare bacterial families (Fig. 36.14).

Regulatory Issues

Currently, metagenomic methods are primarily used in the research setting (77). For a method to be adopted as a benchmark for regulatory compliance, it must be verifiable, sensitive, specific, precise, and accurate. Current metagenomic methods do not meet all of these thresholds, but there is clearly regulatory interest in advancing the technology to meet such goals. The current gold standard for monitoring foodborne outbreaks is PulseNet USA. The aim of the network is to rapidly detect multistate outbreaks caused by foodborne pathogens through pulsed-field gel electrophoresis (PFGE) of outbreak samples with subsequent comparison to a national database of PFGE patterns. While still relying on PFGE, PulseNet also has piloted WGS for use in both outbreak investigations and routine surveillance of foodborne pathogens (78). Though not currently implemented, use of WGS in PulseNet and other regulatory frameworks is likely the precursor to regulation of meat and poultry pathogens from a metagenomics perspective (78). The ability to trace pathogens using metagenomic methods may be the ultimate tool for identifying specific environmental sources of contamination (47, 79).

Future Directions

In relation to other techniques applied to the area of meat and poultry microbiology, metagenomics is still an emerging

methodology. While many laboratories and researchers have demonstrated versatility and utility of metagenomics for advancing knowledge within a specific discipline, there still are many areas in which use of metagenomics has yet to be fully explored. In attempting to forecast what metagenomics may mean for meat and poultry science, it may be useful to understand how metagenomics has advanced scientific knowledge within human-oriented fields. Beyond characterizing different niches in the human body, research was conducted on links between the microbiome and different disease conditions ranging from irritable bowel syndrome to some cancers (80). Broad areas within meat and poultry research that could benefit from further development of metagenomic methods include improved usability and accuracy of bioinformatic and statistical techniques, single-cell sequencing, and intervention-based studies.

					Producers						ripening time/sample type					Starter	
											meat	salami			casing		
Family/ total samples	% of positive samples	n of total sequences	n of genera	most represented genus	A	B	C	D	E	F	0 d	0 d	21 d	49 d	63 d	21 d	yes	no
					11	11	10	10	10	11	1	12	12	12	12	12	24	24
Comamonadaceae	65	116	13	*Pelomonas*	8	7	6	7	8	5	2	11	6	6	7	9	14	16
Moraxellaceae	65	564	3	*Acinetobacter*	5	10	6	7	7	6	2	9	3	9	6	12	12	15
Corynebacteriaceae	60	2588	1	*Corynebacterium*	4	6	7	8	5	8	2	6	6	6	6	12	12	12
Micrococcaceae	48	463	6	*Kocuria*	4	5	5	4	6	6	1	4	5	6	4	10	10	9
Bacillaceae	40	247	3	*Bacillus*	2	1	4	7	5	6	0	4	5	6	4	6	10	9
Enterococcaceae	40	232	3	*Enterococcus*	4	4	6	4	3	4	1	6	2	4	1	11	5	8
Halomonadaceae	30	1463	3	*Cobetia*	2	3	4	4	2	4	2	3	1	1	1	11	4	2
Clostridiaceae	27	493	4	*Clostridium*	2	2	2	2	5	4	0	4	3	0	0	10	6	1
Caulobacteraceae	22	38	5	*Brevundimonas*	4	2	3	0	2	3	3	5	1	3	1	1	4	6
Methylobacteriaceae	22	42	1	*Methylobacterium*	3	2	1	2	3	3	0	4	3	3	2	2	7	5
Campylobacteraceae	21	142	2	*Arcobacter*	3	3	1	2	1	3	0	4	0	0	0	9	2	2
Microbacteriaceae	19	33	3	*Cryocola*	1	2	3	2	1	3	1	6	1	2	1	1	7	3
Pseudonocardiaceae	16	16	4	*Saccharopolyspora*	1	1	2	1	4	1	0	3	3	1	1	2	4	4
Fusobacteriaceae	16	14	1	*Fusobacterium*	3	2	1	0	2	2	0	1	1	0	5	3	4	3
Dermabacteraceae	14	101	1	*Brachybacterium*	0	3	0	2	3	1	0	0	1	1	1	6	3	0
Aerococcaceae	14	34	3	*Aerococcus*	2	2	2	0	1	2	0	0	0	0	1	8	0	1
Lachnospiraceae	14	18	4	*Coprococcus*	0	0	3	0	3	3	0	0	0	3	3	3	3	3
Aeromonadaceae	14	30	0	*nd*	2	0	2	1	4	0	0	4	1	1	1	2	6	1
Weeksellaceae	13	13	3	*Chryseobacterium*	1	0	0	2	3	2	0	5	0	0	1	2	4	2
Carnobacteriaceae	13	153	2	*Carnobacterium*	1	1	3	2	0	1	1	2	2	0	1	2	2	3
Veillonellaceae	11	19	2	*Veillonella*	2	0	0	3	0	2	0	0	0	3	3	2	2	3
Neisseriaceae	11	11	3	*Neisseria*	1	1	0	3	0	2	1	3	0	1	1	1	2	3
Pasteurellaceae	11	16	1	*Haemophilus*	2	1	0	1	1	2	1	0	2	3	0	1	4	1
Christensenellaceae	10	12	0	*nd*	3	1	2	0	0	0	0	2	0	0	0	4	1	1
Tissierellaceae	10	19	3	*Anaerococcus*	2	1	0	0	0	3	0	1	1	3	0	1	2	3
Xanthomonadaceae	10	15	7	*Pseudoxanthomonas*	3	0	1	1	0	1	0	2	0	1	2	1	2	3
Thermaceae	10	20	2	*Thermus*	2	2	0	0	2	0	0	2	0	1	1	2	1	3

Figure 36.14. The rare microbiome in Salame Piacentino DOP samples: distribution of families accounting for less than 5% of the total bacterial diversity in the 63 samples analyzed. The total number of sequences retrieved and the number of genera represented are reported for each rare family. A heat-mapping color scale indicates the relative distribution of positive samples among producers, ripening times, and presence or absence of starter addition. Reprinted from reference 92.

The field of bioinformatics is rapidly expanding, and new techniques are being developed in tandem with rapidly decreasing sequencing costs. An area that has seen growth in the past few years, and that will continue to mature, is the development of graphic user interfaces that will allow more biologists access to informatics tools. In terms of bioinformatics development, advancements in assembly algorithms specific to metagenomic data are continuing to evolve, such as the release of metaSPAdes (81). There are other nonconventional methods for bioinformatics development as well, such as crowd-sourcing development. Through an open call with associated prize money, crowd-sourcing developed 89 new sequence alignment algorithms, 30 more efficient than the previous benchmark, in just 2 weeks (82).

Single-cell sequencing is another area where growth will likely occur. In shotgun metagenomics, diversity of the microbial community decreases the ability to detect strain-level variation and specific genes. Multiple displacement amplification enables amplification of a single bacterial genome. Use of this technique in conjunction with NGS can yield almost complete bacterial genomes (83). This method has been used in built environments where bacteria of interest are low in abundance, such as in hospitals. Use of this method could lead to more complete understanding of low-abundance pathogens and their transmission between the environment and a host or a product (83). Because processing facilities deal with the same dynamics (i.e., a built environment with low pathogen presence, such as a meat plant), the usefulness of this technology is certainly an area worth exploring.

Use of metagenomics will likely continue to transition from primarily exploratory and descriptive studies to causation and intervention-based studies. Examples of this within the human literature include studies of *Clostridioides* (formerly *Clostridium*) *difficile* infections and gene editing. In individuals with *C. difficile* infections, characterized by a proliferation of *C. difficile*, low-volume fecal transplants from healthy donors allow repopulation of the intestinal microbiome with “healthy” microbes. Recent 16S

rRNA sequencing studies have shown high abundance of *Bacteroidetes* and *Firmicutes* in donor feces, leading to the possibility of treating *C. difficile* infections with a probiotic specifically enriched for these taxa (84). Gene editing, specifically via the CRISPR (clustered regularly interspaced short palindromic repeat)-Cas9 system and others, holds promise for targeting specific genomes in a metagenomic sample. This technology allows the deletion, insertion, and modification of the DNA sequences of cells, which, in turn, allows control of function of specific genes and regulatory elements (85). This method already is being examined in the context of food safety research. Using the CRISPRCas9 system, researchers were able to specifically cleave Shiga toxin genes in bacterial cells, leading to significant reductions in numbers of STEC (86). These authors believe that this system could be further harnessed to decrease foodborne pathogens and other genes of interest in certain microbial communities.

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Food Microbiomes: A New Paradigm for Food and Food Ecology

INTERSECTION OF HUMAN AND FOOD MICROBIOMES

Culture-independent next-generation sequencing technologies have fueled a renaissance in our understanding of what it means to be human. Targeted and targetindependent metagenomic data have been used to describe microbial ecologies from soils to clouds and from body surfaces (skin) to internal processes (gut) (1–4). Even the human immune system can no longer be understood without also considering the 100- to potentially 1,000-fold more abundant complement of microbial genes compared to host genes (5, 6). This paradigm shift has also occurred in our understanding of the foods we consume and the complex microbial ecologies along the farm- to fork continuum. These ecologies are important both for food safety (exposure to pathogens) and for issues of nutrition, allergy, and immunology. Despite the emphasis on "purity" in the Pure Food and Drug Act of 1906, which led to the creation of the Food and Drug Administration, the Act was written at a time when the word "purity" completely excluded microbes. It is now understood that almost every food (except, potentially, highly processed foods) has a bacterial, fungal, viral, and potentially archaeal component to its "naive" (pure) state. Food science finds itself at a new and exciting interface of microbiology, nutrition, and immunology. We are reevaluating nutritional sciences, using a more comprehensive inventory of the prebiotic and probiotic potential of foods (Fig. 37.1).

The convenience and affordability of next-generation sequencing technologies, improved bioinformatic pipelines, and converging reference databases have enabled the description of culture-independent microbiota associated with numerous environmental, human, and food microbiomes that were previously poorly understood. The complex network of interactions between the microbiota of mammals, agricultural reservoirs, and food plants is a frontier of exciting research. Years of descriptive characterizations of food and food ecologies are beginning to fuel nutritional

hypotheses at the human-food intersect. Numerous states of human health and disease are already correlated to dysbiotic human microbiomes (7, 8). From 1950 to 2000, we witnessed a significant decline in acute illnesses such as mumps, measles, rheumatic fever, tuberculosis, and hepatitis, the control of many of which can be attributed to the use of vaccines. During the same period and extending to the present, we have been witnessing an increase in chronic illness associated with immune responses, such as type 1 diabetes, Crohn's disease, multiple sclerosis, obesity, and asthma (9). It is possible that some of these chronic diseases are on the rise in response to non-target effects of the use of antibiotics in medicines and foods, unintentionally having disrupted internal ecology and physiology we have yet to even characterize. Additionally, the exposure to an increasingly sophisticated array of chemicals (such as preservatives, pesticides, herbicides, insecticides, sterilizers, etc.) over the past 50 years of food production may have resulted in an unintended antibiotic impact on American microbiomes. This public health phenomenon positions food in a more important role than ever before. Food itself may function as an epigenetic "fix" for numerous states of disease that have been correlated with states of dysbiosis and nutritional deficiency. Thus, the full understanding of the microbial load in our food and the impact of the food matrix itself on the human microbiome (as a prebiotic, probiotic, or antibiotic) is of vital importance to public health.

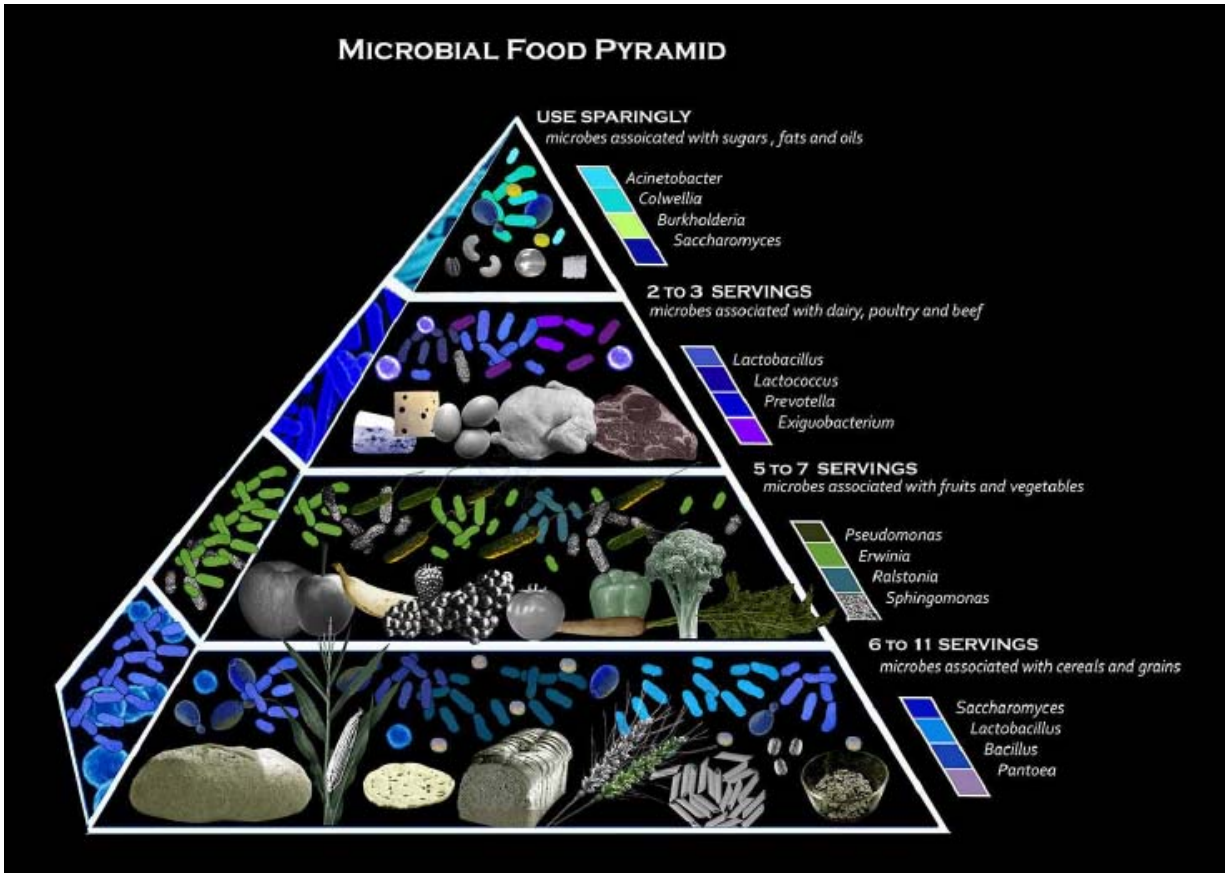


Figure 37.1. Microbial food pyramid. This graphic is designed to highlight the fact that healthy diets must consider not only foods themselves, but also the microbial content of foods. Even if a food is completely devoid of microbes, the food matrix itself will interact with the human microbiome in some manner that will impact nutrition and health.

SEQUENCING TECHNOLOGIES: GENOMETRAKR AND METAGENOMETRAKR

From 2005 to 2011, most metagenomic sequencing endeavors relied on Solexa (the first iteration of Illumina sequencing) and/or Roche 454 pyrosequencers. As early as 2009, Pac Bio platforms were inconsistently providing long reads (20 to 50,000 kb), primarily used to help navigate complex genome assemblies, especially for organisms with AT-rich areas and/or highly repetitive elements.

Recently, newer Pac Bio platforms have been used for higher-throughput long-read metagenomic studies, with exciting results (10). Since about 2011, Illumina platforms have been the workhorses of programs such as GenomeTrakr (11), designed to rapidly identify foodborne pathogens by clustering whole-genome sequence (WGS) data of bacterial isolates following illness outbreaks. New programs such as MetagenomeTrakr, designed to provide more comprehensive ecological fingerprints of foods and rapid metagenomic response to foodborne outbreaks, are also being coordinated by scientists at the Center for Food Safety and Applied Research using the MiSeq platform in the same way that WGS programs have been implemented.

Small state and university labs around the world have the ability to perform their own metagenomic and genomic sequencing due to the relatively affordable cost of MiSeq "desktop sequencers." These machines have become standard equipment in many labs, and targetindependent metagenomic surveys are relatively commonplace for all types of research. Sequencing cores and high-throughput laboratories have relied on Illumina HighSeq platforms (higher-throughput Illumina machines); these are now being phased out, to be replaced by Illumina's newer NovaSeq platforms, which provide billions of bases for half the price of previous machines. From 2006 to 2018, the amount of data produced by a single Illumina run went from approximately 1 Gb to 6 Tb, with newer machines being capable of generating as many as 20 billion reads in a single run. At the same time, long reads are now being produced by a tiny handheld device called the MinION. While not yet financially practical, the technology itself has become much more reliable in past years and holds great promise for field studies and rapid on-site or *in situ* real-time sequencing of full genomes and PCR targets from the palm of one's hand (12, 13). All of these technologies (and more) have contributed to the vast collection of data that now spans numerous food and food environments.

PATHOGEN DETECTION IN THE FARM-TO-FORK CONTINUUM

By the time a fresh fruit or vegetable or even a highly processed food makes it to the point of human consumption, it has traveled through multiple diverse, yet interwoven, ecologies. Despite modern, sophisticated approaches to food handling, pathogen-contaminated foods still result in human deaths, as occurred in Germany with the *Escherichia coli* O104 outbreak of the summer of 2011 (14) and recently in South Africa, where as many as 180 deaths from listeriosis were linked to meat products. The more we learn about the ecologies along food production continuums, the better positioned we will be to identify and mitigate risk factors. Fresh produce is of course a commodity of significant importance, since it is often prepared and consumed without heating or other "kill" steps. Comprehensive surveillance of biological and ecological risks in fresh produce production will help us minimize risks at critical control points in farm-to-fork agroecologies.

Metagenomic Description of Fresh Produce: Tomatoes, Sprouts, and Leafy Greens

Certain cases of *Salmonella* contamination of tomatoes have been hypothesized to occur in agricultural field settings because of the fact that multistate incidents were traced to specific geographic growing regions, suggesting that contamination occurred early in the distribution chain, potentially in the field (15, 16). Thus, there has been much focus on preharvest ecologies. Early metagenomic work focused on providing baselines for numerous crops, where no such information was previously available (17). This type of "profiling" of agricultural environments has been applied to many components within specific agroecologies, including the phyllosphere (plant surfaces), soil, water, air, fungicides, fertilizers, pesticides, biogeography, and other agricultural management practices (18–21).

In a metagenomic survey of tomato plants at harvest time from a geographical region to which outbreak strains of *Salmonella* had been traced, Ottesen et al. (19) described distinct groupings of microbial communities that were associated with different organs of *Solanum lycopersicum* (tomato) and a gradient of compositional similarity that was correlated to the distance of a given plant part from the soil. Unique bacterial phylotypes (at 95% identity), such as *Microvirga*, *Pseudomonas*, *Sphingomonas*, *Brachybacterium*, *Rhizobiales*, *Paracoccus*, *Chryseomonas*, and *Microbacterium*, were associated with fruits and flowers of tomato plants. The most frequently observed bacterial taxa across aerial plant regions were *Pseudomonas* and *Xanthomonas*. Dominant fungal taxa that could be identified to the genus level included *Hypocrea*, *Aureobasidium*, and *Cryptococcus* (19). These types of data for crop ecologies provide a new resolution for our understanding of risk factors along the farm-to-fork continuum. Other work, focused on the study of biogeographical microbiology of crops, documented a completely distinct bacterial ecology for tomato phytobiomes from East-versus West-grown tomatoes (22). These distinct ecologies also appeared to correlate with completely different risk factors for the crop.

Sprouts are another crop with a long history of association with illness outbreaks. Since 1996, the FDA has been involved in investigating 48 outbreaks associated with sprouts resulting in a total of 2,499 cases, including 179 hospitalizations and 3 deaths. Most outbreaks were attributed to alfalfa sprouts, although clover, mung bean, and sprouted chia were also implicated. *Salmonella* was the most common pathogen identified, followed by *E. coli* O157:H7 and then *Listeria monocytogenes* (23). In response to the contamination events associated with sprouts, there has been substantial interest by commercial sprout growers in using metagenomic approaches to examine the entire sprout production process to improve understanding of the growing ecology that has resulted in multiple illnesses. In a recent study, coordinated by a commercial sprout grower, a shotgun metagenomic approach was used to study the microbial dynamics of a sprouts crop over the 4

days the seeds take to mature (24). The diversity of bacteria, fungi, protists, and viruses associated with sprout water was described. (A baseline profile of the water was also taken to better understand how the water itself may seed downstream sprout ecology.) Dominant bacteria included *Klebsiella*, *Pseudomonas*, *Acinetobacter*, *Enterobacter*, *Curvibacter*, *Delftia*, *Flavobacterium*, *Escherichia*, and *Pantoea*. From the first day of growing to the last, there was an increase in the relative abundance of *Curvibacter* and *Delftia*. *Escherichia* was more prominent on days 2 and 3 and then appeared to be moderately suppressed by day 4.

One goal of the research was to identify naturally occurring opportunities for biological control, in the same way that data from metagenomic tomato crop research identified *Paenibacillus* as a potential inhibitor of pathogens like *Salmonella*. Once the dynamic microbiology of all crops is more comprehensively understood, there will be opportunities for more natural, potentially safer, biological controls. In fact, the previously mentioned biogeographic profiling of tomato microbiota from agricultural regions in the eastern and western United States identified a statistically significant enrichment of *Firmicutes* taxa in the phyllosphere of tomatoes that correlated with a lower incidence of *Salmonella* contamination events. This led to the fruitful hypothesis that *Firmicutes* genera with similar antibiotic potential (*Paenibacillus*) that occur naturally in tomato ecologies in the eastern United States might be successfully harnessed for biological control of *Salmonella* in agricultural settings in this region, and more widely, as postharvest biological controls for foodborne pathogens (25).

Another important food type that has been consistently linked to foodborne illness outbreaks is fresh leafy greens. Of all hospitalizations due to illnesses contracted from leafy vegetables between 1973 and 2012, 46% were caused by Shiga toxin-producing *E. coli* (STEC) (26). Fresh bagged spinach was linked to six deaths and more than 100 hospitalizations from hemorrhagic colitis and hemolytic-uremic syndrome resulting from STEC infections. The infectious dose for STEC has been estimated to be 10 to 100 CFU;

thus, methods for detecting STEC in foods require a high degree of sensitivity. Work by Leonard et al. (26) on leafy vegetables demonstrated that shotgun metagenomics can be utilized to detect the presence of *E. coli* contamination in fresh leafy produce amidst the complex microbial native background of spinach, while simultaneously detecting multiple virulence genes as well as the molecular somatic O and flagellar H types (26). This approach relies on a brief enrichment step in order to reliably detect low STEC contamination levels in spinach (0.1 CFU/g) due to the bioinformatic confusion that can occur from the high level of indigenous microbiota and nonpathogenic *E. coli* strains.

METAGENOMICS AND QUASIMETAGENOMICS

While metagenomics has been extremely valuable for the description of numerous food and agricultural ecologies to identify critical control points in the food chain, it is still difficult to use culture-independent pathogen detection for conclusive identification of specific pathogenic strains from diverse and complex food or agricultural environments. Similar to the work with *E. coli* described above, the abundance of organisms like *Salmonella* in environmental settings is low, and they occur among numerous close relatives, which precludes effective source tracking from true metagenomic (culture-independent) sampling. Thus, as with *E. coli* in spinach in the work by Leonard et al. (26), some type of enrichment step is still necessary to obtain high-resolution phylogenetic source tracking of these clonal foodborne pathogens from complex food environments. While PCR targets may confirm the presence of the genus *Salmonella* or *Listeria*, for most source tracking efforts, a much higher-resolution analysis of the serovar or subtype of these highly clonal organisms is needed in order to trace the source.

To address this conundrum, researchers have begun using an approach known as quasimetagenomics for applied metagenomic source tracking (27). Because the word “metagenomics” refers to the “genomics of uncultured organisms” (28), “quasimetagenomics” is now used to describe the application of metagenomic methods like PCR and shotgun sequencing to environmental matrices that have undergone some degree of enrichment. Enriched samples are, by their nature, subsets of true community diversity, so they cannot be accurately described as “metagenomic”; hence the application of the term “quasimetagenomics.”

Proof of Concept for Quasimetagenomic Source Tracking with *L. monocytogenes*-Contaminated Ice Cream

A quasimetagenomics approach was used to retrospectively study samples of contaminated ice cream as they went through FDA, U.S. Department of Agriculture (USDA), and Food Safety and Inspection Service methods to enrich *L. monocytogenes* from ice cream. Results of the study demonstrated that the microbiological enrichment protocols for all agencies performed equally well for efficient recovery of *L. monocytogenes*. Genomic data recovered from the shotgun-sequenced enrichments at specific hours were even capable of identifying all of the strains of *L. monocytogenes* that had been associated with the outbreak (29).

Figure 37.2 shows the enrichment of *L. monocytogenes* over the 48 hours of pre-enrichment, which is the first step in the recovery process.

Levels of *Listeria* were very low in the first hours of the enrichment, as *Listeria* organisms struggled to compete with numerous other taxa during early hours. At hour 20 and 28, it gained a strong foothold and began to dominate the samples. Using shotgun data from hours as early as hour 20, single-nucleotide-polymorphism clustering and multilocus sequence typing trees placed strains of *L. monocytogenes* from quasimetagenomic data in exactly in the same

phylogenetic relationships as genomic data from WGS of pure independent isolates (Fig. 37.3). This is an exciting source tracking frontier for the future, requiring half the time of current state-of-the-art WGS standard operating procedures.

LOOKING TOWARD THE FUTURE OF AMERICAN FOOD

The Food Safety Modernization Act established rules and standards to describe minimum standards for safe growing, harvesting, packing, and holding of fruits and vegetables grown for human consumption (30). The new standards cover requirements for agricultural water quality, employee health and hygiene, exclusion of wild animals from agricultural areas, biological soil amendments of animal origin (such as compost and manure), equipment and tool management, and building sanitation. There are also requirements for the use of biological soil amendments of animal origin, which include manure and compost, which are added to soil to improve its ability to support plant growth. The more data that can be generated for all of these microbially complex ecological components of agriculture production, the more apt future recommendations will be.

One thing that is not mentioned among the risk factors delineated by the Food Safety Modernization Act is the possibility that air pressures in agricultural ecosystems influence the microbiotas of crop plants. Airborne environmental pressures have been documented to play important and previously unrecognized roles in driving the epiphytic microbiome of food plants in field settings (31). The better we are able to fully describe all microbial constituents of agricultural ecologies that contribute to microbiota that ends up on or within our foods, the better positioned we will be to protect those foods from contamination by pathogens. This continuously evolving knowledge base will also facilitate improved management strategies, improved regulatory requirements, and

hopefully more sustainable, less toxic biological controls for the management and preservation of food crops.

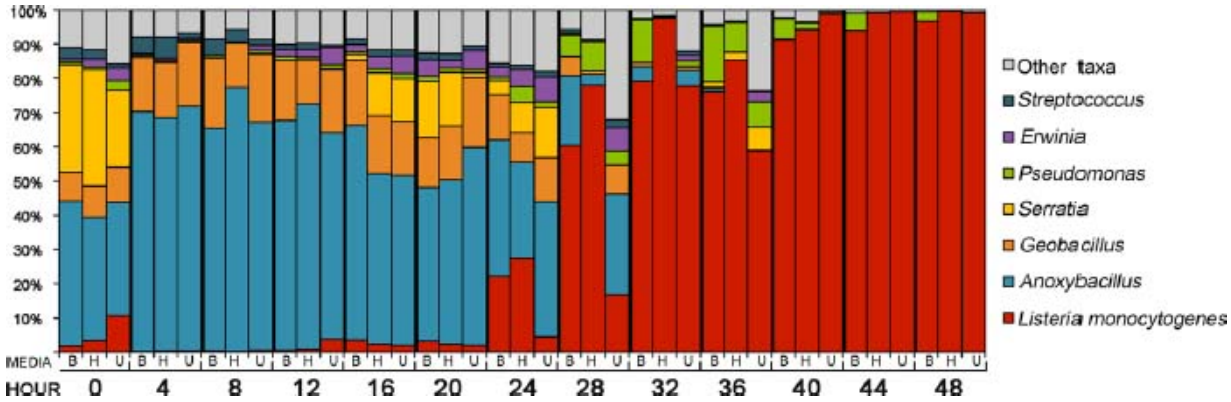


Figure 37.2. Quasimetagenomic survey of the FDA, USDA, and ISO enrichments for the recovery of *L. monocytogenes* from ice cream. B, buffered *Listeria* enrichment broth (FDA BAM method); U, University of Vermont modified broth with Fraser broth (USDA method); H, half-Fraser broth with transfer to Fraser broth (ISO method). All methods performed equally well for recovery of *Listeria*. Exciting source tracking utility was documented as early as hours 20 and 28, when genomic data for *Listeria* could be used to subtype strains with the same precision as WGS methods, in half the time (32). Adapted from reference 32.

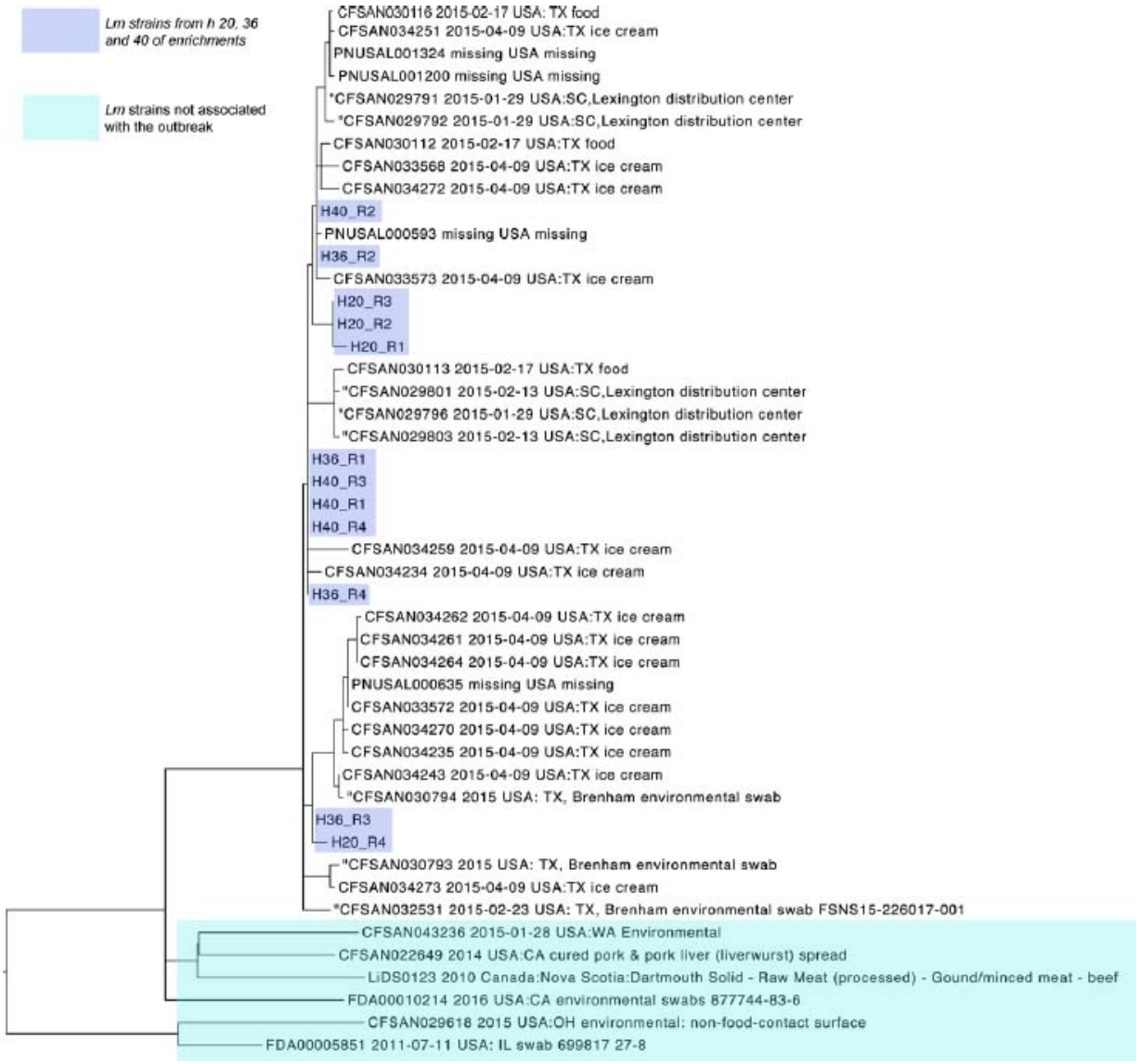


Figure 37.3. Using the NCBI data for the 2015 Blue Bell ice cream outbreak (abbreviated collection shown here) to assess the phylogenetic utility of a quasimetagenomic approach to source tracking, we show that single-nucleotide-polymorphism and MLST data from quasimetagenomic data of enrichments can be used to infer the same phylogenetic source attribution information that was achieved with WGS strains (data in purple are from hours 20, 36, and 40 of *Listeria* enrichments). This approach took half the time of traditional WGS and thus holds great promise as a method to reduce the time needed to source track pathogens from foods (32). *Lm*, *L. monocytogenes*.

Plants, like humans, comprise differentiated cell types, which make up organs. Microbial constituents of human organs such as skin have been shown to be niche driven (2) and unique in comparison to one another, and this is also true for plants (19). As we continue to better understand human microbiomes, a critical complement to public health research is the continued study of food microbiomes. The food microbiota intersects with the human microbiota and influences both health and disease, either by direct introduction of microbes or by the introduction of matrices such as fibers, sugars, and starches that function as prebiotics for resident microbiota. While early work describing phytobiomes and food ecologies emulated methods and protocols pioneered by the Human Microbiome Project, the field is now leading its own path back to human health, not only through improved food safety by reduction of pathogens but also by the characterization of microbiomes, metabolomes, and the vast array of bacterial, fungal, viral, protist, and multicellular eukaryotic organisms that play key roles in the architecture of food and food ecologies. The physics, chemistry, and microbiology of our foods will continue to impact our human microbiomes and influence states of health and disease. Now, more than ever before, the study of foods, ingredients, additives, preservatives, fungicides, bactericides, and all of the other diverse chemistry that is used to stabilize, preserve, and protect our foods must also be considered from the standpoint of the human microbiome. How will these chemistries affect nontarget members of gastrointestinal microbiota? The more data we gather, the more responsibly we can underpin new guidance and recommendations for food handling practices that will contribute to production of safe and nutritious American foods.

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38
Molecular Source Tracking and Molecular Subtyping

Bacterial subtyping may be defined as the characterization of bacteria below the species (or subspecies) level. Such characterization allows bacterial isolates to be placed into groups that are more or less similar to one another based on one or more (usually many) characteristics and may also reveal how such groups relate to each other. Subtyping can thus be used to study the population structure of a particular bacterial species, to determine the possible evolution of the subject microorganism, or to study the molecular epidemiology of a microbe. The types of methods used for subtyping and the approaches to data analysis and interpretation may vary greatly with the reason for specific subtyping. This chapter focuses almost entirely on subtyping for molecular epidemiology.

The term “molecular epidemiology” in the context of foodborne bacteria is usually applied to the subtyping of bacteria that cause foodborne disease and the ways in which such subtyping data contribute to understanding the transmission of those bacteria to humans. Molecular epidemiology can be applied to identifying the source of a particular outbreak or to a broader understanding of the role of certain foods or processes in outbreak-related or sporadic infections.

REASONS FOR PERFORMING MOLECULAR SUBTYPING

Perhaps the most easily appreciated reason for molecular subtyping is to facilitate the identification and investigation of foodborne disease outbreaks. By identifying isolates with the same molecular subtype, one can determine which isolates are most likely to have a common source. When isolates of foodborne pathogens with the same subtype are found to cluster temporally, especially if they also cluster geographically, it may indicate an outbreak of foodborne disease. Such cluster detection is the principal focus of the PulseNet system (1). PulseNet is described in more detail below. Once clusters are recognized, epidemiologic investigations can determine if a cluster actually represents an outbreak. Molecular subtyping can further facilitate epidemiologic investigations by determining which infections, of all those occurring at the same time as the outbreak, are most likely to be outbreak related (those sharing the same subtype or very similar ones). Subtyping contributes to tracking the source of an outbreak in two ways: by facilitating the epidemiologic investigation and by matching the subtype of isolates from food products with those from patients.

Table 38.1. Properties of methods commonly used for molecular subtyping of foodborne pathogens

Method	Strain discrimination	Interlaboratory reproducibility	Supply costs	Specialized equipment	Automatable	Unambiguous output
Plasmid profiling	Moderate	Moderate	Low	No	No	No
PFGE	High	Moderate	Moderate	Yes	No	No
rep-PCR/ERIC-PCR [a]	Moderate	Low	Low	No	No/yes [b]	No/yes [b]
PCR-RFLP	Low to moderate	High	Low	No	No	No
MLST [c]	Moderate	High	High	Yes	Yes	Yes
MLVA [c]	High	High	High	Yes	Yes	Yes

[a] ERIC, enterobacterial repetitive intergenic consensus.

[b] "No" refers to the standard method; "yes" refers to the DiversiLab system.

[c] Comments refer to the method as performed using automated DNA sequencing equipment and software.

There are many reasons for performing molecular subtyping other than cluster detection and outbreak investigation. Public health laboratories use subtyping to identify new and emerging bacterial pathogens, such as *Salmonella enterica* serotype Typhimurium DT104, which was identified in the 1990s. Food processing plants may use subtyping for tracking microorganisms through the plant to determine where they enter or reside within the plant and to monitor efforts to eliminate them. The types of subtyping methods used depend on the microorganism under study and the specific application (Table 38.1). Subtyping methods are described in detail in the next section, and specific applications are discussed in subsequent sections.

Regardless of the subtyping method being used, the importance of considering subtyping data in the context of other available data cannot be overemphasized. Factors such as the discriminative power of the subtyping method, the genetic diversity of the microbes being analyzed, and the occurrence and distribution of common molecular subtypes must all be considered in interpretation of subtyping results. Whenever possible, interpretation should be made in the context of epidemiologic and environmental investigations. Subtyping can only indicate which isolates are more likely or less likely to be related to an outbreak, food, or environmental source. Subtyping data alone cannot prove such a link.

COMMON SUBTYPING METHODS

Phenotypic Methods

Although the focus of this chapter is molecular methods, it is important to consider them in the context of earlier phenotypic methods, such as serotyping, phage typing, biotyping, and antimicrobial susceptibility typing. Most of these phenotypic methods have long and successful histories of use in subtyping for the same purposes for which molecular methods are now used. In fact, most of them are still used prior to, or in conjunction with, molecular

methods. Serotyping of *Salmonella* is an excellent example. Serotyping is so widely practiced that microbiologists tend to think of it more as identification than as subtyping, but serotyping is itself a powerful method of subtyping, with more than 2,500 named serotypes of *Salmonella*. With relatively rare serotypes, serotyping alone may provide a sufficient level of subtyping. For more common serotypes, phage typing systems with good strain discrimination have been developed, but these systems are serotype specific and are available in few laboratories, so molecular methods are generally used to provide further strain discrimination.

Although molecular methods typically provide greater strain discrimination than phenotypic methods, this is not always the case, and it is only one reason why molecular methods are generally preferred. Perhaps the most important reason for using molecular methods is that they do not require specialized reagents or expertise and can thus be performed in almost any laboratory with basic molecular biology capabilities. This ease of performance is also the most obvious drawback to molecular subtyping, as it allows every laboratory to perform and interpret subtyping according to their own criteria, making interlaboratory comparisons difficult unless laboratories can agree to standardized protocols.

Plasmid Profile Analysis

One of the first molecular methods used for strain identification or source tracking is plasmid profile analysis. Plasmids are small pieces of extrachromosomal DNA that are typically circular and supercoiled. Plasmids range in size from a few hundred to several hundred thousand base pairs and are present in most bacterial species. Plasmid DNA is extracted using a method that separates plasmid from chromosomal DNA, and the plasmids are then separated by agarose gel electrophoresis. How the plasmids travel in the gel depends on both size and conformation. Sensitivity can be increased by restriction digestion of the plasmid DNA so that different plasmids of the same approximate size can be distinguished.

One of the first successful uses of plasmid profile analysis in epidemiologic investigations occurred in 1981, when exposure to marijuana was associated with a cluster of *S. enterica* serotype Muenchen infections (2). Early uses of plasmid profile analysis included identifying outbreak-associated strains of *Salmonella* serotype Typhimurium (3, 4) and linking patient and hamburger isolates of *Escherichia coli* O157:H7 (5). Plasmid analysis continues to be useful today. During an outbreak of *Salmonella* serotype Typhimurium DT104 in England and Wales in 2000, all isolates analyzed by pulsed-field gel electrophoresis (PFGE) had the same profile, but 67% of the isolates could be classified as outbreak associated based on plasmid analysis (6). One major drawback of plasmid profiling is that bacteria may gain or lose plasmids during the course of an outbreak, resulting in different plasmid profiles and confounding interpretation.

RFLP Methods

DNA may be digested by restriction endonucleases, which are enzymes that cut the DNA at specific nucleotide sequences (the recognition sequence). The number and size of the resulting DNA fragments (restriction fragments) are unique to a particular strain; different strains yield different restriction fragment patterns. This is the principle behind all restriction fragment length polymorphism (RFLP)-based methods. In the initial methods, the DNA was digested with high-frequency cutting enzymes like EcoRI or HindIII, and the resulting fragments were separated by standard agarose gel electrophoresis (7). The resulting restriction patterns were very complex due to the huge number of fragments obtained,

resulting in poor resolution of individual fragments and difficulty in interpreting results. To overcome this problem, several variations on the basic method were developed, of which PFGE remains critical for surveillance and outbreak investigations of foodborne diseases.

PFGE Methods

The problem of excessively complex RFLP patterns was solved by decreasing the number of restriction fragments by using infrequently cutting enzymes. This way, the actual number of fragments obtained is reduced to a manageable number. While this strategy is obvious, the size of the resulting fragments prevents them from being separated by standard gel electrophoresis. The development of PFGE in 1983 (8) eliminated this drawback. During the following few years, several different types of PFGE were devised, including transverse alternating-field electrophoresis (9), field inversion gel electrophoresis (10), orthogonal field alternation gel electrophoresis (10), and contour-clamped homogeneous electric field electrophoresis (11). Although these methods differ considerably in the physical way in which fragments are separated, they alldepend on changing the direction of current flow (pulsing) over a gradient of time intervals between changes. The contour-clamped homogeneous electric field technology is probably the only one used today.

From the early 1990s to the late 2010s, PFGE was the gold standard for molecular subtyping and source tracking for most foodborne bacteria, as well as many other bacterial agents. PFGE is so widely known and accepted that a lengthy list of applications would be superfluous. The application of PFGE to specific foodborne pathogens is described below. As with any subtyping method, PFGE can present problems in interlaboratory reproducibility and interpretation of results. Such problems can never be totally eliminated, but they can be minimized by using carefully standardized protocols and image analysis software (1, 12).

PCR-Based Methods

Although many different PCR-based subtyping methods have been reported, most methods fall into one of two categories. The first type of PCR-based subtyping methods involves using primers homologous to known, conserved, multicopy sequences. In this case, differences in amplicon lengths are the basis of subtype comparisons. Two such methods, based on the presence of repeated elements, were reported by Versalovic et al. (13). These authors found that amplification of repetitive extragenic palindromic elements or enterobacterial repetitive intergenic consensus sequences could discriminate between laboratory strains of *E. coli*, suggesting that these methods could be used for subtyping foodborne pathogens. Methods targeting repetitive elements are collectively known as rep-PCR. Although rep-PCR methods have not become as widely used as other typing methods, such as PFGE, they do have the advantages of being relatively inexpensive and easy to perform. They have been used to track fecal pollution by subtyping *E. coli* isolates in diverse settings, such as dairy and swine production systems (14) and Apalachicola Bay in Florida (15). As with other subtyping methods, successful source tracking can be improved with the use of appropriate statistical methods (16). The biggest drawback to rep-PCR may be difficulties with intra- and interlaboratory reproducibility. This problem may have been solved by the introduction of a commercial semiautomated platform for rep-PCR, the DiversiLab system (bioMérieux, Marcy l'Étoile, France). In this system, quality-controlled reagents are provided in a kit format with Internet-based computer-assisted analysis, reporting, and data storage (17). Although the system does not provide the same high discriminatory power as PFGE, it seems to be gaining acceptance in the food microbiology community (18, 19).

The second category of PCR-based methods combines PCR with restriction digestion: either PCR amplification followed by restriction digestion of the amplicon or restriction digestion of the genome followed by selective amplification of certain restriction fragments. In the first approach, the target locus is typically 1 to 2 kb, and digestion results in several fragments that are then separated by gel electrophoresis, forming a characteristic RFLP profile. A specific example of the utility of such a PCR-RFLP in subtyping *Campylobacter* is discussed in detail in the *Campylobacter* section.

DNA Sequence-Based Methods MLST

Multilocus sequence typing (MLST) was one of the first subtyping or strain-tracking methods that were based on DNA sequences rather than DNA fragment sizes. All of the RFLP-based methods described above suffer, to different degrees, from the same problems. In addition to the problems of interlaboratory variability in methods and interpretation, RFLP methods have further shortcomings. Fragments from different test strains that appear to be the same size on a gel may originate from different parts of the genome with completely different genetic content. Fragments that are very close in size may not be separated during electrophoresis and thus not recognized. The reasons for differences in patterns are not known, and strains with fewer differences are not necessarily more closely related phylogenetically than strains with more differences. DNA sequence-based subtyping yields unambiguous data that can be readily compared between laboratories and can also be used to quantify differences between test strains.

MLST was derived from the earlier technology of multilocus enzyme electrophoresis, which is a phenotypic method that detects changes in certain housekeeping loci by charting variations in the electrophoretic mobility of their gene products (20). Only changes that result in a different electrophoretic mobility are detected. MLST is a more sensitive method that detects all nucleotide changes by direct sequencing. MLST is typically performed by sequencing 450- to 500-bp internal fragments of seven housekeeping genes. Since these housekeeping genes are under little selective pressure, the accumulation of changes occurring by chance is rather low, making MLST an ideal tool for understanding the evolution of microorganisms or strains. However, this lack of rapid change in the housekeeping genes makes MLST a less-than-ideal method for investigating outbreaks or conducting traceback studies. MLST typing schemes have been described for a number of foodborne pathogens, for example, *Campylobacter* (21), *S. enterica* (22), *E. coli* (23), and *Listeria monocytogenes* (24). In order to increase the discriminatory power, MLST schemes targeting faster-evolving sequences such as virulenceassociated genes or temperate phages have been developed (25, 26). Whole-genome sequencing (WGS) has allowed comprehensive, genome-wide MLST schemes to be developed and implemented, as detailed below.

VNTR-Based Typing, including MLVA

The recent sequencing of several complete bacterial genomes has revealed that much of the bacterial genome consists of repeated short nucleotide sequences. The repeats may vary in size, location, and complexity. Repeats can be classified according to their structure, such as inverted, dyad, or direct repeats. In bacteria, the repeat units are typically 1 to 10 nucleotides, although repeating units of up to 100 bp are recognized. These repeating arrays are commonly called variable-number tandem repeats (VNTRs). Variability occurs both in the sequence of the repeating unit and in the number of repeats between strains. Slipped-strand mispairing is the molecular model that best explains the variability of short sequence repeats (27).

Stretches of relatively short arrays of repeat units, when being copied by DNA polymerase, may engage in illegitimate base pairing. This forces the polymerase to introduce or delete individual repeat units. VNTRs are often found in noncoding regions controlling gene expression, but they are also found within open reading frames (28, 29).

The sequence of the repeat unit and the number of copies repeated are characteristic of each VNTR. For highly clonal microorganisms, there may be little variability for a given VNTR, and multiple loci must be examined. This approach has been termed multiple-locus VNTR analysis (MLVA). In some ways, MLVA may be analogous to MLST, in that multiple loci must be sequenced to provide sufficient strain discrimination. As typically performed, MLVA determines the number of repeats at a VNTR locus by the size of a PCR amplicon using high-resolution capillary electrophoresis rather than by actual DNA sequencing.

The benefits of MLVA include high throughput, technical simplicity, and objectivity of the data. The interlaboratory reproducibility of the data is excellent as long as the same capillary electrophoresis platform is used. However, there are some significant sizing discrepancies between capillary electrophoresis platforms from different manufacturers due to differences in dye and polymer chemistries employed by each platform. When different instrument platforms are employed, interlaboratory data sharing can be facilitated by a calibration strain set that is used to normalize the allele types to be comparable to the actual sequenced copy number (30). The assays also are highly species and even serotype specific, and prior knowledge about the sequence is required for the protocol development.

One of the first, and perhaps one of the most important, applications of MLVA was the subtyping of the highly monomorphic potential agent of bioterrorism *Bacillus anthracis* (31). It has also proven useful for subtyping two other potential agents of bioterrorism for which other methods had not proven adequate, *Yersinia pestis* (32) and *Francisella tularensis* (33). In recent years, MLVA has also been widely used to subtype foodborne microorganisms either as a primary method or as a complementary technique to PFGE. These applications are discussed below.

DNA Microarrays

A DNA microarray is a molecular platform in which a few hundred to thousands of specific DNA oligonucleotides or short sequences (capture molecules) are bound to a matrix. The array is used to detect specific DNA sequences (target sequences) in a test sample of DNA from the microbe being investigated by hybridization to capture molecules followed by detection of the arraybound test DNA.

DNA microarrays once represented an attractive platform for use in bacterial identification and subtyping, especially on the genome scale, because they allow rapid and accurate analysis of large numbers of different DNA molecules. Reported applications of the DNA microarray technology include pathogen identification (34–36), detection of virulence and antimicrobial resistance markers (37–40), single nucleotide polymorphism (SNP) typing (41), and genome comparisons (42). However, the increasingly routine practice of WGS in recent years has made microarrays largely obsolete as a genome-scale subtyping platform. All of the aforementioned subtyping analyses using microarrays can now be readily performed using WGS, as detailed below.

WGS

Advances in sequencing technology over the last 2 decades have made WGS-based subtyping approaches the method of choice for many foodborne pathogens. Sequencing platforms can be roughly categorized into two groups, platforms producing short (<1,000-bp) reads (Illumina platforms; Ion Torrent [Thermo Fisher]) and platforms producing long (>1,000-bp) reads (Pacific Biosciences; Oxford Nanopore MinION). While sequence data generated by long-read platforms are essential for the assembly of closed genomes (i.e., a genome sequence that represents the entire contiguous genome, without gaps in the assembly), at the time of writing, the cost, accuracy, and output of short-read platforms make them the platform of choice for most WGS-based subtyping approaches. Bioinformatics approaches underlying WGS-based subtyping can be either reference mapping based, *de novo*, or a combination of both approaches. For reference mappingbased approaches, reads are aligned (mapped) to a reference genome (typically a closed genome) or a sequence database. Sequence depth and coverage, along with single nucleotide or insertion/deletion differences, are then used to infer genome-wide variants or allelic differences. *De novo* approaches use genome assembly or graph-like representations to infer genomic variants or allelic differences. Reference mapping-based approaches are usually faster and less computationally intensive than *de novo* approaches, while *de novo* approaches are critical to assess variation in regions that are not present in the reference genome or in the sequence database, such as genomic insertions or mobile genetic elements.

SNPs

The emergence of WGS for bacterial subtyping came with the prevalent choice of SNPs as the subtyping targets. A SNP is a change in a single nucleotide in one sequence relative to another caused by nucleotide mutation, horizontal gene transfer, or intragenic recombination events. Many prevalent foodborne bacterial pathogens, especially closely related strains, display a clonal population structure with little recombination within the population. For these organisms, SNPs identified along entire genomes are reliable and high-resolution markers for phylogenetic reconstruction. During outbreaks, the clonal transmission of pathogens can be reflected by phylogenetic trees, with the close phylogenetic distances between isolates suggesting epidemiological association between cases. Therefore, outbreak clusters can be robustly identified from SNP-based phylogenies. Furthermore, the identification of SNPs is more straightforward and accurate than that of other mutations, such as insertions and deletions, using short sequencing reads. High-fidelity SNPs can be reliably detected by mapping short sequencing reads to an appropriate reference genome (43).

While whole-genome SNP typing usually provides maximum discrimination for outbreak investigations and is highly useful for population studies, it is difficult to standardize. The choice of reference genomes and bioinformatics tools can affect the results of SNP analyses (44). For this reason, SNP profiles of analyzed strains are not amenable to standard nomenclature, which is important for data reporting and exchange among surveillance laboratories. Additionally, the tools used for SNP calling require specialized bioinformatics skills and access to local high-performance computing, both of which are not readily available to allend users, particularly public health laboratorians performing surveillance. Specific examples of utilizing SNPs for characterization of foodborne microbes are given below.

wgMLST

Whole-genome MLST (wgMLST) is a natural extension of classical MLST, in that it extends the number of loci interrogated from typically seven gene fragments to hundreds or

thousands of genes in the whole genome, depending on the organism or wgMLST scheme. The tremendous increase of the number of interrogated loci naturally brings an increase in resolution, making it a subtyping method that is relevant for both species-level population structure inference and potential outbreak cluster detection. In its most extended application, variations in the genes in the whole pangenome of the set of reference strains used to create the wgMLST schema are assessed. In core genome MLST (cgMLST), only genes that are shared by allgenomes (core genes) are used. It is also possible to create schemas tailored to specific tasks (e.g., source attribution of sporadic illness) and containing any number of genes from the core and/or accessory genome that vary in presence within a bacterial species (45). wgMLST is the most discriminatory of the approaches; however, cgMLST is often more practical, as it allows a database with a small but stable set of genes that are found in most newly sequenced genomes. cgMLST and wgMLST approaches provide a higher level of discrimination than older subtyping methods, such as PFGE, and allow standardized nomenclature of cgMLST and wgMLST types (43) when centralized databases are used, which may improve communication between national and international public health centers.

Using wgMLST reduces the complexity of wholegenome analysis from an entire genome (3 to 5 million positions in a typical foodborne pathogen genome) to hundreds to a few thousands of genes. Because the complexity is reduced, the analysis can be run on a local computer instead of a powerful centralized server. Furthermore, the analysis itself is simplified (in the proprietary software packages BioNumerics and SeqSphere+ and numerous open-source packages), allowing most biologists without advanced knowledge of bioinformatics to run the analysis. Typically, a wgMLST analysis using an assembly-free approach takes only several seconds on a local computer while retaining correlation with SNP analysis (Fig. 38.1). Because of the reduced complexity, wgMLST delivers the power of a whole-genome analysis but in less time, with less required expertise, and on a less powerful computer.

WGS for Other Subtyping Schemes

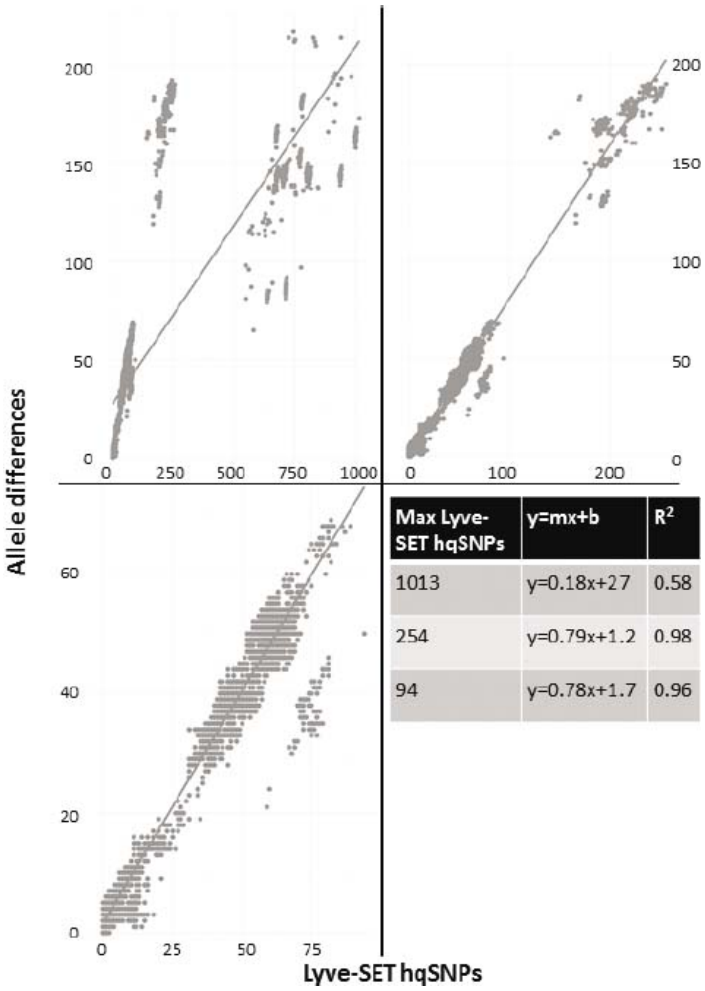
By providing genetic information of the entire genomes, WGS can be used to predict a variety of genotypic and phenotypic subtypes. A growing list of WGS-based subtyping tools is making WGS an inclusive platform for molecular subtyping. For example, traditional MLST (46–49), plasmid profiling (50), serotyping (51–53), and antibiotic resistance profiling (54) can all be performed using WGS data. These bioinformatics tools extract subtyping markers from assembled genomes (46, 47, 52–54), raw sequencing reads (48, 49), or both (51). The ability to predict subtypes directly from raw reads avoids the computationally intensive and sometimes error-prone process of *de novo* genome assembly, leading to more rapid turnaround and better scalability for high-throughput applications.

While in theory any genotypes can be determined from WGS, the challenge of assembling complete genomes without gaps from short sequencing reads (i.e., between 100 and 300 bp) prevents the incorporation of certain molecular subtyping schemes into a single WGS platform. For instance, draft genomes assembled from short reads are often too fragmented to predict PFGE patterns, which depend upon a few large fragments of the genomes, and MLVA profiles, because the short repeats do not assemble accurately.

Web Resources for WGS Databasing and Subtyping Analyses

WGS-based subtyping and source tracking of foodborne pathogens require reference databases and related informatics tools. Integrated, Web-based resources for WGS data depository and subtyping analyses include, among many others, EnteroBase

(enterobase.warwick.ac.uk) and National Center for Biotechnology Information (NCBI) Pathogen Detection (www.ncbi.nlm.nih.gov/pathogens/). EnteroBase currently houses databases for *Salmonella*, *Escherichia*/*Shigella*, *Yersinia*, and *Moraxella*. It provides both classic seven-gene and genomewide schemes for MLST analyses. The NCBI Pathogen Detection system is focused on active and real-time surveillance of foodborne pathogens using sequencing data. A Web-based portal (www.ncbi.nlm.nih.gov/pathogens/isolates#/search/) integrates genotypic (e.g., SNP, sequence, and resistance gene) and phenotypic (e.g., antibiotic susceptibility) information and metadata to facilitate the detection of potential foodborne contamination sources. This system collects data deposited at NCBI, including genome sequences submitted by a number of public health agencies in the United States and other countries that use WGS for routine pathogen surveillance. Besides EnteroBase and NCBI Pathogen Detection, PATRIC (www.patricbrc.org) (55) is another major Web resource of pathogen genomes and analytical tools. Unlike the former two, PATRIC is geared for general purpose query and bioinformatics analysis.



Max Lyve-SET hqSNPs	y=mx+b	R^2
1013	y=0.18x+27	0.58
254	y=0.79x+1.2	0.98
94	y=0.78x+1.7	0.96

Figure 38.1. MLST correlates strongly with SNP analysis when the number of SNPs is lower than 255. A scatterplot was generated using all allelic distances from wgMLST and SNP distances from SNP analysis for three *L. monocytogenes* outbreak clusters. The top left plot shows all pairwise distances, the top right limits the data points to those with <255 SNPs, and the bottom left limits the data points to those of <100. Adapted from reference 43.

APPLICATION OF MOLECULAR SUBTYPING METHODS TO SPECIFIC FOODBORNE BACTERIA

Salmonella

Bacteria of the genus *Salmonella* are so widespread in animals, humans, and the environment that virtually every commonly used subtyping method has been applied to the molecular epidemiology of these bacteria. Although it is not generally thought of as a subtyping method, serotyping of *Salmonella* is actually a very sensitive subtyping method, with more than 2,500 recognized serotypes. With rare serotypes, simply determining the serotype may be sufficient to at least suggest a source or epidemiologic connection. For example, an outbreak of *Salmonella* serotype Hartford infections occurred among visitors to a theme park in Orlando, Florida, in 1995 (56). Serotype Hartford is a sufficiently rare serotype that further subtyping was not needed to suggest an association with drinking orange juice at the park. The association was further strengthened by the isolation of another rare *Salmonella* serotype, Gaminara, from the implicated brand of orange juice as well as from a person with a serotype Hartford coinfection. Ironically, the PFGE subtypes of serotype Gaminara from the patient and the orange juice were different, which could have led to erroneous conclusions if the epidemiologic evidence had not been considered.

Since maintaining the wide panel of diagnostic antisera that is required to perform serotyping is time-consuming and difficult, several molecular methods have been developed to predict the serotype. These methods may be grouped into two types: methods that target the serotype-determining genes (*rfb*, *fliC*, and *fljB*) and surrogate methods that provide subtypes that may correlate with the serotype but do not target the serotypedetermining genes. If a method that targets the serotypedetermining genes is used, the system will be compatible with the Kauffman-White scheme, which contains the phenotypic descriptions of all*Salmonella* serotypes (57), and hence backwards compatible with historical data sets based on the Kauffmann-White scheme. Two protocols based on PCR amplification of the serotype-determining genes with subsequent detection of the targets using a DNA array have been published (36, 58, 59). The surrogate methods are methods that were developed for other purposes but happen to correlate with the serotype, e.g., PFGE (60) or rep-PCR (61), or methods that target non-serotype-specific sequences present in some but not all serotypes (62). The surrogate methods reliably identify a serotype only if its molecular profile is already present in the correlation database. Any isolate with an unrecognized profile by a surrogate method has to be confirmed by a method that utilizes the Kauffmann-White scheme, i.e., traditional serotyping or its molecular counterpart. As mentioned above, *Salmonella* serotypes can now be accurately predicted from WGS data. SeqSero (51) is the first reported bioinformatics tool for bacterial serotyping using WGS. By exclusively targeting serotype determinant genes, SeqSero is able to predict more than 2,200 serotypes and provide close continuity with the Kauffman-White scheme.

For the more common serotypes, such as *Salmonella* serotypes Enteritidis and Typhimurium, serotyping alone typically does not provide sufficient strain discrimination for source tracking. Some of the earliest descriptions of molecular strain typing involved the application of plasmid profile analysis to *Salmonella* serotypes Muenchen (2), Typhimurium (3), and Enteritidis (63).

Since the establishment of PulseNet (1), PFGE has been routinely used to support surveillance and outbreak investigation of *Salmonella* serotype Typhimurium (64) and many

other serotypes (65). In addition to outbreak investigations, PFGE has been successfully applied to tracking *Salmonella* in production environments. Liljebjelke et al. used PFGE to reveal that isolates of *Salmonella* serotypes Typhimurium and Enteritidis from processed poultry carcasses from one farm were indistinguishable from isolates obtained in the poultry house environment and at a company breeder farm, suggesting vertical as well as horizontal transmission (66).

MLVA has also been used to subtype *Salmonella* serotypes Typhimurium (67, 68) and Enteritidis (69, 70). In Denmark and Norway, MLVA has been included in the routine surveillance of *Salmonella* serotype Typhimurium since 2004 and has proven to be superior to PFGE in both surveillance and outbreak detection (71, 72). Dyet et al. investigated the ability of MLVA to discriminate between different serotype Typhimurium phage types in New Zealand (73). They observed good discriminatory power among DT104 isolates but limited discrimination among phage types DT101, DT160, and RDNC (reacts but does not conform to a recognized Typhimurium phage type). PulseNet USA has also used MLVA as a complementary technique to PFGE to further characterize PFGE-defined clusters of *Salmonella* serotypes Typhimurium and Enteritidis with common PFGE patterns and temporarily associated PFGE-defined clusters with closely related patterns (74).

Besides PFGE and MLVA, MLST (25, 75, 76) and DNA microarrays (35, 37–40) have been evaluated for *Salmonella* subtyping and characterization. It was found that MLST was not as discriminating as PFGE when applied to *Salmonella* serotype Typhimurium isolates (76).

The first application of WGS in a retrospective investigation of a *Salmonella* outbreak was reported in 2011 (77). Between July 2009 and May 2010, a multistate outbreak of *S. enterica* serotype Montevideo occurred in the United States. Nearly 300 illnesses were reported in 44 states and the District of Columbia (https://www.cdc.gov/salmonella/2010/montevideo-5-4-2010.html). Epidemiologic evidence suggested that black and red peppers used to prepare salami were the source of the infections, but PFGE was unable to distinguish food and clinical isolates of this outbreak from previous isolates associated with a pistachio contamination. However, pistachios were quickly excluded as a possible source of the outbreak, since no patients had been exposed to them. WGS afforded sufficient subtyping resolution to confirm the results of traceback investigation and identify pistachios as a confounding food vehicle of the outbreak strains.

PFGE is also not discriminatory enough for some strains of *S. enterica* serotype Enteritidis, one of the most prevalent serotypes in the United States and worldwide (78). Among the *Salmonella* serotype Enteritidis isolates reported to PulseNet, approximately 45% display a particular PFGE pattern (JEGX01.0004) using XbaI (79), making PFGE ineffective for strain-level subtyping of these isolates. WGS has addressed this issue. In a retrospective investigation that comparatively evaluated WGS-based SNP typing and other molecular subtyping methods, such as PFGE and MLVA, WGS-based SNP typing was the only method able to resolve all16 outbreak clusters of *Salmonella* serotype Enteritidis detected between 2001 and 2012 in the United States (79). Rapid surveillance of *Salmonella* serotype Enteritidis using WGS was demonstrated by conducting both retrospective and prospective investigations at the state level in the United States (80). Besides detecting additional putative outbreaks, the investigators were able to attribute additional putative cases to confirmed outbreaks. Some of these isolates would have been excluded from the corresponding outbreak by PFGE because their PFGE patterns differed from that of the outbreak strain.

In the United Kingdom, real-time nanopore sequencing was recently evaluated for outbreak investigation (81). By retrospectively analyzing *Salmonella* Enteritidis isolates from a 2016

hospital outbreak, the authors demonstrated rapid species identification, serotype prediction, and phylogenetic placement within 20 min, 40 min, and 2 h after the start of sequencing, respectively. At the international level, WGS was used to confirm cases of a multicountry outbreak of *Salmonella* serotype Enteritidis illness linked to eggs in Europe (82). Besides surveillance and outbreak investigation, the ability of WGS to provide robust phylogenetic inference with high epidemiological correlation has also promoted the understanding of population structure and evolution of this pathogen (83–85).

STEC O157 and Non-O157 STEC

One of the first applications of PFGE to foodborne disease investigations occurred during the large hamburger-associated outbreak of *E. coli* O157:H7 in the western United States in 1993 (86). The demand for PFGE subtyping to support investigations of *E. coli* O157:H7 outbreaks was the driving force behind the creation of PulseNet USA, the national molecular subtyping network for foodborne disease surveillance (1). PFGE was the PulseNet standard method for subtyping *E. coli* O157 until WGS took over that role in 2019. A standardized PFGE protocol for the six most prevalent serotypes of non-O157 Shiga toxin-producing *E. coli* (STEC) was released by PulseNet in 2009 (https://www.cdc.gov/pulsenet/index.html). The relative clonality of the bacterium limits the applicability of MLST (87). Foley et al. compared PFGE, MLST, and rep-PCR for typing isolates from food, cattle, and humans and concluded that PFGE was the method of choice (88).

Multiple partially overlapping MLVA protocols for subtyping STEC O157 have been reported in recent years (65, 67, 89). All reports indicate the ability of MLVA to discriminate between apparently unrelated isolates with indistinguishable PFGE patterns. In the United States, MLVA data have been used in outbreak investigations since 2008 to narrow the case definition and strengthen the association with the vehicle, particularly when the outbreak strain displays a common PFGE pattern and the presumptive source of exposure is a common one. Also, Dyet et al. reported a better epidemiologic concordance of MLVA data than of PFGE data when they used the PulseNet standardized MLVA protocol to characterize 118 isolates from New Zealand (73). A more generic MLVA protocol covering all pathogenic *E. coli* strains was recently published by Lindstedt et al. (90). However, this assay appears to have limited discriminatory power, particularly among non-O157 STEC isolates (91).

WGS-based approaches have been developed that make it possible to rapidly identify putative STEC, subtype isolates using cgMLST, and obtain SNP information to infer putative outbreak clusters. *E. coli* serotypes can be inferred from the presence of alleles of O-antigen processing genes *wzx*, *wzy*, *wzm*, and *wzt* and alleles of the flagellin genes *fliC*, *flkA*, *fllA*, *flmA*, and *flnA* in a genome (52). Additionally, the presence of Shiga toxin genes can be inferred from WGS to confirm the STEC pathotype (92). Lindsey et al. further demonstrate that WGS-based inference of serotype, pathotype, and resistance profile is more efficient timewise and economically than traditional subtyping methods (92). While *E. coli* O157:H7 is considered relatively clonal, SNP diversity is large enough to be valuable for outbreak investigations. A pre-WGS, microarray-based study reported 906 SNPs in 523 chromosomal genes (41). A recent WGS-based study included isolates from seven North American outbreaks and found 3,313 SNPs (93). That study further assessed within-outbreak SNP diversity and proposed criteria for WGS data in outbreak investigations. Crowe et al. recently reported using high-quality SNP data as part of the case definition in order to improve specificity in a large high-profile STEC O121 outbreak associated with flour (94). Based on the WGS data, the

investigators were able to exclude background cases from the case definition and link cases with variant PFGE patterns to the outbreak.

L. monocytogenes

Since the inception of PulseNet and until 2013, PFGE was the gold standard for molecular subtyping of *L. monocytogenes* (95, 96). As the standard method for PulseNet, it has contributed to identifying clusters of *L. monocytogenes* infections and tracking the source of outbreaks (95, 96). It also has been and still is used to track *L. monocytogenes* strains in food processing plants (97). Gudmundsdottir et al. used PFGE to identify potential sources of contamination of cold-smoked salmon in a processing plant in Iceland (98).

Other subtyping methods have been developed for *L. monocytogenes*. Multiple partially overlapping MLVA protocols have been reported for subtyping *L. monocytogenes*, but none of them appear to provide discriminatory power comparable to that of PFGE, particularly among lineage I isolates (71, 99, 100). MLST was also reported to identify a larger number of subtypes of *L. monocytogenes* than PFGE, but this study did not determine the epidemiologic utility of the method (101). A variant of MLST named multi-virulence-locus sequence typing included virulence and virulence-associated genes to better distinguish closely related isolates, such as those with indistinguishable PFGE patterns (26, 102). Increasing the number of genotyping markers was another strategy to improve subtyping resolution for *L. monocytogenes*. A multilocus genotyping assay examined 60 SNPs, indels, or truncations in 22 genes distributed across the *Listeria* genome (103). The method identified strains belonging to the known epidemic clones ECI, ECIa, and ECII in *L. monocytogenes* lineage I. This method was subsequently expanded to lineage II, III, and IV strains (104) and optimized for lineage, serotype, and epidemic clone identification of *L. monocytogenes* (105). DNA microarrays have also been used for genome-wide (34, 106) and pangenome-scale subtyping of *L. monocytogenes* (42). Despite the wide array of reported methods, PFGE remained the method of choice for highly discriminatory subtyping of *Listeria* before the arrival of WGS.

In 2013, a collaboration was initiated among the Centers for Diseases Control and Prevention (CDC), the U.S. Food and Drug Administration (FDA), the Food Safety Inspection Service (FSIS) of the United States Department of Agriculture (USDA), and the NCBI to carry out WGS of all*L. monocytogenes* isolates found in routine surveillance and share the WGS data publicly in real time. SNP subtyping was used at the onset of this collaboration (43, 107). Through international collaboration, a cgMLST scheme was developed (108). Because of its ease of use, amenability to nomenclature standardization, and concordance with high-quality SNP analysis (43), wgMLST replaced SNP subtyping in routine *L. monocytogenes* surveillance. Since implementation of real-time WGS of *L. monocytogenes* in combination with epidemiologic surveillance, more potential outbreak-related clusters are being detected (109).

Sequencing all isolates from routine surveillance has informed us about the use of PFGE patterns to define clusters. In some cases, WGS clusters contained diverse PFGE patterns, while in other cases, multiple WGS clusters were found in PFGE-defined clusters. Furthermore, WGS can aid in finding the food vehicle for “cold” cases and sporadic cases, as WGS can phylogenetically link isolates from human cases and food. Furthermore, because of sequencing of both food product- and patient-derived isolates, outbreaks can be confirmed following product testing, allowing an early association of an outbreak with a contaminated food. Finally, WGS can help create a rapid and precise definition of outbreak cases and thus productively redirect epidemiological resources (109).

Campylobacter jejuni

Unlike the situation for *Salmonella*, STEC, and *L. monocytogenes*, there has been a lack of consensus on the method for subtyping *C. jejuni*. The plasticity of the *Campylobacter* genome, caused by horizontal genetic exchange, and the relative lack of identifiable outbreaks have led some authors to question the utility of real-time molecular typing (110, 111) for outbreak detection.

PFGE was suggested as an epidemiologic tool for *C. jejuni* as long ago as 1991 (112). It was used to discriminate between outbreak and sporadic isolates of *C. jejuni* within a single serotype as early as 1994 (97) and continues to be used (113). Despite these successes, several investigators have expressed concerns about the effect of genomic instability on PFGE type. There was clear evidence that genomic rearrangement and genetic exchange between strains alter PFGE patterns *in vivo* in poultry (114, 115), although at least some strains appeared to be genetically stable (116). This lack of stability made it more difficult to use PFGE to trace the source of *C. jejuni* contamination or to establish links between animal sources and human infections. Unfortunately, PFGE was not the only method affected by genetic instability.

Another early molecular typing method for *C. jejuni* was based on amplification of the flagellin gene *flaA*, followed by restriction digestion of the amplicon and separation of the fragments by gel electrophoresis (117). The sensitivity and specificity of this method were improved by sequencing of the *flaA* gene, and typing was further simplified by focusing on the short variable region within the gene (118). Both of these approaches were affected by recombination within the flagellin locus (119) and are generally not as sensitive as other available methods.

MLST has also been reported to be useful, especially when combined with antigen gene sequence data from targets such as *flaA* (120, 121), *flaB*, and *porA* (122). Some alleles have host specificity, and for that reason MLST has emerged as a tool for microbiological source attribution of campylobacteriosis (123) and for study of the population dynamics and geographic spread of *Campylobacter* (124, 125).

To improve subtyping resolution, another genotyping method has been developed to examine the presence and absence of a larger set of genes than that of typical MLST assays. One example, termed comparative genomic fingerprinting, targets a total of 40 genes (126). Another similar approach, called PCR binary typing, included a total of 68 genes (127).

As with other foodborne pathogens, molecular subtyping of *C. jejuni* and other *Campylobacter* species is being transformed by WGS. Cody et al. reported a retrospective evaluation of WGS for routine subtyping of *C. jejuni* and *Campylobacter coli* (128). WGS data from 384 clinical *Campylobacter* isolates collected over 4 months in Oxford, United Kingdom, were analyzed using a 52-locus ribosomal MLST scheme and a 1,643-locus wgMLST scheme when additional resolution was needed (129). Discrimination among *Campylobacter* clonal complexes and between closely related isolates was achieved by the whole-genome gene-bygene subtyping approach that was reference genome free and scalable to large data sets.

FUTURE OF MOLECULAR TYPING OF FOODBORNE PATHOGENS

With the increasing international trade of food and food animals, a foodborne infection in one country may have its origin in another country, even one on another continent. It is therefore

crucial that molecular subtyping methods for foodborne pathogens be harmonized worldwide to facilitate the rapid comparison of strains isolated in different countries. This harmonization for comparison is best done in the framework of surveillance networks. Such networking systems have been in place for 2 decades in Europe—first as the Salm-net, then the Enter-net (130, 131), and now the European Centers for Disease Prevention and Control Food and Waterborne Diseases and Zoonoses Network (FWD-Net) (https://ecdc.europa.eu/en/about-us/partnerships-and-networks/disease-and-laboratory-networks/fwd-net)—as well as in the United States with PulseNet (98), and most recently with the extension of the PulseNet network internationally onto six continents (132). The WHO Global Foodborne Infections Network (formerly Global Salm-Surv) also focuses on surveillance of foodborne infections internationally, building capacity through training workshops (http://www.who.int/gfn/en/). The future success of these networks will largely depend on the development and adoption of cutting-edge subtyping methods. The GenomeTrakr network, founded by the FDA in 2012, is a network for surveillance of foodborne pathogens in food and the food production environment. The network uses a publicly available database of raw sequences for foodborne pathogens from different sources and of diverse geographic origins housed as bioprojects at the NCBI (National Institutes of Health) (133).

An ideal subtyping method should type all strains, should discriminate between all isolates that are epidemiologically unrelated while identifying all related isolates, and should be 100% reproducible, universally applicable, and inexpensive. The results obtained should be definitive, fully portable, and easy to interpret. New methods should correlate with previously used methods so that the data obtained with the new methods can be related to historical data obtained with the previous methods. It is evident that no currently used subtyping method fulfills all these requirements, and it is likely that none ever will. Nevertheless, new methods are constantly being developed and evaluated and are bringing the science closer to the ideal.

With the ongoing implementation of WGS, we now have an opportunity for the first time in the history of foodborne disease surveillance to establish universal global standards for subtyping foodborne bacteria that will result in easily exchangeable data and global nomenclature. PulseNet International recently laid outits vision to adopt wgMLST as its primary surveillance tool, replacing both PFGE and traditional reference characterization assays with a single efficient workflow (134). Their goal is to place the allele and strain nomenclature databases in the public domain so that all participants, regardless of the analysis software platform they use, will detect the alleles in the same way and use a common nomenclature. Thorough validation studies using well-defined sets of historical isolates with global geographic representation are required to evaluate the allele databases. Additionally, the level of natural variation in the genome sequence that occurs *in vitro* as a result of culture manipulations (e.g., single colony picks from a pure culture or serial culture passages) and *in vivo* during short-term, single-point source outbreaks or zoonotic outbreaks and long-term, recurring single-source outbreaks needs to be determined.

The level of expected variation among isolates not epidemiologically related to one another needs to be defined, too. Tedious validation studies, as described above, are necessary in order to properly analyze and interpret WGS data from the context of molecular epidemiology. Without a doubt, the use of WGS data for cluster detection will result in detection of more clusters than PFGE. Resources for the epidemiological follow-up of all clusters are currently not available in most countries, and for that reason, defining rules to ensure epidemiological follow-up of clusters that represent outbreaks that are most likely to be solved is crucial (135).

Appropriate quality assurance standards and the need for accreditation will also need to be addressed, given that information that can be extracted from the WGS data, such as species identification, serotype, and virulence and antimicrobial resistance profiles, could be used for making decisions about patient management. While the current short-read technologies are still too slow to be used for real-time diagnostics at a physician's office, the Oxford Nanopore MinION sequencer holds some promise for rapid field diagnostics (136). However, the platform currently suffers from a high sequence error rate, so advances in technology need to take place before wider application in public health surveillance is possible.

The WGS technology currently being implemented for bacterial foodborne disease surveillance is dependent on the availability of pure cultures. The increasing use of culture-independent diagnostic tests by clinical laboratories has decreased the availability of isolates for surveillance, and this trend is likely to continue. Therefore, methods that may detect and subtype foodborne bacteria directly from the primary samples are urgently needed. Shotgun metagenomic analysis of the primary sample is likely going to be the long-term solution for the lack of pure cultures. The analysis method needs to provide detection, virulence characterization, and subtyping of the pathogenic organisms at the same time, thereby creating the potential to detect public health events, e.g., outbreaks, in real time, with no delays caused by isolating and growing the organisms as pure cultures. Additionally, outbreaks due to more than one pathogen or strain can be characterized.

While some proof-of-concept studies have already shown the feasibility of this method for characterization of the types of primary samples encountered in foodborne disease surveillance (137, 138), the technology is still too complex, slow, and expensive for routine use and faces formidable challenges that need to be tackled. There are also challenges due to low levels of pathogen in comparison to background microbiome and human DNA and similarity between pathogens and members of the background microbiome (e.g., *E. coli*). Some of the challenges can be addressed using clutter mitigation (removing human and background flora DNA from samples/sequences), read binning to separate pathogen and microbiome sequences, building comprehensive sequence reference libraries containing not only a representative diversity of pathogenic microbes but also the human microbiome, and building bioinformatic analysis pipelines capable of handling metagenomics data in local public health laboratories.

Addressing these challenges is expected to take several years, requiring a shorter-term "bridge gap" solution if the demise of culture-based methods progresses rapidly. Amplicon-based targeted sequencing directly from the clinical or food sample, while not optimal because prior knowledge or hypothesis is required about the etiologic agent, may provide such an approach. Additionally, when amplification is performed, pathogens present at low levels in the specimen are more likely to be detected. Similar to WGS-based isolate subtyping using wgMLST, amplicon targets can be designed by tiling primers along loci that provide enough resolution to do strain-level subtyping. There are enzymatic and microfluidics-based approaches that can be used to multiplex hundreds or thousands of potential loci to be amplified, which can then be used as a library to be sequenced on a next-generation-sequencing machine. However, whatever advances the new technologies provide for characterizing pure cultures or primary samples, it must be remembered that subtyping data will continue to be just one part of the picture and cannot replace epidemiological or environmental investigations.

Note in Proof

Since the writing of this chapter, PulseNet USA has gradually (2018–2019) implemented WGS, primarily cgMLST, as the standard subtyping method for all major foodborne pathogens. At the same time, WGS was implemented for routine identification, serotyping, detection of virulence and antimicrobial resistance determinants, and plasmid profiling of pathogens under PulseNet surveillance whenever relevant.

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39
Predictive Microbiology and Microbial Risk Assessment

PREDICTIVE MICROBIOLOGY

Predictive microbiology is often considered to have its origins in the early 20th century, when Bigelow and Esty (1), Bigelow (2), and Esty and Meyer (3) proposed a log-linear model to describe bacterial death kinetics by thermal processing (i.e., heat). This model was widely applied in the food industry, particularly in the canning industry. Now, about 100 years later, these results are still applied by the food industry to reduce pathogens such as *Clostridium botulinum* in low-acid canned foods. During this period, other areas of research, such as fermentation microbiology, have also contributed to the development of predictive microbiology. The term "predictive microbiology" in the food safety domain was coined by Roberts and Jarvis (4), establishing the conceptual basis of modern predictive microbiology.

In predictive microbiology, mathematical models are used to predict the behavior, such as the growth, survival, and inactivation responses, of microorganisms under different environmental conditions. Mathematical models are developed by culturing microorganisms under a variety of conditions, such as different temperatures, pHs, and water activities (a_w), and fitting the data to a mathematical equation. A database of predictive models can be established, which allows the microbial behavior to be predicted under conditions not specifically tested in the original experiments. Bacterial numbers can change during allstages of food production and processing, depending on the way the food is handled. Predictive microbiology can be used to assess the effects of processing, product composition, storage conditions, etc., on the final contamination of a product at the time of consumption. This permits comparison between the efficacies of individual control steps to limit the numbers of pathogens consumed. For instance, the overall impact of steps to limit raw product contamination at the farm or processing facility could be contrasted to the ability to prevent growth during storage (5). The focus has also shifted from the number of pathogens consumed to the probability of illness during the last few decades. This integration of predictive microbiology and the likelihood of illness enables researchers and other concerned stakeholders to conduct risk assessments and other food safety management procedures, such as hazard analysis and critical control points (HACCP).

Types of Models

Models can be classified into three categories: primary, secondary, and tertiary (5). Primary models measure the response of the microorganism over time to a single set of conditions. The response can be changes in microbial population density with respect to time. These primary models include growth models (6–8), the growth decline model (5, 9, 10), *D* values (or thermal inactivation) (11), inactivation/survival models (10, 12), growth rate values (13), and even subjective estimation of lag time (14). The most common types of primary growth models are the three-phase linear model (7), Baranyi model (15, 16), Gompertz model (17), Huang model (18), and logistic model (6, 19). The modified Gompertz model is an empirical sigmoidal relationship, while the Baranyi model is a differential equation based in part on the concept that the rate of bacterial growth is controlled by the rate of a bottleneck biochemical reaction. The three-phase linear model is a simple approach that assumes a constant bacterial population during the lag phase and the stationary phase and log-linear growth in the bacterial population during the growth period. The Huang model is based on the fundamental observations of the classical bacterial growth process, which exhibits the three phases—lag, exponential, and stationary—as well as the bacterial adaption process in the lag phase. This model

utilizes a transition function to simulate the bacterial adaption process and to define the lag phase of a growth curve, allowing the model to transit smoothly from the lag phase to the exponential phase.

The secondary models indicate how parameters of primary models change with respect to one or more extrinsic or intrinsic factors. Examples of extrinsic factors include temperature, relative humidity, and storage atmosphere, whereas water activity, pH, naturally occurring organic acids, added preservatives, and interactions between groups of microorganisms are some of the examples of intrinsic factors. The secondary modeling may represent simple situations (e.g., one where the storage temperature is the only important factor influencing microbial kinetics), but in many foods, the environmental parameters that influence microbial kinetics are complex and dynamic and include the combined effects of extrinsic and intrinsic factors. Response surface (20), Arrhenius (21), squareroot (22), and Bělehradek (13, 23) models based on the gamma concept, such as those described by Rosso et al. (24), are some examples of secondary models. Arrhenius and square root relationships are suitable where temperature is the primary factor of concern. When other factors, such as organic acid or nitrite concentration, are also considered, polynomial regression equations are more suitable. The response surface or polynomial models are very flexible, with squared, cubic, and cross-product terms, but they have a less mechanistic interpretation than other secondary models.

In the tertiary-level models, the process of primary and secondary models is reversed to obtain the prediction or estimation. Intrinsic- and extrinsic-factor values of interest are entered into the secondary models to obtain the parameter values of the primary models. The primary model is then solved with respect to time to obtain the growth, survival, or inactivation curves expected from the corresponding combination of extrinsic and intrinsic factors.

Lag Phase Duration and Growth Rates

Since the transition from the no-growth phase to the exponential growth phase is continuous rather than abrupt, the lag phase (λ) has no clearly or uniquely defined duration. Traditionally, the lag time has been determined in two ways; graphically as the intersection of the tangents to the growth curve at the lag and exponential phases, as shown in Fig. 39.1, or through nonlinear regression by fitting the experimental data with a growth model that has lag time as one of its adjustable parameters. Although what follows might be considered a hair-splitting argument, both lag time determination methods are conceptually problematic. One potential difficulty with the lag time, determined by either method, will always arise when the growth curve has a steep slope at the inflection point and a very moderate climb in the region preceding it. The problem with the curve-fitting method is that different growth models might yield very different values when used to fit the same experimental data. These problems should not come as a surprise. They are the inevitable penalty of attempting to assign special significance to a point on a segment of a curve that rises (or falls) monotonically—the inflection point being a notable exception. Although in many cases either method provides a marker of the onset of the accelerated growth regimen, the rendered value need not accurately indicate when the acceleration of the growth started.

Many food microbiologists consider the maximum specific growth rate (μ_{max}) the measure of the growth potential of an organism and hence of its physiological vigor. As a result, most secondary models, notably the Arrhenius equation, Ratkowsky's square root model, and their various modifications and combinations, have been developed exclusively for this parameter and how it is affected by temperature and the properties of the habitat. Mathematically, μ_{max} is defined as the slope of the (sigmoid) growth curve at its inflection point. If allmicrobial growth curves were symmetric or approximately symmetric and if they had the same time range and asymptotic growth level, μ_{max} would indeed be a universal growth parameter. This refers to comparisons of the growth of the same organism at different temperatures or under different conditions, such as different pHs, different a_w values, different salt or sugar concentrations, the presence or absence of antimicrobials, etc. The μ_{max} issue always arises in comparison to the growth patterns of the same or different organisms if the degree of symmetry of their growth curves, or asymmetry, is not the same and/or if they achieve different growth levels and have different lags. In all such cases, reference to μ_{max} alone might be of

very limited value, if any, because the difference in the other growth characteristics might be much more significant in practice. Here again, an attempt to establish the universality of a link between the μ_{max} and the lag time (λ), which might not even exist, would be difficult to justify (25).

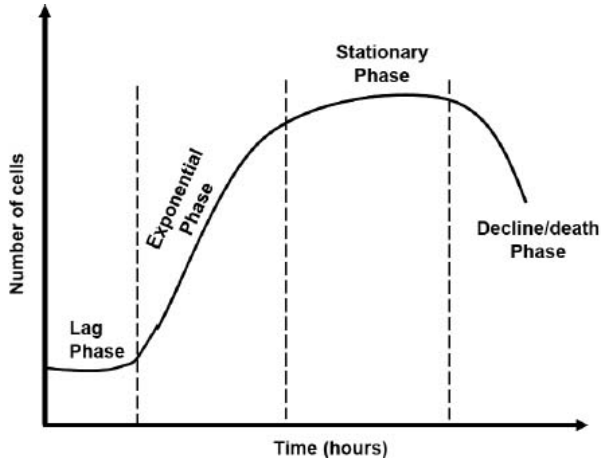


Figure 39.1. Bacterial growth cycle.

Most growth models consider the lag phase in their basic framework. A correlation between the generation time or growth rate and lag phase is often presumed (26, 27). The lag phase is clearer when the inoculum cells are in the same physiological state and have the same cultural history (8). The lag phase duration can be modeled independently of the growth rate with a modified Arrhenius relationship, which assumes the log of the growth rate to be inversely proportional to the reciprocal of the absolute temperature (13). This relationship is based on the control of the rate by a single rate-limiting enzymatic reaction:

$$\ln(1/\lambda) = C_o + C_1/T + C_2/T^2$$

Where λ is the lag phase duration, T is temperature (in kelvin), and C_O, C_1, and C_2 are coefficients to be determined.

Zwietering et al. (17, 28) investigated the effectiveness of several models for describing the effect of temperature on the growth kinetics of *Lactobacillus plantarum*, an organism for which they had a large database of experimental values. They concluded that two reparameterizations of the square root equation were the optimum way to model growth rates and maximum population densities. However, they concluded that the effect of the lag phase was better described by a hyperbolic function:

$$\ln(\lambda) = p/(T - q)$$

Where λ is the lag phase duration, T is temperature (in kelvin), p is a parameter that accounts for the decrease in lag phase duration as the temperature is increased, and q is the temperature (in kelvin) at which the lag phase is infinite.

The square root model (22) is probably the most studied and widely used for the effect of temperature on microbial growth. Below the microorganism's optimum growth temperature, the square root of the growth rate constant (R) and the reciprocal of the lag phase duration (λ) are linearly related to the temperature according to the following relationship:

$$R^{0.5} = b(T - T_{min})$$

$$\lambda^{0.5} = b(T - T_{min})$$

where b is the slope of the regression line, T is the incubation temperature (in kelvin), and T_{min} is the notational minimum growth temperature (in kelvin), which is derived by extrapolating the regression

line to zero. As the incubation temperatures increase beyond the microorganism's optimum growth temperature, growth rates begin to decline. Ratkowsky et al. (29) further expanded their model to include a term that takes into account this depression of growth rate:

$$R^{0.5} = b(T - T_{min})\{1 - \exp[c(T - T_{max})]\}$$

where T_{max} is the notational maximum growth temperature (in kelvin), and c is constant.

When more than one variable has to be considered to predict the growth rate of a microorganism, the type of model employed is dependent on the number and independence of variables. If temperature and another variable are independent of each other, modification of the Ratkowsky model is effective. For example, Miles et al. (30) studied the effect of temperature and water activity on the growth of *Vibrio parahaemolyticus*. The combined effects of the two variables could be described by a simple multiplicative expression:

$$R^{0.5} = b(T - T_{min})(a_w - a_{w\,min})^{0.5}$$

While it is assumed that additional variables could be included using the above approach, the response surface technique has been the primary method for developing models for more complex foods that are dependent on four or more primary variables, particularly if the variables are interactive. This approach employs the regression analysis techniques to generate the best-fit, multidimensional response surface equations that describe the effects and interactions of the experimental variables.

Fluctuating Temperature Conditions

Bacterial populations exposed to sudden temperature variations can experience adaptation delays in growth, known as intermediate lag phases (31). It has been reported by several authors that a combination of several temperature shifts might cause intermediate lag phases of significant duration (16, 32). In general, investigators have found that growth rate adapts immediately to the new temperature as long as the temperature changes are within the range for growth of the organism (15, 16). However, Baranyi et al. (16) found that during fluctuations to temperatures below the minimum for growth, predictions for *Brochothrix thermosphacta* became unsatisfactory, and it was hypothesized that cells had undergone a physiological change. In contrast, Mitchell et al.(33) found that when *Salmonella enterica* serovar Typhimurium was exposed to cyclic temperature changes between 48°C and 22°C, lag periods were not induced and growth rate adapted instantaneously to the new temperature, although some loss of viability was observed at the lower temperature. They concluded that the time periods below the minimum temperature for growth in their experiments were insufficient to perturb growth.

Parameters for Predictive Model Performance

Various indices have been proposed to indicate the quality or goodness of fit of a model. Error mean square, F, and R^2 values from regression analysis are frequently reported that use the absolute difference between the predicted and observed values. Simultaneously plotting data and estimates from the model is necessary to observe whether there are any specific areas within the interpolation space where the model is poorly fitted. The regression analysis assumes that data are normally distributed. If they are not, data should be transformed before modeling. This requirement also applies to nonlinear curvefitting procedures. Determining whether a data set is normally distributed, either before or after a transformation, is not always easy, particularly in cases where only a few replicates are conducted. Because variation clearly increases with increasing value for microbiological parameters, such as generation time or lag phase duration, the logarithms of these values are typically taken.

Although formal hypothesis tests provide a convenient framework for displaying the statistical results of empirical comparisons, standard tests should not be used without consideration of underlying measurement error structure. As part of the validation process, predictions of microbial

concentrations are often compared with the microbial concentrations measured in the experimental studies. Measurements of these microbial concentrations are subject to variability from several sources, including temporal and spatial sampling, sample preparation and analysis, and data entry or recording. Adjustments for measurement error must be made before statistical tests can be used to empirically compare experimental data with model predictions. It is important that users of the predictive model have an understanding of both the limits of the predictive model and the range of its applicability. When considering performance, of most immediate interest is whether the model is fail-dangerous or fail-safe, i.e., whether it underestimates or overestimates the risk of spoilage or extent of pathogen growth. In general, it is accepted that models should predict as closely as possible the observed behavior to avoid wastage of product, but it is better to overestimate the risk rather than underestimating. Ross (34) suggested accuracy and bias factors to assess the level of predictive model performances.

$$\text{Accuracy factor } (A_f) = 10^{(\sum |\log(\text{predicted/observed})|)}$$

$$\text{Bias factor } (B_f) = 10^{(\sum \log(\text{predicted/observed})}$$

These indices have been widely used in predictivemicrobiology research. There is currently no consensus as to what values of these indices constitute a model that provides acceptable predictions of pathogen growth in broth or on food. Although Ross et al. (35) recommended *Bf* values from 0.7 to 1.15 for growth rates, different researchers use different ranges as acceptable performance levels. However, these indices have a few major limitations, as suggested by Oscar (36):

1. These indices are mean values that do not detect some forms of prediction bias.
2. These indices are subject to bias by outliers.
3. Prediction cases involving no growth are excluded from the calculation of A_f and B_f In such cases, these indices overestimate the performance of the models because the “no growth” prediction cases are excluded from the calculation of A_f and B_f.
4. A_f and B_f values are not consistent with each other. More specifically, acceptable values for B_f are independent of the number of model variables, and the criteria for acceptable bias factor consider whether predictions err more in the fail-safe direction (value < 1). In contrast, acceptable A_f values are dependent on the number of model variables, and A_f does not consider whether the model errs more in the fail-safe or fail-dangerous direction.

Oscar (36) introduced a simplified and graphically represented acceptable prediction zone method, which overcomes some of the shortcomings of the A_f and B_f indices. Although this method is fairly simple and easy to interpret, the drawback is that it provides the number of observations falling inside the acceptable range, without giving any statistical information. This method does not provide any information on how close the observed values are to the predicted values.

Predictive-Microbiology Application Software Tools

Current applications of predictive microbiology are broad but can be summarized into three groups of activities (37): product innovation, operational support, and incident support. These three categories of tools or software provide food safety information during new-product development, manufacturing of food, and foodborne-illness outbreaks or recalls. The product innovation-associated applications assess the speed of microbial proliferation, growth limits, or inactivation rate associated with particular food formulations and/or process conditions in order to develop new products and processes. Based on this information, these tools can be used to reformulate existing products and determine storage conditions and shelf life. The operational support tools support food safety decisions that need to be made as part of establishing or running a food manufacturing operation, such as designing infactory heating regimens, setting critical control points in HACCP, and assessing the impact of process deviations on microbiological safety and quality of food products. The incident

support tools estimate the impact on consumer safety or product quality in case of problems (such as an outbreak of foodborne illness or a recall) due to potentially contaminated products reaching the market. Table 39.1 summarizes the common software and tools used in predictive microbiology. Some of these tools are available at no cost, whereas others are commercially available.

Limitations

Even though predictive models are widely used after being appropriately validated, there are several limitations because of the complex behavior of microorganisms and food systems. Some of these limitations are as follows:

1. Predictive models are simplified representations of complex microbial behavior in food and environment systems, and in many cases, not every important variable or factor that affects microbial behavior is included in the model.
2. Predictive models should not be extrapolated outside the range in which they are developed, because these models are derived from experimental data and thus do not consider microbial behavior outside the parameters studied (e.g., temperature range). For example, the square root growth model (Ratkowsky model) for bacteria growth cannot be extrapolated to temperatures above the optimum temperature for growth. An extrapolation will provide a high growth rate, whereas in reality the growth rate actually declines, as represented by the modified Ratkowsky model. Thus, predictions outside the experimental range are not only inaccurate but nonsensical in many instances.
3. Some sigmoidal curves, such as Baranyi and modified Gompertz models, represent growth rates that are higher than the actual growth rates. This is because of the calculation of maximum specific growth rate (μ_{max}) (growth at the maximum rate actually occurs during a limited period during the exponential growth phase) and not simple growth rates.
4. Predictive models are derived from experimental data that do not always reflect real food systems. Usually, predictive models are not designed for the specific conditions that microorganisms may be exposed to in foods. Thus, several models tend to give consistent underpredictions (fail-dangerous) or overpredictions (fail-safe).
5. Most of the predictive models describe the changes in microbial behavior for homogeneous populations, whereas in reality, microbial competition affects the food environment.
6. Models developed under static conditions may or may not be applicable in the fluctuating conditions of temperature, pH, or relative humidity. More research needs to be done on how the lag phase duration or the growth rate changes with the fluctuations in temperature and other relevant conditions.
7. The preinoculation history of bacteria has a considerable impact on their lag phase duration and growth rate.

Table 39.1. Software and tools commonly used in predictive microbiology

Software or tool	Source	Year of creation	Accessibility	Brief description
Baseline	http://www.baselineapp.com/	2012	Free; Internet access	Simulates the growth and inactivation of 5 pathogens in different food systems, using predictive models from the literature
ComBase	http://www.combase.cc/	2004	Free; Internet access	Provides a database with >50,000 records to describe the evolution (growth and inactivation) of several microorganisms in culture media or foods. It also provides some fitting and simulation modules for growth and inactivation. 15 microorganisms are accounted for.
Dairy Products Safety Predictor	http://www.aqr.maison.dulait.fr/	2012	Commercial; Internet access	Simulates an exposure or risk assessment model previously developed for a specific microorganism in a specific dairy product. Users have personal accounts where their model(s) is uploaded and they can then test new parameters.
FDA-iRISK	https://irisk.foodrisk.org/	2012	Free; Internet	Allows

			access	prioritizing food risk by implementing risk assessment models for different pathogens and food matrices. Templates for risk assessment models are proposed.
FILTREX	http://genome.jouy.inra.fr/logiciels/filtrex/	2013	Free; downloadable	Assesses the parameters of primary growth and inactivation models and provides support for optimal sequential design of challenge test experiments
FISHMAP	http://www.azti.es/fishmap	2011	Free; downloadable	Simulates the growth of 8 spoilage species on fish matrix
Food Spoilage and Safety Predictor (FSSP)	http://fssp.food.dtu.dk/	1999	Free; downloadable	Predicts the growth of 3 pathogens, 3 spoilers, and generic microorganisms in meat and seafood products; 12 environmental parameters can be integrated in the simulation in dynamic conditions.
GInaFiT	http://cit.kuleuven.be/biotec/software/GinaFit	2003	Free; downloadable	Allows fitting of 10 inactivation models on experimental data and selection of the best model
GroPIN	http://www.aua.gr/psomas/gropin	2013	Free; downloadable	Simulates the behavior of 66 microorganisms,

				including pathogen and spoilage species, in different food matrices. A database of predictive models is also provided and can be implemented with new models. Monte Carlo simulations are also proposed to take into account the variability.
iPMP	www.ars.usda.gov/northeast-area/wyndmoor-pa/eastern-regional-research-center/docs/ipmp-2013/	2013	Free; downloadable	Analyzes experimental data commonly encountered in predictive microbiology and in the development of predictive models
Listeria Meat Model	http://www.cpmf2.be/software.php	2012	Commercial; downloadable	Simulates the growth of the pathogen in meat and different meat products
MicroHibro	http://www.microhibro.com/	2011	Free; Internet access	Assesses and validates the parameters of a primary model based on experimental data. Implementation of risk assessment models, followed by a sensitivity analysis of the model, is also possible based on the

				combination of several basic processes and using the database of predictive models provided.
MRV (Microbial Responses Viewer)	http://mrviewer.info/	2008	Free; Internet access	Growth/no-growth interface based on ComBase database. It provides a visualization of the specific growth rates.
NIZO Premia	No Internet access	1995	Commercial; no Internet access	Predicts behavior of pathogens and spoilage microorganisms. Predictions are performed mainly in dairy products; other substrates are possible if their characteristics are provided by the user.
PMM-Lab	https://sourceforge.net/projects/pmmlab/	2012	Free; Internet access	Simulates the behavior of pathogens and spoilers in different food matrices.
PMP (USDA)	https://pmp.errc.ars.usda.gov/default.aspx	1990s	Free; downloadable; Internet access	Predicts the growth and inactivation of foodborne bacteria, primarily pathogens, under various environmental conditions
Prediction of Microbial Safety in Meat Products	http://dmripredict.dk/	2006	Free; Internet access	Predicts and simulates the behavior of four pathogen

				species on meat products
SHELF	Mishra et al. (75)	2016	Free from authors on request	Optimizes the temperature for leafy greens considering refrigeration cost, sensory quality, and microbial safety
Sym'Previus	http://www.symprevius.org/	2003	Commercial; Internet access	Simulates growth and inactivation of 6 pathogens and 13 spoilers in several food matrices in both static and dynamic conditions; deterministic and probabilistic approaches can be used.

MICROBIAL RISK ASSESSMENT

Risk Analysis

In food processing, a hazard is a biological, chemical, allergenic, or physical substance that has the potential to cause harm to consumers. Not all hazards are equally serious or pose equally immediate threats. Some situations can be more hazardous depending on the levels, sizes, quantities, or doses of unwanted substance or conditions. When it comes to food safety, how hazardous a substance or condition is may vary greatly. The level of danger can depend not only on the type of hazard but also on who might consume a food product. Usually, there is a threshold level below or above which the presence of a hazard is considered tolerable, or acceptable.

In the past 2 decades, research in the food safety discipline has addressed the assessment of hazards in foods within the framework of risk analysis, a new science-sed paradigm intended to ensure human health protection. The objectives of risk analysis are to estimate the risk to human health of a hazard associated with food consumption and, most importantly, to assess appropriate management strategies in the "farm-to-fork" food chain capable of mitigating the risks. Risk analysis represents a structured decision-making process with three closely connected components: risk assessment, risk management, and risk communication (Fig. 39.2).

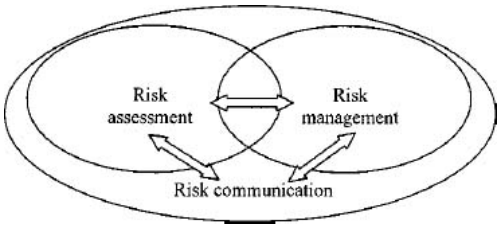


Figure 39.2. Codex Alimentarius risk analysis framework.

Risk assessment is the central scientific component of risk analysis. Building a risk assessment model is central not only for the characterization or estimation of the potential adverse effects on health resulting from exposure to hazards (i.e., risk) but most importantly for the prediction of the risk reduction attained by each of the potential intervention strategies under consideration. On the other hand, risk management is the process of evaluating policy alternatives in consultation with stakeholders and is generally performed by a food safety authority. It encompasses the identification and selection of risk management options or intervention strategies as well as their implementation. Risk managers are responsible for verifying that the risk mitigation measures that were selected and implemented are in fact achieving the intended results, which they carry out by monitoring and surveillance activities (38). Lastly, risk communication refers to the sharing of goals, information, and data between risk assessors and managers in addition to other stakeholders, such as the public and industry. Risk communication plays an important role in ensuring that the results and other important details of a risk assessment are clearly understandable and communicated to all parties; it is essential in ensuring transparency in risk analysis. Experience has shown that regular communication between all parties is necessary to ensure that the risk assessment has maximum value to the risk managers and that risk management decisions are understood and accepted by the public and various stakeholders; thus, risk communication should encompass both risk management and risk assessment as proposed by the risk analysis framework published by the Codex Alimentarius Commission (39). The main goals of risk communication are as follows:

1. promoting awareness and understanding of risks (among researchers, government officials, consumers, and other concerned stakeholders)
2. promoting consistency and clarity about the risk analysis process
3. providing an understanding for risk management decisions
4. strengthening good working relationships and promoting respect
5. promoting appropriate involvement of all stakeholder groups
6. exchanging information, knowledge, attitudes, practices, and perceptions among those involved

Microbial Risk Assessment

Microbial risk assessments can be either qualitative or quantitative. The risk management questions typically address the approach and spectrum of risk assessment (40). Qualitative risk assessments express risk as a grade or category (e.g., low, moderate, or high risk) rather than as a number, as in a quantitative risk assessment. Quantitative risk assessments can be either deterministic or stochastic (probabilistic). The mean growth rate is multiplied by the mean storage time to predict the amount of microbial growth. In real-life conditions, there is inherent variability in such parameters, often represented by standard deviations or confidence intervals, but the algebraic calculations consider only the means for both input parameters and the calculated values. This approach is a deterministic, or point estimate, analysis.

Making a worst-case estimate is another example of deterministic analysis. Quantitative risk assessment involves the distributions of the model parameters into the calculations. The sources of the distributions about the mean values are from variability and uncertainty (41). Variability refers to the inherent differences in outcomes obtained from a process. Uncertainty is lack of knowledge. Uncertainty and variability are often described using distributions, such as normal, lognormal, beta, binomial, triangle, histogram, or program evaluation and review technique distributions. If the input parameters of a model or a food process are distributions, the calculated output will also be a distribution. To make these calculations, Monte Carlo simulation is generally used. In this method, a value is randomly selected from each distribution according to the characteristics of that distribution and the model output is calculated. A new set of values from each distribution is selected and the process is recalculated (iterated). Its mean or median can describe this distribution, but the entire shape and spread of the distribution are important in the interpretation of the process (5). For a

disinfection calculation, for example, the disinfection received by the lowest 10% or 1% of the model simulations may be more relevant to setting critical control point parameters than the mean or median values. This type of analysis is called “probabilistic” or “stochastic” and provides a more comprehensible description of the risk being assessed than the deterministic analysis (42).

Quantitative microbial risk assessment (QMRA) is a science-based process used to quantitatively estimate the adverse health effects resulting from exposure to microorganisms. In particular, QMRA is used to tackle food safety problems caused by the intake of food products contaminated with potentially hazardous microorganisms. Risk assessment, which is an integral part of the risk analysis process, is interrelated with two other components: risk management and risk communication (43).

Risk Assessment Steps

The Codex Alimentarius Commission (39), established by the Food and Agriculture Organization of the United Nations and the World Health Organization and recognized by the World Trade Organization as the relevant organization for international food safety standards and guidelines, defines risk assessment as “a scientifically based process consisting of the following steps: (i) hazard identification, (ii) hazard characterization, (iii) exposure assessment, and (iv) risk characterization” (39).

Hazard Identification

The first step of risk assessment describes a microorganism and the disease it causes, including symptoms, severity, and death rates from the microorganism. This step also identifies sensitive populations that are particularly susceptible to infection.

Hazard Characterization

Hazard characterization describes the nature and extent of the adverse health effects known to be associated with the specific hazard. If possible, a relationship is established between the different levels of exposure to the hazard in food (i.e., the dose) at the point of consumption and the likelihood of different adverse health effects such as infection or illness. It describes the dose-response relationship.

Exposure Assessment

The aim of exposure assessment is to determine the frequency and magnitude of exposure to pathogens via food consumption. In exposure assessment, quantitative information about pathogen concentration in food is needed. Additionally, data on the exposure of humans (the size of the exposed population, the nature of the exposed population [e.g., vulnerable groups], and frequency of exposure) are also required.

Risk Characterization

During risk characterization, the results of the two previous steps, exposure assessment and hazard characterization, are integrated to generate a risk estimate. If a risk assessment is quantitative, the risk may be characterized in terms of risk per serving, risk per annual rate of consumption, or even in number of cases for a target population. In microbial risk analysis, the derivation of efficient management strategies should be assisted by three elements: food chain data availability, predictive microbiology, and sampling strategies.

Uncertainty and Variability

Uncertainty is defined as the lack of knowledge or availability of data concerning inputs for the risk assessments, appropriate models, and assumptions (44). Both subjective and objective aspects are important in assessing the degree of uncertainty, which is defined as the degree of the lack of confidence that one has concerning the validity of the information obtained (45). A document

outlining the general guidelines for characterizing and communicating uncertainty in exposure assessment was released by the World Health Organization in 2008 (46). A more detailed guideline for uncertainty assessment and uncertainty communication has been developed for risk assessment by the Netherlands Environmental Assessment Agency (47–49). Unlike uncertainty, variability refers to the inherent heterogeneity or diversity of data in an assessment. It is "a quantitative description of the range or spread of a set of values" (50) and is often expressed through statistical metrics such as variance, standard deviation, and interquartile ranges that reflect the variability of the data. The primary difference between the two is that uncertainty can be reduced or eliminated with more or better data, whereas variability cannot be reduced, but it can be better characterized.

In many cases both variability and uncertainty are present. For example, a particular drinking water may be contaminated with *Cryptosporidium* oocysts. The number of oocysts in different water samples will vary. If the oocysts move randomly in the water—that is, they move independently, neither clustering together nor dispersing—then the probability that a given number of oocysts will be found in a given water sample can be described by a Poisson distribution (51):

$$\text{Prob}[x \text{ oocysts}] = \lambda^x \exp(-\lambda)/x!$$

Where λ is a parameter. One property of this distribution is that the mean is equal to λ. One can think of λ as the long-run mean, that is, the mean if an infinite number of samples from the same Poisson distribution are averaged. Because a finite sample is not guaranteed to be perfectly representative of the population from which it is drawn, even after the mean (median, variance, etc.) for a sample is obtained, there is still uncertainty as to the population mean (median, variance, etc.). It is uncertainty due to this sampling variability which is quantified through statistical methods.

It is common to first assess an appropriate functional form for $f(x)$ and then, given the chosen form of $f(x)$, assess the uncertainty in the parameters of $f(x)$. In the classical statistical framework, these parameters are fixed values. Based on a sample, estimates are obtained for these parameters. However, given sampling variability, these parameter estimates have variability in them. This variability can be assessed and the standard deviation of the estimate of the parameter quantified. This standard deviation of a parameter estimate is termed a standard error. Thus, uncertainty in model parameters (due to sampling variability and only sampling variability) is captured by these standard errors. In the Bayesian framework, model parameters are treated as themselves being random variables. In this framework, the λ value for a Poisson process would follow a probability distribution with a mean (reflecting the central tendency of λ) and a variance (reflecting uncertainty as to the true value of λ). In the classical framework, it is not quite correct to describe the parameters of models as following uncertainty distributions. Instead, the standard errors are used to include or exclude different possible values of the model parameters with various levels of confidence. It is not clear that any harm is done in simply treating model parameters as random variables with means equal to their estimates and standard deviations equal to their standard errors. This view essentially adopts the Bayesian framework even for models estimated in a classical framework. It is often a convenient approach to adopt in risk assessments where probability is used quite generally to describe a wide variety of uncertainties (51).

Uncertainty and Variability in Hazard Identification

The hazard identification step involves the determination that a health hazard is or may be associated with a biological, chemical, or physical agent present in foods. This step provides a dichotomous answer—that is, the factor is or is not thought to be a human health hazard. The uncertainty involves the correct classification of the agent (i.e., it is or is not a human health hazard). If the agent is tested for prevalence in the food sample multiple times, it is predicted to be either positive or negative with a certain degree of precision that is related to the performance of the microbial test. Uncertainty associated with the analysis of a microorganism in this study derives from knowing whether the sampling method is actually capable of predicting a positive (or negative) response.

Three issues are considered potentially significant contributions to uncertainty and variability in hazard identification. First is the misclassification of an agent—either identification of an agent as a hazard when it is not one or failure to identify an agent as a hazard when it is one. Second is the issue of the reliability of the screening method for appropriately identifying a hazard and the reliability of the microbial sampling to give the same result each time the sampling is performed. Third is the issue of extrapolation, because all screening methods are used to extrapolate the information provided by the test to predict human hazards. Epidemiological studies are used to predict the impact of exposures on human populations in the future.

Uncertainty and Variability in Hazard Characterization

Hazard characterization is the process of defining the site, mechanism of action, and at minimum the dose-response relationship (proportion responding or severity of response). In this step, it is likely that a series of models may be developed. The models vary from purely mathematical representations to biologically based representations. As a result, each has varying degrees of representation of the actual human disease process and thus varying degrees of uncertainty (52).

Model uncertainty is likely to be an important issue in the hazard characterization step. Mathematical dose-response relationships have the greatest uncertainty in actual representation of the biological processes. Despite the admittedly large uncertainty, dose-response models are currently the most commonly used methods for predicting human health effects and have often proved useful in establishing policy. As interest in risk assessment has grown, the sophistication of the models, including the accuracy and completeness of their representation of the biological processes, has also grown. An important issue of both variability and uncertainty that arises in hazard characterization is in the variance in the dose response at the dosages for the species studied. To increase the power and the value of a negative study, large exposures are typically used in studies. These exposures are generally substantially greater than usual human exposures. That means that models including exposure response information gathered at high exposures may not be accurate at the low exposure levels of concern for human risk assessment. In addition, there is variance among animals' responses at a given dose, despite the fact that most experimental animals are genetically in Model uncertainty is likely to be an important issue in the hazard characterization step. Mathematical dose-response relationships have the greatest uncertainty in actual representation of the biological processes. Despite the admittedly large uncertainty, dose-response models are currently the most commonly used methods for predicting human health effects and have often proved useful in establishing policy. As interest in risk assessment has grown, the sophistication of the models, including the accuracy and completeness of their representation of the biological processes, has also grown. An important issue of both variability and uncertainty that arises in hazard characterization is in the variance in the dose response at the dosages for the species studied. To increase the power and the value of a negative study, large exposures are typically used in studies. These exposures are generally substantially greater than usual human exposures. That means that models including exposure response information gathered at high exposures may not be accurate at the low exposure levels of concern for human risk assessment. In addition, there is variance among animals' responses at a given dose, despite the fact that most experimental animals are genetically inbred and are therefore expected to be genetically identical. If outbred animals are used, the variability in the dose-response relationship is expected to be larger, and if humans are exposed, the variance is also expected to be large. Another issue of both uncertainty and variability that arises in hazard characterization is the need to extrapolate between species. Approaches used for extrapolation between species include both uncertainty about the appropriate model for performing the extrapolation and variability in the parameters used for extrapolation (53).

Uncertainty and Variability in Exposure Assessment

Any model used to represent exposure should include several pieces of information (52):

1. the level of an agent measured in a commodity or the levels measured in soil, plants, irrigation water, or animals that supply this commodity
2. the depletion/concentration ratio, which defines changes in the level of an agent as a result of processing, preparation, and dilution
3. the frequency and magnitude of human intake of a commodity
4. the level of exposure, or the amount of the commodity an individual is likely to ingest
5. the average time for the type of health effects under consideration to be clinically detectable

These factors typically converge in the process of defining the distribution of population exposure. The population at risk for exposure refers to the population that consumes food containing the hazard. An exposure assessment is the key input to the assessment of dose, which reflects the amount of the agent delivered to the target organ or tissue, where the adverse effect can be induced. Defining exposure pathways is an important component of the exposure assessment. An exposure pathway is the course a biological, chemical, or physical agent takes from a known source to an exposed individual. In the case of agents in food, concentrations of chemicals and/or organisms (microbes, parasites, etc.) can change between what is measured in soil, plants, water, animals, and raw food and what is ingested by an individual. In the case of chemicals, there can be some increases of contaminant concentration due to processing (i.e., distillation), but more likely, the storage, processing, and preparation of the food product will result in a reduction of contaminant concentration. For microorganisms, there might be significant increases of microbe or contaminant concentration due to replication under favorable environmental conditions (temperature, pH, and water activity). Thus, significant Uncertainties might be expected in the ratio of the concentration of a bacterial agent in food at the time of consumption to the concentration measured in raw foods or measured in animals, plants, or soil (**54**).

Uncertainty and Variability in Risk Characterization

Once hazard characterization and exposure information has been collected, risk characterization is carried out by constructing a model for the distribution of individual or population risk. This is done by summing the effect over all exposure routes. Because of the uncertainties and variabilities involved in its constituent steps, the overall process of risk characterization might involve potentially large uncertainties.

An important final step in the risk characterization process is the characterization of uncertainties. In order to directly characterize uncertainties in risk assessments, it is necessary to take a tiered approach to uncertainty analysis. Three tiers can be used. First, the variance of all input values should be clearly stated and the impact of these variances on the final estimates of risk assessed. Second, a sensitivity analysis should be used to assess how model predictions are impacted by model reliability and data precision. The goal of a sensitivity analysis is to rank the input parameters on the basis of their contribution to variance in the output. Finally, variance propagation methods should be used to carefully map how the overall precision of risk estimates is tied to the variability and uncertainty associated with the models, inputs, and scenarios (55).

Separating Uncertainty and Variability by Two-Dimensional Monte Carlo Simulation

A two-dimensional (or second-order) Monte Carlo simulation was proposed to estimate the uncertainty in the risk estimates stemming from parameter uncertainty (56, 57). A two-dimensional Monte Carlo simulation is a Monte Carlo simulation in which the distributions reflecting variability and uncertainty are sampled separately in the simulation framework, so that variability and uncertainty in the output may be estimated separately (55). This framework has been used in various domains of risk assessment (58, 59) and is now used in the food safety domain (60, 61). Nevertheless, variability and uncertainty are gathered in some risk assessment studies through a one-dimensional

Monte Carlo simulation (62, 63), because separating uncertainty and variability is computationally challenging and often confusing. In order to enable two-dimensional Monte Carlo simulations such as the one proposed by Cullen and Frey (57), one needs to quantitatively describe the uncertainty associated with the parameters in the risk assessment model. The bootstrap resampling method, Bayesian derivation, and expert elicitation are some of the available methods (64).

Bootstrapping

The bootstrap method is a fairly easy tool for numerically calculating the uncertainty of a data set of measurements, by repeatedly drawing results randomly from the data set. In some cases, an analytical formula is available to estimate the standard error of a parameter. For complicated model forms, such formulae may not be available. Bootstrapping is a standard method to assess the uncertainty in parameter estimates due to sampling variability. The concept underlying bootstrapping is that of treating the sample obtained as the probability density distribution function of the model. Each point has probability $1/N$, where N is the number of observations in the sample. This assumption allows us to create alternative samples from the data. One randomly draws N observations from the observed data set to create an alternate data set. These observations are drawn with replacement, meaning that a particular observation is not eliminated from subsequent draws after it is sampled. It is this "sampling with replacement" that allows each alternative data set to be slightly different from the original data set, since some observations will appear multiple times in the alternative data set and some will not be sampled at all (65). Uncertainty can be described using this method, with the assumptions that the sample is unbiased and random and represents the entire population.

The next step is to estimate the desired parameter (or summary statistics, such as mean, variance, 90th percentile, etc.) for each of the alternative data sets. Each combination of generating an alternative data set and estimating the quantity of interest is termed an iteration of the bootstrap procedure. The values for each data set are then considered discrete samples from the probability distribution of the parameter. Means, variances, and percentiles for the parameter estimate can be found from this discrete sample. Thus, the standard error of the parameter estimate can be estimated as the standard deviation of the different parameter estimates obtained from each iteration. This is essentially a Monte Carlo approach to assessing parameter uncertainty, and, as with all Monte Carlo analyses, the number of iterations conducted should be large, preferably as large as 10,000 (64). A smaller number of iterations may be acceptable if convergence of estimates can be observed. In this case, one would track the estimate of interest across different iterations. Values will fluctuate greatly at first, as the estimate is based on a small number of iterations, but these fluctuations will decrease as a larger sample size is obtained. Convergence is achieved when the estimate no longer shows fluctuations large enough to be of concern to the analysis (55).

Expert elicitation

Expert elicitation is the process of eliciting subjective judgments from experts. It is used to provide input for risk assessments when empirical data are lacking, are of poor quality, or are difficult to obtain. Since the elicitation of expert judgment involves subjectivity, sometimes it is prone to bias on the part of the expert, as well as the elicitor (person collecting the expert opinion) and the elicitation protocol used, all of which may ultimately have an impact on the validity of the decisions based on a risk assessment. Havelaar et al. (66) state that "an expert opinion may not be expected to provide an unbiased estimate of the relative importance of different transmission routes. Rather it should be regarded as a structured way to obtain consensus opinion, based on available data." The goal of an expert elicitation procedure is to reduce this bias as much as possible, and this requires thoughtful preparation. A structured expert elicitation involves the selection of the experts, explanation to the experts of the problem and the elicitation procedure, a clear definition of the quantity to be assessed, a discussion of the gaps in the knowledge, specification of the experts' opinions in a distribution, and the decision whether to aggregate the distributions of the different experts. A successful expert elicitation also implies solid training in elicitation techniques.

Well-defined procedures, including the identification of target, query, and performance variables, the selection of experts, and a systematic combination or synthesis of expert judgment, can yield valuable information on the questions posed. Formal (and sometimes complex) methods should be used to determine overall probabilities with classical or Bayesian approaches to data aggregation. Whatever method is used to assess overall probabilities, it is critical that expert elicitation contain some estimate of uncertainty (67).

Sensitivity Analysis

The risk estimate of a risk assessment model is the most important piece of information that may be presented in a risk assessment report. Often, the risk managers are more interested in the results from a sensitivity and scenario analysis that show the relative importance of different parameters and determine the impact on the output from changes in various input parameters. Sensitivity analysis is used to determine the degree of influence that a given input has on the value of the output. Most risk assessment models focus on stochastic sensitivity analysis; however, less detailed methods to conduct deterministic and worst-case sensitivity analysis have been described (68). The results, usually displayed in the form of tornado graphs, are a result of statistical analysis (stepwise least-square regression or rank order correlation) done using the input and output data generated during the Monte Carlo iterations (69). An example of a tornado graph was taken from the risk assessment of *Escherichia coli* O157:H7 in leafy greens and is shown in Fig. 39.3 (62). The results from a sensitivity analysis are extremely helpful in identifying points in the systems where additional data collection is most useful (high impact of uncertainty for a parameter) and where mitigation strategies aimed at impacting risk could be most efficient (high impact of a variability parameter).

There is no universal standard for conducting sensitivity analysis (70). In fact, multiple approaches may be legitimately used, because each approach may examine a different type of influence. The typical sensitivity analysis examines the magnitude of change in exposures for some change in inputs (i.e., Δoutput/Δinput). One challenge is determining how a change in exposures should be measured. In some cases, change in the average dose value may be satisfactory (e.g., measuring conditional expected dose). In other cases, the change in the variability of doses may be of interest. If sensitivity is measured using analysis-of-variance techniques, then changes in the average and variance can be assessed together. Correlation or other quantitative measures of association (e.g., spider plots and tornado charts) are commonly available in commercial software packages used for Monte Carlo simulation (64). Another approach used in risk assessments is regional sensitivity analysis, which estimates the impact of a number of parameters contemporaneously. Feasible parameter ranges and parameter distributions are defined prior to the analysis. Then, a Monte Carlo analysis, sometimes combined with a Latin hypercube sampling scheme, is used to produce a large number of different parameter combinations and to obtain a model response for each combination. These multiple model responses are then analyzed to obtain information about the impact of single parameters on the model response (42).

The major challenge for sensitivity analysis is that it is difficult to separate sensitivity from uncertainty in most exposure assessment models. Uncertainty about model components can result in a very mixed description of the importance of model components. Theoretically, a model input may be highly influential across part of its uncertainty range but much less influential across another part. At the least, acknowledgment of such discrepancies should be communicated to risk managers. Again, focused sensitivity analysis facilitates deeper analysis of a few components instead of superficial analysis of many components.

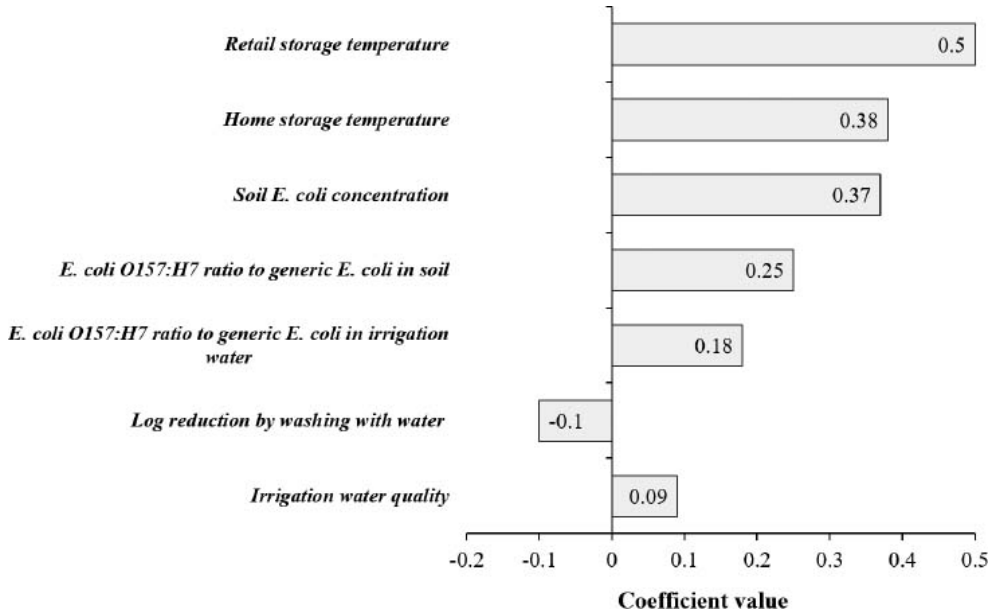


Figure 39.3. Tornado graph showing the most important parameters and variables affecting the estimated number of illness cases per year due to consumption of *E. coli* O157:H7-contaminated fresh-cut lettuce. Reprinted from reference 62.

Scenario Analysis

A scenario analysis consists of a series of "what if" options for mitigation measures, interventions, or policy changes. This type of analysis allows the evaluation of health benefits of various measures that prevent or mitigate exposures. Scenario analysis begins the process of identifying scenarios that constitute a risk as well as those that are not a risk. It simplifies the next stage of exposure model development, quantifying the likelihood of scenarios that end in an exposure dose. In developing scenarios during the conceptual development phase, one should begin by defining the mathematical relationships between process steps. Are these additive or multiplicative? Are there correlations between different inputs? What other factors influence the relationships among inputs?

Compared to scenario development, determining likelihood and dose is usually a more mathematical exercise The relationships between model inputs need to be mathematically described; statistical methods are often employed to quantify these relationships. Also, the process of converting conceptual relationships into explicit mathematical relationships commonly involves additional assumptions beyond those represented in the conceptual model. Transparency is particularly important during this process. Before data availability or analysis is considered, the mathematical development needed to determine likelihood and dose should be determined. Once a tentative mathematical model is completed, its structure can be communicated with outside reviewers and risk managers. Typically, the mathematics will provoke discussion about the data needs of the model. A clearly defined mathematical model provides an opportunity for specialists to review and comment on the course of the project. If scrutiny of the mathematics indicates the need for change, the changes should be made before a large investment in data acquisition and analysis is completed. Frequent outreach and feedback is one manifestation of effective exposure assessment (42).

Recent Developments in Food Safety Risk Assessment

Various risk assessment approaches have further matured through application of complex computational modeling and availability of more microbiological data. One limitation of the traditional risk assessment approach is that it is static. For example, if one is estimating the growth of *Salmonella* in chicken during storage, the values of storage time and temperature from a distribution are used. This temperature is usually constant for a given iteration. In reality, the temperature is not

fixed during storage, and there is possibility of cross-contamination at any time during the storage. These variations are usually not addressed in traditional risk assessments. There have been several recent developments and studies related to food safety risk assessment; we cite some of them as examples.

Recently, a system modeling approach was introduced which reported the probability of contaminated leafy greens harvest in a major leafy-greens-producing region in California (71). A system modeling approach considers any system to comprise several subsystems which can better be understood as being interdependent, rather than isolated. Pathogen pathways in food systems such as a leafy greens farm are very complex, but the system modeling approach is very promising with regard to understanding this complexity. The system model (71) calculated the survival and contamination of *E. coli* in a leafy greens farm system continuously throughout the year and predicted the months at highest risk for contaminated harvests. These results were in good agreement with the recent reported outbreaks.

Similarly, another model was developed to describe a hypothetical cattle farm in order to compare the relative importance of five routes of exposure of leafy vegetables to pathogens. The routes were aquatic recreation downstream of the farm, consumption of contaminated ground beef processed with limited interventions, consumption of leafy greens, direct animal contact, and the recreational use of a cattle pasture. To accommodate diverse environmental and hydrological pathways, existing QMRAs were integrated with novel and simple climate and field-level submodels (72). FDA-iRISK (irisk.foodrisk.org/) is a Web-based system designed to analyze data concerning microbial and chemical hazards in food and return an estimate of the resulting health burden on a population level. The data required to execute this analysis include the food, its associated consumption data and processing and preparation methods, the hazard and its dose-response curve, and the anticipated health effects of the hazard when ingested by humans. Each of these elements contributes an essential piece of information to the model on which the final estimate of risk is based.

Strengths and Limitations of Microbial Risk Assessments

Risk assessments applied to pathogens in food products can provide valuable information for risk managers. In addition to providing estimates of the overall probability of illness or infection associated with a food product, microbial risk assessments help to identify food processing and handling steps that affect the overall risk associated with the consumption of a particular food product. For example, the USDA Food Safety and Inspection Service (FSIS) developed a QMRA for *Listeria monocytogenes* in delicatessen meats (73) in response to risk management questions regarding the effectiveness of food contact surface testing and sanitation regimens in reducing the risk of contamination by *L. monocytogenes*. Results showed that a decline in the concentration of *L. monocytogenes* may be achieved by increasing surface testing and sanitation efforts. Another FSISsponsored risk assessment, for *Clostridium perfringens* in ready-to-eat and partially cooked meat and poultry products (74), demonstrated that storage temperatures used by retailers and consumers had a significant effect on the estimated risk of disease caused by this organism.

The quality of the data sources used to develop a microbial risk assessment is very important, as it determines the accuracy of the risk estimates produced. How closely the risk assessment model describes the actual scenario being studied also affects the accuracy of the conclusions. The risk estimates may be validated by using epidemiologic data; however, the usefulness of epidemiologic data is often limited, especially if the risk assessment considers specific food products in a larger food product category (such as fresh-cut lettuce in the fresh produce category) or if specific food-handling conditions (such as consumer behaviors regarding home storage) are being evaluated. Lack of information regarding any of the supply chain steps considered in the risk assessment can also increase the uncertainty of risk estimates.

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Kelly Stevens
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40
Food Safety Management Systems

INTRODUCTION

What Is an FSMS?

A food safety management system (FSMS; also called a food safety risk management system) ensures food safety across the supply chain by setting and documenting standards and expectations for activities focused on the production of safe foods. The International Organization for Standardization specifies requirements for an FSMS where an organization in the food chain needs to demonstrate its ability to control food safety hazards to ensure that food is safe at the time of human consumption (1). An effective FSMS will contain several key elements, including a hazard analysis and critical control point (HACCP) program, preventive controls, prerequisite and environmental monitoring programs, hygienic zoning, testing programs, consumer and customer feedback, traceability and recall, and documentation. Elements of an FSMS include specifications, procedures, processes, verifications, validations, and documentation that demonstrate commitment and execution of a manufacturer's FSMS.

Why Is an FSMS Needed?

Consumers expect safe food. Food producers must be committed to providing safe food 100% of the time. This is true for minimally processed foods, such as fresh produce, and for formulated, complex foods that provide consumers with a wide range of convenient options. Food holds a unique place as a consumer product.

Customs, culture, and rituals all play a part in the importance of food in society. While people interact with consumer products in many ways, all food products are intended to be ingested either in the form sold or after further preparation.

While consumers expect safe foods all the time, the statistics on foodborne illness indicate that there is significant room for improvement. The U.S. CDC estimates that each year there are about 46 million cases of foodborne illness and that about 8 million of those illnesses are caused by about 30 known pathogens (2). A global burden estimate by the World Heath Organization suggested that there are about 600 million illnesses each year attributed to 31 foodborne hazards (3). Norovirus and *Campylobacter* are the most common foodborne hazards. Determining the exact burden of foodborne illness is challenging. While these estimates consider all food sources, when the attribution is reviewed by food category, it is likely that only a small fraction of these illnesses are attributed to processed foods. However, issues with processed foods do cause numerous recalls and illnesses each year.

The best way to meet the challenge of providing safe food 100% of the time is to implement an effective FSMS. It is an internationally recognized comprehensive approach to managing food safety and compliance with regulatory, customer, and internal requirements.

What Factors Shape the Design of an Effective FSMS?

The FSMS must use a holistic approach to identify hazards, understand risks, and implement controls to reduce risks to acceptable levels. It is an understatement to say this is easier said than done. Figure 40.1 highlights the sources of information that should be used to create an FSMS. The level of rigor for a food safety risk management system will be dependent on a variety of factors. Every FSMS must consider the intrinsic risk of the food, the processing conditions, and the characteristics and behaviors of the consumers. Additionally, the FSMS must also ensure that customer expectations and regulatory requirements are met. All of this cannot

be achieved without an effective management program, including documentation for the FSMS. The final key factor for the success of any FSMS is the fundamental expectation of continuous improvement, which is a strategic approach to understanding an FSMS and identifying opportunities to drive down risks to food safety and quality. The programs that are necessary for an effective FSMS are discussed below.

Role of Regulations

Regulatory agencies are responsible for setting food safety standards and verifying compliance. In many cases, food manufacturers are under the jurisdiction of multiple regulatory bodies (federal, state, and local) charged with the oversight of safe food production. In developed countries and in most developing countries, regulations form the minimum operating standards for any food production facilities and should form the base of any food safety risk management system.

Regulations have played a historically important role in food safety. Many regulations are based on the best available science in setting standards to produce safe foods. Other regulations are in place to provide clear directions on how food should be produced and held. Regardless of philosophical approach, regulations can be considered the minimum expectations that must be met to make safe food, and compliance is expected.

Meeting the regulatory standard may not always be sufficient to ensure food safety, because many regulations do not set standards to the level of detail needed for an effective program. For example, the FDA Food Safety Modernization Act (FSMA; https://www.fda.gov/food/guidanceregulation/fsma/) sets the expectation for safe food. However, the FSMA does not mandate how to achieve the safe manufacture of any specific product. Manufacturers are expected to demonstrate that the food safety plan in place is appropriately implemented and executed to achieve

safe food. The burden of proof lies with the manufacturer, and the role of the regulator is to verify.

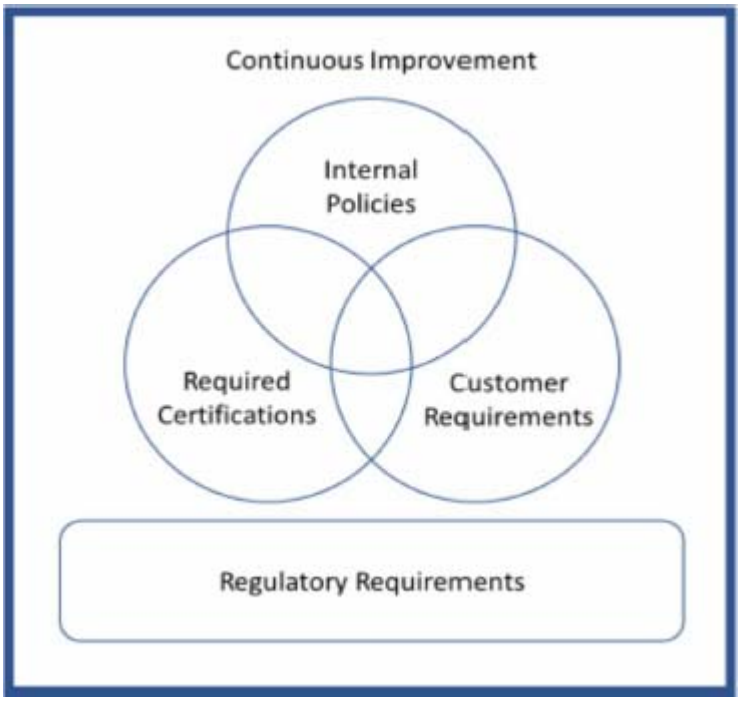


Figure 40.1. Factors to consider in developing an FSMS.

Role of Customer and Retailer Requirements

There is a distinction between customers and consumers. From the perspective of the manufacturer, consumers are the individuals who ultimately eat food products. Customers of a manufacturer may include retailers, such as grocery stores, convenience stores, club stores, and online marketers. Customers may also include food service operators and distributors as well as industrial users. In the current commercial environment, customer requirements play a role in the development of a food safety risk management system. Retailers have an intimate relationship with their customers (consumers) and play an active role in verifying that the products offered for sale meet the food safety and quality requirements of the consumers that they serve. While individual consumers are not able to set specific requirements, customers may set requirements as a part of doing business. Retailers have food safety and quality expectations of their suppliers and an associated verification

strategy. Retailer verification often includes an auditing component. In some cases, customers just accept recognized audit certification schemes. In others, customers have their own set of specific requirements. Finally, there are customers that want recognized certifications and have additional requirements. A manufacturer that supplies multiple customers will have to manage multiple sets of requirements and have a strong program in place to ensure compliance. An FSMS must take customer requirements into account and demonstrate compliance.

Role of Certifications

Nongovernment organizations that set standards for FSMSs provide a means for food production facilities to receive certifications from an independent source. These certifications can be used by the facility and customers to verify the facility has acceptable food safety procedures in place. In some regions of the world, food safety certifications are the price of entry into the business of supplying customers. The Global Food Safety Initiative (GFSI) is an example of a global FSMS certification program that has its roots in the retail sector. In 2000, the GFSI was established with a mission to provide continuous improvement in FSMSs and to ensure confidence in the delivery of safe food to consumers worldwide. There are multiple benchmarked schemes under the GFSI umbrella, including the British Retail Consortium, the Safety Quality Food (SQF) Institute, FSSC 2200, and several other organizations. In addition to informing companies of the elements of an FSMS, certifications can be a tool for manufacturers to demonstrate effectiveness of their FSMS to customers and regulators.

Role of Internal Policies

Policies provide the basis for governance of an entity such as a food company. Policies may not be needed for very small entities, but as an entity grows, at some point there will be a need for policies. Developing a standard set of rules and standards provides a framework for consistent operation. This is needed if there are

many facilities engaged in the production of food products. Even small entities can benefit from internal policies that help set the direction for safe food production.

Continuous Improvement

An FSMS is not a static set of programs and procedures. In Fig. 40.1, continuous improvement is presented as an overarching theme that should influence all aspects of an FSMS. The science of food safety continues to evolve, and new understanding of hazards should be reflected in all elements of the FSMS. In addition, food safety is generally considered a "precompetitive" activity. This allows the sharing of information across the industry. Large and small processors should be willing to allow others to learn from their mistakes. This sharing can help all processors improve. Any food safety event can damage the reputation of the category or whole industry. Therefore, sharing will have a positive impact on the industry by helping to build trust with customers and consumers.

Summary

When food safety risk management systems are discussed, it may be easy to think only about HACCP. HACCP is a tool used around the globe by companies that are committed to producing safe food. While HACCP may be the central tool for food safety, there are several other programs that also play a role in the overall food safety risk management system. The overall system also needs to address the details of programs such as approval of vendors, auditing of facilities, prerequisite programs, and traceability and recall procedures.

In this chapter, we provide an overview of the most important elements of effective food safety risk management systems. The detailed development and execution of these key elements may vary based on the intrinsic and extrinsic factors that apply to any specific food and production system. In addition, we use case studies as examples of approaches to food safety risk management systems.

The activities of Company X are fictional, but the examples are based on real activities and incidents that have occurred in the food industry.

RISK ANALYSIS FOR FOOD SAFETY

This chapter focuses on management systems for food safety. Ultimately, any operation engaged in producing foods should understand the risks associated with food. Before we introduce some key elements of an FSMS, it is important to emphasize that an FSMS is a specific type of risk management system focused on food safety. Since risk management is critical to the FSMS, it is important to understand the concept of risk.

Risk is often described as the combination of the probability that a hazard may exist and the severity of that hazard. While risk is a stand-alone term, it is implied that risks are balanced by benefits. This definition can apply across a wide range of activities, including financial transactions, medical procedures, and personal safety measures. In the financial world, hazards are based on the money or wealth that may be gained or lost. Medical procedures have the potential for life-saving outcomes, but hazards may include severe negative consequences. In the modern world, daily activities such as driving a car and using machinery have significant benefits to the user, but also include hazards such as personal harm. In the world of food production, we think of biological, chemical, and physical hazards.

This book focuses on biological hazards, and therefore, this chapter focuses on the risk analysis of biological hazards in food production. Risk analysis is composed of the following separate but linked elements: risk assessment, risk management, and risk communication (4). It is the risk assessment that informs the risk management decisions. Risk communication is the interaction and exchange of information between groups doing risk assessment and risk management.

Risk assessment is focused on the inherent or intrinsic risks that exist for food ingredients and finished products. These risks may be impacted by many factors, including the ability of pathogens to survive growth in a product or ingredient and the intended consumer population for a product. Risk managers need to decide what is an acceptable level of risk, acknowledging that even after risk management is applied, there is still some residual risk.

To simplify the risk equation, in dealing with microbiological risk, we know that, by definition, if a microorganism is a pathogen, it can cause illness. While some illnesses are more severe than others, people and companies that produce food should not be willing to accept any illnesses. Therefore, severity of the hazard is generally fixed.

Due to this approach, risk assessment and risk management are focused on the probability of a hazard being present and on ways to significantly reduce that probability. This is the foundation for building an FSMS.

Case Study—Risk Assessment

Company X sells fresh vegetables, frozen vegetables, and canned vegetables. The product format, pathogens of concern, and mitigation strategies are summarized in Table 40.1.

Vegetables are agricultural products and in the raw state are known to harbor a variety of pathogens. It is known that nonsporeformers such as *Salmonella*, *Escherichia coli*, and *Listeria monocytogenes* are likely to be present at some level. Sporeformers such as *Clostridium botulinum* are also potentially present. In addition, viruses such as hepatitis A virus may be present due to contaminated water and workers. While it is easy to identify the hazards, talking about risk gets a little more complicated.

Fresh vegetables may be sold as ready to eat (RTE) (e.g., lettuce, carrots, and tomatoes) or not normally RTE (potatoes). Risk management for RTE fresh vegetables starts with good agricultural practices, and for some commodities, washing and sanitizing may occur. Fresh vegetables can be considered a high-risk category for pathogens such as *E. coli* and viruses.

Most frozen vegetables are blanched prior to freezing. Blanching was originally designed to stop enzyme activity, but blanching is a heating step and can provide a reduction of pathogens. However, freezers have been shown to harbor *L. monocytogenes*, so risk management plans may focus on validated blanching and the exclusion of *Listeria* in the postblanching environment.

Canned vegetables go through a heating step that easily reduces the nonsporeformers and viruses to an acceptable level. Sporeformers such as *C. botulinum* are much harder to kill with heat. If these sporeformers are not killed, they may easily grow in the anaerobic environment of a can. Underprocessed cans of vegetables are likely to injure or kill many people. Therefore, the rigor of risk management is significant.

Table 40.1. Summary of vegetable case study

Consumer format	Pathogens of concern	Risk mitigation strategy
Fresh, refrigerated	*Salmonella*, *E. coli*, *L. monocytogenes*	Good agricultural practices; washing and sanitizing; finished-product testing
Frozen	*Salmonella, E. coli, L. monocytogenes*	Blanching; environmental control postblanching
Canned	*C. botulinum*	Validated and verified process for low-acid canned food

This case study demonstrates that risk assessment includes much more than just being aware of the pathogens that might be present in a particular agricultural product. It also important to know how the food will be processed and used by consumers to be able to fully understand how to implement a food safety risk mitigation system.

Risk Assessment across the Supply Chain

When a food product is at the concept stage, it is time for the first risk assessment. Even before the first prototype is made, it is likely that there is a vision for the finished product. There are several initial questions that should help inform a preoperational risk assessment.

1. How will the product be distributed and sold? (At ambient temperature, refrigerated, or frozen?)
2. Will the finished product support the growth of pathogens?
    a. If not, how will this be accomplished?
        i. water activity
        ii. pH
        iii. antimicrobials

3. What are the key ingredients for the product? a. Do these ingredients present microbiological risks?

During this stage of product development, there may still be some unknowns, but answering these questions will help inform the early risk assessment and indicate the mitigations that will be needed. If possible, it may be useful to set some design parameters to build safety into the product during this stage of development.

Once the basics of the product have been determined, it is time to take the concept to a facility where the product can be made. The next level of risk assessment considers the processing conditions. In addition to the questions about the product, additional questions must be asked to inform on risk.

4. Are there any processing steps where microbial growth might occur?

5. Are there steps that will reduce or eliminate any microbial hazards?

6. How is the product packaged?

    a. Is there an opportunity for postprocess contamination?

The questions posed above should help align the details of the site HACCP plan and the FSMS. Up to this point, the risk assessment discussion has focused on the product and the process. Every effort is made to identify risks that are reasonably likely to occur and to design mitigation of those risks through the FSMS.

We must recognize that events may occur that will compromise the FSMS. When an event or incident occurs, risk assessment must be done to determine the impact of the event. When assessing impact (risk assessment), a qualified team must assess whether the event is significant and what the required action is. Required action can range from none to product reprocessing, product destruction, or product recall.

Case Study—Event Risk Assessment

Company X routinely tests finished product for the presence of *Salmonella* as a verification of their FSMS. The firm received a positive result limited to a 24-hour production code. The site conducted a robust investigation. As part of that investigation, the site learned that there was a roof leak on the day of the positive result. The roof leak had been identified and appropriate short-term mitigations had been put into place, including an environmental swab of the leak site. The leak site was determined to be near the product zone. Genetic analysis of the roof leak swab and the product isolate determined that the organisms isolated were a genetic match. The final investigation showed that the roof leak was over the product zone and that the leaked material had made its way into the product zone. In this case, product testing was successful at identifying an issue and preventing the release of potentially contaminated product into commerce.

A new roof leak emerged at Company X. In this case, the roof leak was located away from the product zone and the product zone was protected with shielding. The firm tested samples from the roof leak, and all samples were negative for *Salmonella*. Finished product samples were also negative for that day. Appropriate investigation and mitigation were conducted in response to the roof leak, and no impact on public health was anticipated.

This case study illustrates the importance of having the following programs in place:

- procedures for identification, remediation, and mitigation of roof leaks
- corrective action response plans for finished product positive results
- appropriate sampling plans

KEY ELEMENTS OF EFFECTIVE FSMSS

An effective FSMS contains several key elements, including HACCP, preventive controls, prerequisite programs and environmental monitoring programs (EMPs), hygienic zoning, testing programs, consumer and customer feedback, traceability and recall, and documentation. In this section, we review the components and the importance of each key element.

Hazard Analysis and Critical Control Points

HACCP (pronounced “hassip”) is a simple and effective way to ensure food safety. It allows one to evaluate potential risks to food safety and to prevent them before they happen.

HACCP has its origins in collaboration between Pillsbury and NASA. In 1959, Pillsbury joined forces with NASA and began working on ensuring safe foods for astronauts. Howard Bauman, a trained microbiologist and Pillsbury’s lead scientist, and NASA’s Paul Lachance worked together to impose strict microbiological requirements, including pathogen limits, on all foods destined for space travel. Dr. Bauman and Dr. Lachance soon realized, however, that traditional quality control methods would be inadequate to guarantee the food’s safety. NASA’s further involvement stipulated the use of critical control points (CCPs), so it was a logical step to apply this same process to food manufacturing. Applying this concept to a food system was a new development and a NASA requirement.

During 1972, Pillsbury also established new internal specifications systems and training programs and hired research microbiologists to conduct the first hazard analyses and validations for the production of consumer products. The new systems and HACCP-based ingredient and product specifications were computerized and completed in 1975. Progressive food companies started recognizing the advantages of HACCP over traditional quality control

procedures in food safety management and began to adopt the HACCP system of food safety management.

A HACCP system has seven basic steps. Each one is necessary for the overall program to work.

1. Identify potential food safety hazards associated with ingredients and the manufacturing process (hazard analysis).
2. Identify CCPs—steps critical for food safety.
3. Set critical limits at CCPs.
4. Monitor CCPs—develop monitoring procedures.
5. Identify corrective actions.
6. Document and verify.
7. Conduct periodic audits to ensure that the HACCP system works properly.

More than 50 years later, HACCP continues to be the primary FSMS used globally to ensure the safe manufacture of food products. Implementation of effective HACCP programs allows manufacturers to proactively identify and manage food safety risks, prioritize resources where most impactful, and demonstrate regulatory compliance for food safety.

Preventive Controls

The FSMA introduced the term “preventive controls” to the food safety framework. Preventive controls are defined by the FDA as steps taken by a food manufacturer to reduce or eliminate food safety hazards. Examples of preventive controls include processing controls, allergen controls, sanitation controls, and other controls.

Prerequisite Programs

HACCP programs will not be successful without the successful implementation and execution of prerequisite programs.

Prerequisite programs are foundational programs that are not specific to a product or a manufacturing process. Example prerequisite programs include good manufacturing practices (GMPs), sanitation, allergen control, raw-material management, pest control, maintenance, foreign-material control, and others as appropriate. Prerequisite programs are covered in detail in other sources, so we cover just a few in this chapter.

GMPs

GMPs are the minimum requirements to produce safe foods. GMPs form the foundation for standard procedures for dealing with people, buildings, equipment, and documentation for food safety and quality.

Sanitation Programs

Sanitation programs are foundational programs for establishing and maintaining the hygienic conditions necessary to produce safe food and to maintain quality attributes of that food. Robust sanitation programs enable a manufacturing facility to manage hygiene, pest, microbiological, and allergen risks. Sanitation programs take into consideration equipment product contact surfaces, equipment non-product contact surfaces, the processing environment (floors, walls, drains, etc.), facility and equipment design, and janitorial activities.

Sanitation activities typically include a combination of cleaning activities, which remove the food debris, and sanitization activities, which serve to decrease microbial populations. Cleaning methods vary based on the equipment being used and the nature of the hazards that need to be controlled. Hazard controls may include control of microbial growth, reduction of spoilage microorganisms, pathogen elimination, allergen removal, pest control, and control for quality attributes. Cleaning methods include wet cleaning, dry cleaning, and a combination of wet and dry cleaning, sometimes referred to as controlled wet cleaning.

Wet cleaning uses a combination of water and cleaning chemicals to remove food residue and to return the food contact surfaces to a hygienic state. It is important to match the cleaning chemistry and cleaning method to the material being cleaned and the type of soil that needs to be removed. The use of appropriate cleaning chemistry facilitates the removal of organic materials and can decrease effort needed to effectively clean processing equipment. Conversely, the use of inappropriate cleaning chemistry may result in creation of challenging soils that are difficult to remove and potential increased risk. Wet cleaning is typically used for systems that produce high-water-activity or sticky products to control microbial and/or allergen risks. Types of wet cleaning methods include manual cleaning, clean-in-place methods, and clean-out-of-place methods. Regardless of method, all wet cleaning procedures include the critical elements included in Fig. 40.2. In addition, it is important to have the correct concentration of cleaning chemicals, the right temperature, for the right amount of time, and the appropriate mechanical action during the wash step.

- prerinse (removal of food debris)
- wash
    - concentration of cleaning chemicals
        - Detergent helps the removal of food from the equipment surfaces.
    - temperature
        - Cleaning chemicals have an optimal temperature for maximum effectiveness.
    - time
    - mechanical action
- rinse
    - removal of residual detergent and loose food debris

    - inspection for visible cleanliness
- sanitize
    - microbial destruction
    - System must be clean prior to application of sanitizer. Sanitizers are not effective on dirty surfaces.



Figure 40.2. Critical elements of a sanitation procedure.

Dry cleaning methods are best used for low-water-activity systems or dry systems where microbial growth is not a concern and the environment should be kept dry to prevent introduction of microbial risks. Dry cleaning methods use mechanical action to physically remove food residue from the processing lines. Dry cleaning methods may include the use of vacuum cleaners, scrapers, and brushes. High-pressure blasting and air hoses can be used; however, these methods are not effective at removing the food residue; rather, they relocate it to another part of the processing environment. An area that has been dry cleaned may not be shiny; however, it should meet the visually clean standard with little or no

remaining residue on the equipment. When allergen removal is a risk to be managed, dry cleaning effectiveness must be verified and validated to ensure that allergen removal was effective. Aqueous sanitizers are typically Not used after dry cleaning to prevent an increased microbiological risk. Some novel nonaqueous sanitizers are available on the market, and their use may add value to a dry cleaning system.

Regardless of cleaning methods utilized, the results of all sanitation activities should be monitored and verified for effectiveness. Monitoring activities may include measurement of sanitizer concentration, visual inspection, and analytical testing. Verification activities include a second level of visual inspection and document review. Periodic review of program documentation, records, and corrective actions can be useful in finding and eliminating potentially harmful trends.

Cleaning and sanitation are fundamental to the food safety system of a manufacturing facility. Effective cleaning is necessary before sanitizer application can be effective. Cleaning methods should be selected to best control the risks that are being managed.

Sanitation definitions

Below are some terms that are commonly used in discussing sanitation, with their definitions.

cleaning: the physical removal of organic material from processing equipment and the processing environment (Food Safety Preventive Controls Alliance [FSPCA] definition: the removal of soil, food residue, dirt, grease, or other objectionable material)

sanitizing: the destruction of microorganisms on previously cleaned surfaces. Sanitizing is different from disinfection. (FSPCA definition: adequately treating cleaned surfaces by a process that is effective in destroying vegetative cells of pathogens and in substantially reducing numbers of other undesirable microorganisms but without adversely impacting the product or its safety for consumers)

monitoring: a planned sequence of observations or measurements to assess whether a system or process is under control

verification: activities that determine whether a system is operating as intended

validation: the scientific process demonstrating that the system as designed can adequately control the identified hazards to produce a safe product

Environmental Monitoring Programs

EMPs are not generally considered prerequisite programs. EMPs are included in this section because of the important role that they play in the verification of the efficacy of sanitation and hygienic practices.

Every manufacturing facility has its own unique microbial population. Microorganisms are introduced to the processing environment via vectors such as ingredients, people, materials, and transport vehicles. GMPs and other control programs help in microbial control. EMPs play an important role in monitoring the processing environment for the presence or absence of microbial pathogens of concern. EMPs are designed to proactively find

potential sources of pathogens in the manufacturing environment. The best-designed programs leverage a seek and-destroy approach. Identification of potential sources of contamination allows a prudent manufacturer to mitigate and remediate before environmental pathogens can become established in the environment and potentially cross-contaminate product. It is important to note that an EMP that never yields a positive result is not a program to celebrate; rather, it is a program to reevaluate for effectiveness.

EMPs can be used to verify the adequacy of sanitation programs and to assess the potential for environmental pathogen contamination of RTE foods. EMPs are designed to monitor the pathogen pressure in the manufacturing environment and focus on finding and eliminating potential sources of contamination. Manufacturing environments are not sterile and are subject to constant change and influx of microflora from ingredients, materials, personnel traffic, equipment traffic, and transient equipment. Contaminating microorganisms are sometimes classified as either transient or resident microorganisms. Transient microorganisms do not persist or become established in the processing environment. It is expected that robust EMPs will find transient microbes periodically. Resident environmental pathogens become established in the processing environment and are challenging to remediate and eliminate. Resident environmental pathogens may persist in harborage points for long periods of time.

When an EMP is being designed, it helps to think of a manufacturing environment as having four zones, as defined by the International Commission on Microbiological Specifications for Foods and shown in Table 40.2 (5).

Once an environmental pathogen becomes established in the processing environment, remediation efforts must target identification of the root cause and elimination of the source of contamination via procedural changes, behavior changes, and potentially capital investment. Conventional wisdom would say that

finding and eliminating sources of contamination in zones 3 and 2 will decrease the likelihood of product contamination. This is illustrated in the following case study.

Table 40.2 Environmental monitoring zones

Zone	Definition	Example
1	Product contact surfaces	Conveyor belt surface
2	Non-product contact surfaces near product zone	Conveyor supports
3	Remote processing areas	Drains
4	Nonprocessing areas	Employee welfare areas

Case Study—EMP

Manufacturer X produces an RTE savory dip product. The facility performs a validated wet clean sanitation cycle every 24 hours. The facility environmental pathogen monitoring program is performed weekly, and samples are taken primarily in zones 2 and 3. One of the zone 3 samples, a drain swab, was positive for *Listeria* species. The facility investigated and reviewed the sanitation procedures. The root cause was determined to be an error in the sanitizer concentration. The drain swab being positive was an early indicator, and prompt action allowed the facility to identify the problem and remediate.

EMP Design Considerations

EMPs should not be statistically based. The programs should be biased to find potential sources of contamination. There is not a magic formula or a one-size-fits-all approach.

One of the first steps in developing an EMP is to determine what target organism to test for in a given facility environment. Environmental pathogens as defined by FDA include *L.*

monocytogenes and *Salmonella* species. *Listeria* species are recognized as an indicator organism for *L. monocytogenes*. Some manufacturers choose to test for *Listeria* species, as the test casts a wider net for locations where *L. monocytogenes* may grow. Processing conditions should be evaluated when one is deciding which organism(s) to test for. As a general rule, it is appropriate to test for *Salmonella* and *L. monocytogenes* in wet-cleaned processing environments. In dry environments, testing for *Salmonella* would be appropriate. In some cases, it is also appropriate to test for *L. monocytogenes* in dry processing environments.

Design principles for a robust EMP start with a "find it, fix it" approach. There are several factors that should be considered in advance of program development, and thoughtful development is critical to success. Factors that should be considered during plan development are listed in Table 40.3.

All positive results should be investigated and appropriate corrective action implemented. Understanding the sample details, such as sampling site, time of sampling, hygienic status, test organism, and site location, will aid in the investigation. Some examples of positive samples and possible explanations that should provide a starting point for investigation are listed below.

- positive postsanitation sample: sanitation protocol may not have been effective
- positive sample during production in raw processing area: either sanitation was not effective or raw ingredients are a source of contamination
- positive sample during production in postlethality, RTE-food processing area: cross-contamination, presence of a harborage site, or ineffective hygienic zoning

Routine sanitation programs should be effective at removing transient microorganisms before they become resident microorganisms. EMPs as a verification program are best

approached with a “find it, fix it” mentality, also known as “seek and destroy.” The purpose of EMPs is to find and eliminate environmental pathogens and any potential harborage points.

Hygienic Zoning

Sanitation, environmental monitoring, and hygienic zoning are three program elements that work together to ensure that processing facilities are designed, maintained, and monitored in a way that allows safe food to be produced.

Processors that manufacture RTE foods may employ hygienic zoning as a tool to reduce the risk of cross-contamination from raw processing areas to postlethality processing areas (Fig. 40.3). Hygienic zoning differs from zoning for environmental monitoring in that hygienic zoning differentiates facility hygiene requirements to minimize product cross-contamination, whereas zones in EMPs differentiate degrees of proximity to product contact surfaces. In an RTE-food processing facility, different areas, such as nonmanufacturing, basic GMP, transition, primary pathogen control, and sensitive high hygiene, have different hygiene requirements based on the risk of potentially contaminating product. Examples of nonmanufacturing areas include employee comfort areas, offices, and maintenance areas. Examples of basic GMP areas include raw-food, receiving, and warehousing areas. Examples of transition areas include locker rooms. Examples of primary pathogen control areas include postlethality processing areas, open product zones, and final packaging areas. Examples of sensitive high-hygiene areas include areas where food for at-risk populations is manufactured.

Table 40.3. Key elements to consider when designing an EMP

Element	Example or comment(s)
Processing area	Processing area, warehouse, packaging area
Sanitation method	Wet, dry, controlled wet
Hygienic zone	RTE, not RTE
Sampling sites Where to sample	Zones 4, 3, 2, 1
Site selection	Random sites, fixed sites, rotating sites, investigational sites
Quantity	Defined in advance
When to sample	After sanitation, at production startup, during production, at end of cycle
Target organism	*Listeria* spp., *L. monocytogenes*, *Salmonella*, quality indicators
Testing frequency	Daily, weekly, monthly, periodically
Reaction to positive results	Action plan defined in advance

Hygienic zoning tools include color-coded maps that detail hygiene zones and expected traffic patterns (Fig. 40.4). Color-coded employee uniforms, dedicated footwear, and controls for entry into primary pathogen control areas are useful for the success of hygienic zoning.

Testing Programs

Testing programs can provide valuable data and insights into the quality and safety of food products. Before any testing program (microbiological, chemical, or physical) is implemented, it is important to clearly understand the purpose of the test, to know how to interpret test results, and to understand the implications of an out-of-tolerance result.

Microbiological testing can be a strong verification tool for a well-managed food safety system; however, microbiological testing of finished food products is not a feasible tool to ensure food safety. Test results can provide valuable information; however, it is important to understand the statistical nature of the sample plan and appropriate interpretation of results. Some important limitations of finished product microbiological testing include the facts that microbiological testing is destructive and that test results are limited to the sample tested. What this means is that in order to ensure that 100% of the product is pathogen free, all product must be destructively tested.

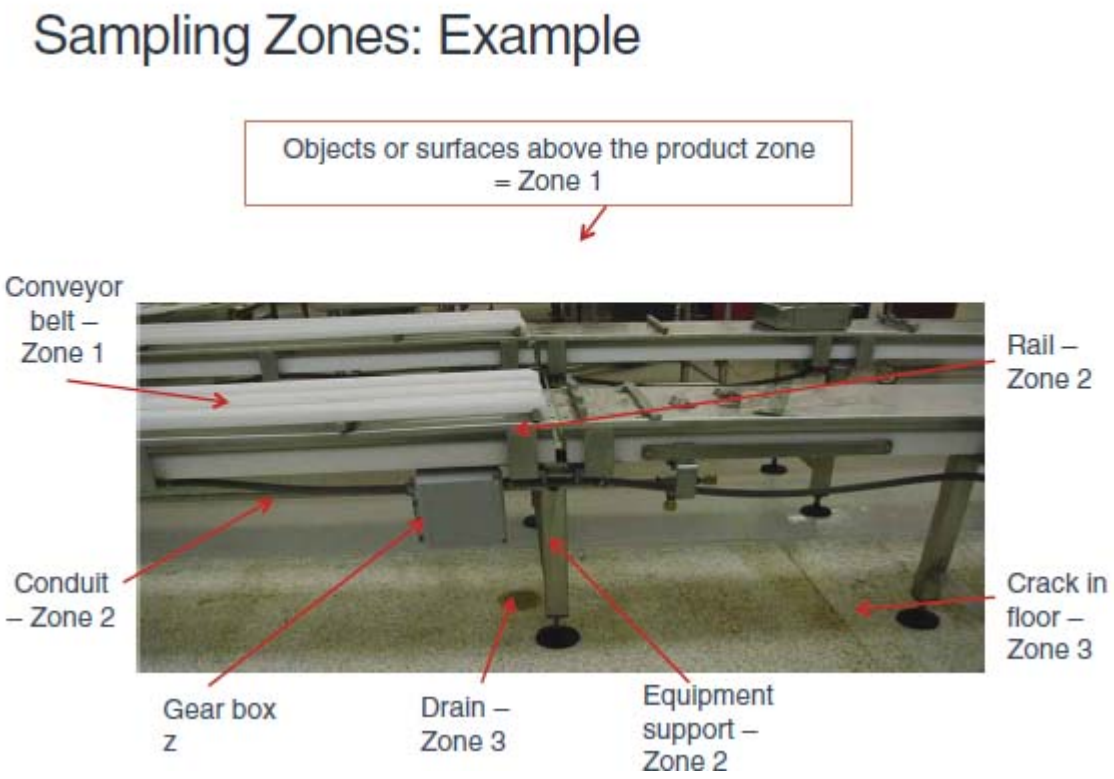


Figure 40.3. Examples of EMP zones using a single piece of equipment. This picture provides some examples. Surfaces above this conveyor belt and the conveyor belt itself are zone 1. The conduit below the belt, the gear box, equipment support leg, and rail adjacent to the belt are zone 2. The drain and the crack in the floor are zone 3.

Case Study—Finished-Product Microbiological Testing

Company X manufactures RTE rice-based meals for airline catering. The firm has a comprehensive FSMS that includes microbiological testing of finished products. Each lot produced is tested for the presence of *Salmonella* and *Bacillus cereus*. Any sample that tests positive is destroyed. All product associated with the sample that tested positive is also destroyed. The firm also has an investigative procedure that is performed any time a positive result is obtained. The firm will sample additional product as part of the investigation. It is important to note that the additional data can be used to guide the investigation; however, negative results cannot be used to negate the initial positive results.

Role of Auditing—Trust but Verify

Auditing plays an essential role in ensuring that FSMSs are functioning as expected and that employees are following expected food safety practices and behaviors. Audits can be an effective way to test the FSMS and to identify improvement opportunities. Just as testing results reflect the sample tested and not necessarily the entire lot produced, audit results are reflective of the point in time at which the audit was performed. Audit results, like test results, are a snapshot. Effective and successful audits rely on three components: (i) a trained, skilled, objective auditor, (ii) transparency and openness on the part of the auditee, and (iii) a clear robust standard and scope to be audited against.

A layered approach to auditing can be an effective approach to verifying FSMSs and fostering an environment of continuous improvement. A layered approach may include a combination of self-audits (first-party audits), second-party audits, third-party audits, customer audits, and regulatory audits. Self-audits are usually performed on a regular basis (e.g., monthly) by a crossfunctional team from the manufacturing location. The purpose is to evaluate the execution of programs against program requirements and directly observe the performance of food safety-critical tasks. Self-audits also provide an opportunity to connect

with employees on the manufacturing shop floor. Internal audits can also be performed by an internal audit team that is not connected to the manufacturing facility. Second-party audits are often used to assess suppliers and manufacturing partners. Third-party audits are audits contracted out to an independent third-party audit provider. Third-party audits can also result in a certificate which can be shared with customers, retailers, and regulators.

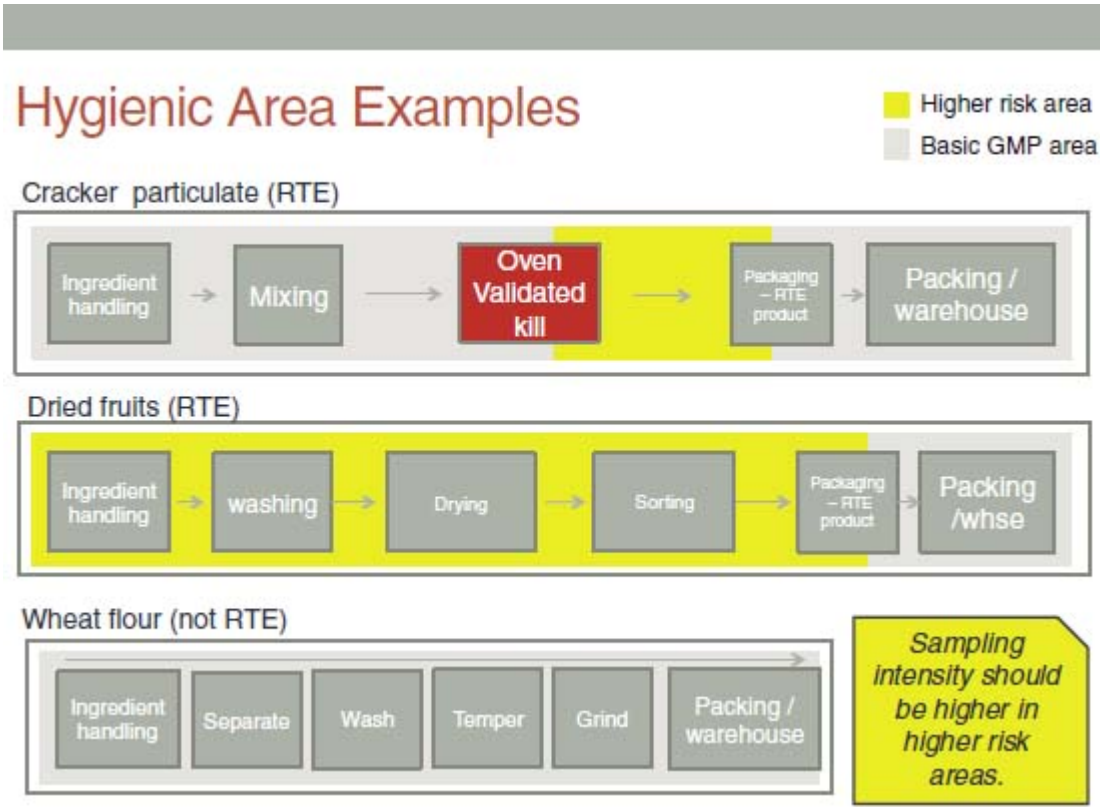


Figure 40.4. Hygiene area site selection. Shown are examples of hygienic area designations in three generic process flows. The yellow background indicates an area of high hygiene risk, and the gray background indicates a basic GMP area. (Top) In the cracker (RTE) production process, the cooking step is a validated kill step, so areas prior to the cooker are considered basic GMP areas. After the cooker, but before packaging, the product is exposed and susceptible to contamination from the environment, so this area is considered a high-risk area. After packaging, the product is no longer exposed, so this area is a basic GMP area. (Middle) In a dried-fruit production process, there is no lethality step and the product is RTE. Therefore, the entire line before packaging is a high-risk area. (iii) In a wheat flour (non-RTE) production line, the entire line is a basic GMP area because there is no RTE product. Sampling intensity should be higher in high-risk areas than in basic GMP areas to distribute verification resources in alignment with risk.

Consumer and Customer Contacts

Food manufacturers should have a way to get feedback from consumers and customers. The most common methods for feedback are the use of toll-free phone numbers and websites. In addition, the role of social media continues to evolve, and it has become a forum for consumers to share their experiences with a product. Monitoring of social media should be considered as part of developing an effective consumer contact system. Customer feedback can be both positive and negative. In a food safety risk management system, negative feedback or complaints are an important source of data.

The consumer contact system should be designed to align with the size and complexity of the food producer. Any complaints that suggest a food safety issue need to be reviewed. This can be very complicated. The system should be set up to collect the critical information needed to properly investigate an issue. The information should include a number of important details (Table 40.4). The product name, size, and code date are the most important pieces of information for investigating potential issues. Any illnesses, reactions, and incidents of physical hazards should be evaluated for the need of further review.

Each company should set up a standard operating procedure for the review of complaints alleging foodborne illness or physical harm. Common standards might include investigating all cases of alleged allergic reactions.

Table 40.4. Key information to collect for consumer and customer complaints

Product name
Package size
Code date
Date of purchase
Location of purchase
Date of consumption
Details of the issue/reaction

Case Study—Consumer Feedback

Company X produces dry, RTE snacks. Each consumer unit includes a toll-free number for consumers to reach a voice mailbox to provide feedback. Each day, all consumer contacts are categorized and entered into a database. Alleged illnesses and injuries are reviewed daily by a trained specialist. Company X sells a high volume of product and has a protocol that escalates the investigation of any alleged allergic reaction or any two alleged illnesses that are recorded over a 1-month time frame. The escalation process includes additional review by a senior food safety specialist familiar with the operation of the processes for the specific product. The investigation may include review of production records and requests for follow-up with the consumer if possible.

Traceability and Recall

Food traceability is important for food manufacturers, and increased transparency is expected by customers and consumers. Understanding the journey food takes from the farm to the consumer's eating experience can impact quality, food safety, and social responsibility. At a minimum, a manufacturer must be able to trace one level forward and one level backward. This means that a

manufacturer must be able to trace any product back to the lot codes of ingredients used and be able to trace product delivery to customers or consumers (when sold directly).

Digital technology can be used to simplify the traceability exercise and can be used to encode product information such as farm locations, harvest date, lot code, transportation journey, processing lot code, and final product distribution. All these data can be shared with consumers to aid in purchase decisions.

It is considered best practice to test the functionality of a traceability program on a regular basis. Mock recalls allow manufacturers to error-proof this traceability program and to assess procedure robustness and efficacy in tracing product one step forward and one step backward in the absence of a food safety incident. Data and insights gained from the mock-recall process can provide valuable insights and allow program improvements. Mock-recall exercises should be designed to challenge the systems as opposed to setting up the participants for success.

Documentation and Records

Documentation management can be considered the backbone of an effective FSMS. A predefined document control procedure will help ensure that appropriate processes are followed, the correct forms are completed, data are reviewed, and follow-up occurs as necessary. Review of critical paperwork should take place in a timely manner to ensure that the processing records clearly indicate that the food was produced within the framework of the established HACCP or food safety plan. A good rule of thumb is that anything that is not recorded, never happened. Whatever is documented, happened exactly in the way that it was written down.

CULTURE AND MANAGEMENT COMMITMENT

In the last decade or so, the concept of food safety culture has emerged as a potential risk factor beyond the traditional hazards and risks known in the food industry. Achieving a consistent, well-functioning food safety system requires commitment of people throughout an organization that produces food (6).

Culture is defined as an unwritten set of values, shared assumptions, and behaviors that are acted upon by both management and employees in any organization. Food safety culture is the subset of these values, shared assumptions, and behaviors that apply to the important issues related to food safety. All organizations have a culture. In some cases, the culture is positive and fosters the right behaviors and practices at all levels within an organization. That statement clearly implies that there are also cultures that foster the wrong behaviors.

Since the study of food safety culture is a relatively new area, it is still not clear how to move an organization from a bad culture to a good culture. While there are few data in this area, it seems reasonable that organizations that have a strong ethical foundation are likely to be able to have a strong food safety culture. However, ethics alone will not achieve all the right behaviors. There are very specific technical aspects of the processes that ensure food safety.

In addition to strong ethics, there must also be a culture of learning and continuous improvement. Together, ethics, learning, and continuous improvement are likely to result in a very strong food safety culture. In larger organizations, the people managing the organization are not likely to be involved in the day-to-day production of food products. Those managers must have an appreciation of what is needed to produce safe foods, so they can ask the right questions and allow money to be spent in the right areas. People involved in the direct production of food need to know the what and why of basic food safety. More importantly, they need to have the power to ask questions and challenge assumptions. In simple terms, they need to know that they can stop the line when something is not right.

EMERGING TRENDS AND TOOLS IN FOOD SAFETY

Rapid Microbiological Methods

Traditional microbiological testing methods are very slow and often require several days to complete. The development of rapid methods is not an emerging trend, and it is likely to continue until results can be achieved in near-real time. The movement from culture-based methods to newer technologies such as enzyme-linked immunosorbent assays and PCR has been evolving for decades. This trend has not slowed. Advancements in enrichment media, concentration techniques, and improved sensors have taken rapid methods to new speeds. Methods that required several days a decade ago can now be done in less than 24 hours. New and improved methods will continue to be developed and introduced into the market. It is important to understand that all methods must be validated against current standards and for the food matrix where they will be used.

Strain Characterization—WGS

In 1996, the U.S. CDC formally launched PulseNet. This approach to foodborne illness investigation radically improves the ability of public health officials to identify and stop outbreaks of foodborne illness. It has been estimated that PulseNet prevents more than 270,000 illnesses in the United States annually (7).

PulseNet relied on pulsed-field gel electrophoresis (PFGE) to characterize pathogens from clinical isolates, food sample isolates, and facility environmental isolates. While PFGE was very successful, new methods have been developed to identify and characterize isolate strains. Sequencing the whole genome can provide much greater differentiation of strains than PFGE. Sequencing methods have become easier and cheaper, and whole-genome sequencing (WGS) (or next-generation sequencing) has

emerged as an even more powerful technology for foodborne-illness surveillance.

The role of WGS in food production is still evolving. It is clear that an FSMS should include environmental monitoring as a verification of sanitation. When positive samples are found in the environment of a food production facility, it is important to understand whether the organism is transient or is an established population. Characterizing the isolate and comparing the data to historical information from the facility is the best way to determine if there is an established population present.

WGS is the most powerful technology available to characterize strains. There are other technologies, such as PFGE, ribotyping, and 16S rRNA gene sequencing techniques, available for the characterization of strains, but these technologies have less power to discriminate strains compared to WGS. It is likely that strain characterization will continue to emerge as an important technology, and companies should consider including strain characterization in the FSMS when pathogens are isolated from environmental and food samples.

Digital Technology and Traceability—Blockchain

Traceability continues to be a challenge across many sectors of the food industry. Slow and inaccurate traces are likely to put more consumers at risk for foodborne illness when events occur within the food industry. In addition, a lack of trust on the part of consumers regarding the source of ingredients can pose a challenge to the food industry. Technologies that allow more accurate, more rapid, and more transparent traceability will likely gain adoption across the industry.

One technology that is getting a lot of attention is blockchains. A blockchain can be described as a decentralized, distributed, and public digital ledger. The ledger is used to record transactions across many computers resulting in records that if altered retroactively

would trigger notification of the breach. Blockchain is the ledger technology used for cryptocurrencies such as Bitcoin.

The use of blockchain technology has been limited to pilot studies used to investigate the viability of this technology (8). Widespread adoption of blockchain or similar digital technologies is likely many years away.

SUMMARY

Producing safe food, all the time, must be a foundational goal for any individual or corporation in the business of making food. It is critical to understand the risks associated with food and take actions to mitigate those risks. FSMSs include multiple, interrelated elements that help to ensure that risks are mitigated to an acceptable level. GMPs and HACCP programs are key elements of an FSMS, but there are other elements based on policy and culture that are also important. A robust FSMS that is effectively implemented, continuously monitored, verified, and subject to continuous improvement is essential for the success of food manufacturers.

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Microbiological Constraints for Use of Reclaimed and Reconditioned Water in Food Production and Processing Operations

Water is a vital component for living organisms, with humans meeting their daily requirements either by consuming it directly from freshwater sources or abstracting it from ingested foods. However, more than 4 billion people live in parts of the world where freshwater is scarce, and this scarcity threatens water security (1). In other parts of the world, there may be a mismatch between demand for and availability of water resources on a temporal scale, with water scarcity being observed more often in the summer months (2). Even in areas where water was recently considered plentiful, both climate and societal changes have been putting a strain on those resources (3). With regard to societal changes, population growth and accompanying economic development have led to competing demands for the finite water resource. The global shift in dietary preference from a vegetarian-based diet to a meat-based diet is another societal change that is also expected to lead to a higher water demand in the future (4).

Currently, on a global basis, agriculture, industry, and domestic households account for 76%, 15%, and 9% of the total freshwater withdrawals, respectively (5). Although withdrawals for irrigation constitute only 31% of the total in the United States (6), the state of California, having had an extended drought, has been forced to limit its agricultural water withdrawals (7). Therefore, to maximize existing water resources under these circumstances, two main steps have been taken by society and industry: water conservation and water reuse. This chapter focuses primarily on water reuse. As there have been many other terms used interchangeably with "reuse," Table 41.1 provides a list and definitions of those terms as well as other terms that arise from these endeavors.

On a global scale, reuse of wastewater is very low, representing only 0.59% of the total water used in 2011 (8). However, across the world, there are disparities in the amount reused by individual countries. For example, both Australia and the United States reused approximately 8% of their wastewater generated. In contrast, 16% and 30% of wastewater generated by Saudi Arabia and Singapore, respectively, was reused, whereas the countries of Israel and Cyprus reused 70% and 89%, respectively, of their domestically generated wastewater (6, 8). Disparities in efforts devoted to reuse of wastewater are also apparent within individual countries or continents. To exemplify this point, Spain and Italy jointly accounted for about 60% of the total European Union's treated wastewater reuse volume in 2006 (8). Moreover, within the United

States, it was estimated that in 2012, 90% of water reuse occurred in just four states, Arizona, California, Florida, and Texas, although other states, such as Nevada, Colorado, New Mexico, Virginia, Washington, and Oregon, were starting to increase their efforts to reuse water (6).

Table 41.1. Definitions of terms used to describe water status or the activities to alter that status

Term	Definition
Direct potable reclaimed water	Reclaimed water that is immediately added to the potable water distribution system and no further treatment is applied
Gray water	Wastewater arising from residential domestic sites that includes wastewater from sinks, showers, and laundry facilities but excludes toilet or urinal wastes
Indirect potable reclaimed water	Reclaimed water that is used to recharge groundwater aquifers or augment surface water reservoirs. The mix of reclaimed and "natural" source water receives additional treatment at a conventional treatment facility before distribution
Potable water	Water that is safe for drinking
Reused/reconditioned/reclaimed water	Wastewater or water used in a manufacturing/processing facility that is treated to reduce or eliminate microbiological, chemical, and physical contaminants that may then be used for an intended purpose
Recycled water	Water that is passed through the same series of processes, pipes, or vessels more than once (the water can be treated or untreated)
Wastewater	Water discharged from homes, businesses, and industries that enters either a septic or sewer system

At present, the dominant wastewater reuse applications worldwide are irrigation of agricultural lands, parks, and golf courses (9). Other reuse applications include groundwater recharge, industrial reuse, and urban uses other than as potable water. As for the potential to directly employ reused water as potable water, there are two main forces preventing wide-scale adoption of this application, including concerns regarding public opinion and the safety of the final product. These impediments are such that only a few small-scale facilities are practicing direct potable-water reuse in the United States (10). However, as potential evidence that such utilization does not jeopardize the health of the public consuming reused water, an operation based in

Windhoek, Namibia, has been mixing reclaimed water with conventionally treated water for potable reuse for over 45 years (11).

Although there are a host of environmental, economic, and social benefits associated with water reuse, there are also potential drawbacks. Hence, it is important to mention that while guidelines for reused water have traditionally focused on health protection measures, there is increasing recognition of the need to also ensure that the impact of reused-water applications on soils and groundwater is such that they are environmentally sustainable for the long term. To address this issue, factors to be considered are the salinity, alkalinity, and the presence of toxic ions in the reused water, as well as the hydraulic and nutrient loading rates. It is also acknowledged that the presence of chemical contaminants is a major concern with regard to the safety of these water sources. However, the focus of this chapter is on the microbiological risks to the health of individuals exposed either directly or indirectly to wastewater or different grades of reused water. Information and discussion pertaining to the presence of chemicals are included only when those chemicals affect the pathogens directly (i.e., antibiotic resistance) or indirectly (i.e., through modification of the indigenous competitive microflora).

MICROBIAL PATHOGENS OF CONCERN IN WATER

Microorganisms occur in every niche of the environment; however, a small proportion of microorganisms cause human diseases. When present in water, these pathogens can infect humans through skin and mucous membrane contact or through ingestion of contaminated water or consumption of food that has come into contact with contaminated water. Waterborne pathogens are differentiated into four groups: bacteria, viruses, protozoan parasites, and helminth parasites. Specific pathogens that may be found in each of these groups are listed in Table 41.2, along with the delineation of their relative infectivity and their ability to persist in untreated freshwater supplies (6).

This list of infectious microorganisms for which water serves as a transmission route is not exhaustive, and the complete list will continue to expand as our ability to detect and link specific isolates to human illness improves. In addition, another group of aquatic microorganisms that are not infectious but are of concern comprises aeromonads and cyanobacteria. Under conditions in which excess nutrients are present in the water systems, these organisms may multiply to densities at which they produce toxins in sufficient concentrations to adversely affect human health (12, 13).

Table 41.2. Waterborne pathogens and relevant characteristics associated with risk of causing human illness[a]

Pathogen group	Pathogen	Persistence in water supplies[b]	Relative infectivity[c]
Bacteria	*Burkholderia pseudomallei*	May multiply	Low
	Campylobacter jejuni, *C. coli*	Moderate	Moderate
	Escherichia coli, pathogenic[d]	Moderate	Low
	E. coli, enterohemorrhagic	Moderate	High
	Francisella tularensis	Long	High
	Legionella pneumophila	May multiply	Moderate
	Mycobacteria (nontuberculosis)	May multiply	Low
	Salmonella Typhi	Moderate	Low
	Salmonella enterica	May multiply	Low
	Shigella dysenteriae	Short	High
Viruses	Adenoviruses	Long	High
	Astroviruses	Long	High
	Enteroviruses	Long	High
	Hepatitis A virus	Long	High
	Hepatitis E virus	Long	High
	Noroviruses	Long	High
	Rotaviruses	Long	High
	Sapoviruses	Long	High
Protozoan parasites	*Acanthamoeba culbertson*	May multiply	High
	Cryptosporidium hominis/parvum	Long	High
	Cyclospora cayetanensis	Long	High
	Entamoeba histolytica	Moderate	High
	Giardia intestinalis	Moderate	High
	Naegleria fowleri	May multiply	Moderate
Helminth parasites	*Dracunculus medinensis*	Moderate	High

[a] Adapted from Table 7.1 in reference 13.

[b] Detection period for infective stage in water at 20°C: short, up to 1 week; moderate, 1 week to 1 month; long, over 1 month.

[c] Based on experiments with human volunteers, epidemiological evidence, and experimental animals studies. Low, 104 organisms or particles; moderate, 102 to 104 organisms or particles; high, 1 to 102 organisms or particles.

[d] Includes enteropathogenic, enterotoxigenic, enteroinvasive, diffusely adherent, and enteroaggregative.

The primary source of waterborne pathogens is thought to be fecal pollution from humans and other animals, with transmission of disease being determined by the concentration of the pathogen and an individual's natural resistance (i.e., infectious dose). Prior to being imbibed or to contaminating food which is subsequently ingested by an individual, waterborne pathogens may travel a number of routes through the environment that include outfalls from sewage treatment plants, septic tanks, meat packing plants, and dairy waste lagoons. More diffuse sources of fecal contamination include storm runoff from urban and rural land that could transfer fecal matter from wildlife, domestic pets, and farm animals into surface waters. Given such a diverse range of sources of fecal contamination, contamination is watershed specific, although there are differences in the major groups of organisms that primarily contribute to disease in developed and developing nations. For example, in the United States, enteric viruses and protozoan parasites contribute to the majority of waterborne-illness outbreaks, whereas in developing countries where helminths are endemic, they are considered the major pathogens of concern in improperly reused and drinking water (14).

TREATMENTS EMPLOYED TO REDUCE OR ELIMINATE PATHOGENS FROM WATER

Given the large number of pathogens that could potentially be present in water, it is not surprising that there would be dramatic variations in their elimination from contaminated water, as they exhibit inherently different properties (e.g., size, nature of protective outer layers, and physicochemical surface properties). Hence, a variety of chemical, physical, and biological treatments are employed to treat contaminated water sources. In order of increasing treatment level, these include preliminary, primary, secondary, and tertiary (or advanced). Since the first three levels of treatment are actually considered water pollution control requirements (steps that would need to be taken regardless of any additional treatment), tertiary treatment is the distinguishing step for producing reused water (9). However, depending on the specific reuse application and associated water quality requirements, the degree of treatment required varies. Details as to the specific treatments that are currently employed to remove microbial contaminants are provided in the following sections, along with the effectiveness of each process. Treatments that are applied solely to remove organic contaminants are not covered. In any event, prior to establishing the treatment requirements that are to be applied for pathogen removal, an understanding of the pathogen occurrence in the untreated water source is essential. To accomplish this task, pathogen analysis should be conducted over a period of time, with care taken to include consideration of seasonal variation and storm events. When

the occurrence and distribution of human pathogens in a water source, such as wastewater, are not available, concentrations may be inferred from observational data on fecal concentrations that are being contributed to the wastewater.

Preliminary and Primary Treatments (Particulate Removal) Applied to Water

Chemical coagulation and flocculation with aluminum (2 to 5 mg/liter) or iron salts (4 to 10 mg/liter) are very important steps to remove colloidal particles and turbidity from wastewater (13). Such processes are known to aggregate wastewater constituents within the size ranging from 0.1 to 10 □m (15) by mechanisms of charge neutralization, adsorption, and entrapment (16–18). However, effective operation of the coagulation process does depend on an optimum coagulant dose that is a function of the raw water quality and pH (13).

Generally, sedimentation is applied prior to filtration to remove the bulk of the solids floc, with horizontal flow and floc blanket clarifiers being the most common forms applied. Alternatively, in dissolved-air flotation, fine air bubbles are dispersed into the floc. The solids within the floc then attach to the air bubbles, which float to the surface of the tank, where they are removed periodically as a layer of sludge. In general, coagulation and sedimentation is an ineffective process for *Cryptosporidium* removal. In support of this statement, there was only a 0.6- to 0.8-log removal when oocyst-seeded water was subjected to coagulation and sedimentation in bench-scale studies (19).

Table 41.3. Effectiveness of filtration for removal of pathogens from coagulant-treated water[a]

	Minimum–maximum reduction (log)	
Pathogen group	Slow sand filtration[b]	Rapid granular filtration[c]
Bacteria	2–6	0.2–4.4
Parasites	0.3–>5	0.4–3.3
Viruses	0.25–4	0–3.5

[a] Adapted from Table 7.7 in reference 13.

[b] Dependent on presence of schmutzdecke (biological film that develops on the surface of the filter), grain size, flow rate, and operating temperature and pH.

[c] Dependent on filter medium and coagulation pretreatment.

To improve the removal of microorganisms following coagulation and sedimentation, the treated water is then filtered (13). Two major types of filtration based upon residence time are used in wastewater facilities. Slow sand filtration is highly efficient for *Cryptosporidium parvum* removal (Table 41.3), with typical flow rates of 0.1 to 0.4 m3/m2 • h, using fine sand with an effective size of 0.15 to 0.4 mm. However, limitations in land requirements prevent many facilities in North America from using slow sand filtration. Alternatively, the majority of facilities operate rapid filtration

processes that require considerably less space. With flow rates of 5 to 30 m3/m2 • h through sand or multimedia with an effective size of 0.5 to 2 mm, rapid filtration is much less efficient than slow filtration for removal of parasites and other pathogens (20). The process, on the other hand, does remove nonmicrobial organic constituents that would otherwise reduce concentrations of disinfectant applied during secondary treatments.

Secondary Treatments (Disinfection) Applied to Water

Chlorine

An important component of many treatment regimens for removal of pathogens in water is chemical disinfection. As the most common type of chemical disinfectant, chlorine is typically applied at dosages ranging from 5 to 15 mg/liter, with a recommended exposure time of 30 min to 2 h (9). Applied either as liquefied chlorine gas, sodium hypochlorite solution, or calcium hypochlorite granules or through on-site chlorine generators (13), dissolution of chlorine in water forms hypochlorous acid (HOCl) and hypochlorite ion (OCl^-). Together, the concentrations of HOCl and OCl^- are termed free-chlorine concentrations. HOCl is a more effective biocide than OCl^- but dissociates into OCl^- at pH values greater than 7 (21). With respect to the mechanism of inactivation, chlorine reacts with both cell membrane components and nucleic acids, causing lysis and irreversible damage.

Three techniques of chlorination may be employed for disinfection. The first technique, termed breakpoint chlorination, is intended to provide a dose wherein there is sufficient chlorine to oxidize both microbial and non-microbial organic matter present in the water (designated chlorine demand) but leave a suitable amount of free residual chlorine to protect the water against reinfection from the point of chlorination to the point of use. Superchlorination followed by dechlorination also achieves the same objective as breakpoint chlorination in being able to oxidize all organic matter, but it does so with an excess of chlorine to rapidly achieve this result followed by reduction of the excess chlorine residual. The final method of chlorination, termed marginal chlorination, is typically applied to water supplies of high quality. In this method, chlorine is dosed to a desired level of free residual chlorine, as the chlorine demand is very low (13).

Regardless of the method used to achieve chlorination, the efficacy of chlorine and other chemical disinfectants is predicted based on knowledge of the residual concentration of disinfectant, temperature, pH, and contact time. This relationship is commonly referred to as the C_rT concept, where C_rT is the product of C_r (the residual concentration of disinfectant, measured in milligrams per liter) and T (the disinfectant contact time, measured in minutes). Therefore, a concentration of 1 mg/liter residual chlorine applied for 30 min corresponds to a C_rT value of 30 mg•min/liter (6).

An important characteristic of an effective disinfectant is the ability to persist in the system and protect against contamination that might occur at a later time. Consequently, to improve this property, ammonia may be added to the system, either concurrently with, prior to, or following chlorine addition to form monochloramines (NH_2Cl), dichloramines ($NHCl_2$), or trichloramines (NCl_3). Monochloramines are preferred and may be maximized by controlling the chlorine-to-ammonia ratio and pH (22). However, despite their persistence, chloramines are not as potent a disinfectant as free chlorine, as evidenced by the observation that a C_rT for free chlorine outperforms the same C_rT for chloramines (6).

Disparities exist in the inactivation of pathogens by chlorine, with bacteria being the least resistant to chlorine, viruses being more resistant, and parasite ova and (oo)cysts being the most resistant. Even among viruses, there exists a wide variation in their susceptibility to free chlorine, with rotavirus being less resistant than hepatitis A virus, which in turn is less resistant than norovirus. For example, when human volunteers were used to measure infectivity of noroviruses, a 3.75-mg/liter dose of chlorine was effective against rotavirus but failed to inactivate norovirus. More importantly, concentrations commonly used in disinfecting drinking water failed to inactivate norovirus (23).

In contrast to bacteria and viruses, which are susceptible to chlorine, protozoan parasites are considered resistant. For example, the C_rT value for a 2-log inactivation of *C. parvum* by free chlorine is 7,200 mg•min/liter at pH 7 and 25°C (24). Moreover, the resistance of *Cryptosporidium*, *Giardia*, and *Cyclospora* to chemical disinfectants such as chlorine is consistent with the recovery of oocysts from drinking water treated at facilities performing properly (25–27). However, the limitations of chlorine for inactivation of protozoan parasites may be circumvented partially by applying a two-step disinfection process. For example, increasing levels of free chlorine pretreatment were shown to reduce the subsequent monochloramine concentration and/or contact time required for a given level of inactivation, thereby demonstrating synergism (28). Chlorine disinfection was also enhanced by the application of electroporation prior to the application of chlorine, creating pores in the walls of the organisms and thereby increasing the permeability of the organism to chlorine (29).

Application of higher chlorine doses to water is not a viable option to improve protozoan inactivation. Disinfection by-products, including trihalomethanes (THM), are formed between organic materials and chlorine, and these by-products pose risks of chemically induced cancer. To minimize their presence, regulations on maximum contaminant levels (MCL) are in force. The current MCL for total THM, the sum of the concentrations of chloroform, bromodichloromethane, dibromochloromethane, and bromoform, is 0.08 mg/liter (30). Interestingly, differences between groundwater and reclaimed water in their composition of dissolved organic matter (DOM) have been demonstrated. In native groundwater, DOM consists of natural fulvic acids, while DOM in reclaimed water is a mixture of aromatic sulfonates (degradation products from bacterial activity) and natural fulvic acids. DOM isolated from groundwater produced significant amounts of THM and total organic halogen, while

DOM in reclaimed water produced minimal amounts of THM and total organic halogen (31)

Chlorine Dioxide

Existing as an undissociated gas dissolved in water, chlorine dioxide (ClO_2) is a potent disinfectant. One shortcoming of this disinfectant, however, is its volatility: if it is left in an open tank, the concentration in solution will decrease slowly.

Penetrating the organism and reacting with vital amino acids in the cytoplasm to kill the organism, ClO_2 is generally considered to have a biocidal efficiency greater than that of free chlorine or monochloramine (32). Further, in contrast to aqueous chlorine, whose effectiveness decreases as pH increases from 6 to 9, the reactivity of aqueous ClO_2 does not change greatly from pH 6 to 8 and is only approximately 20 to 30% lower at pH 10 (32). As with other chemical disinfectants, inactivation with chlorine dioxide is dependent on temperature. In the case of *C. parvum*, C_rT requirements for inactivation were found to increase by an average factor of 3.4 for every 10°C decrease in temperature.

Chlorine dioxide is a relatively stable disinfectant and forms little or no halogenated by-products (33, 34). It does react with compounds such as humic and fulvic acids to form carboxylic acids and aldehydes in the parts-per-billion concentration range. However, the major inorganic by-product of the reaction of chlorine dioxide in water is chlorite, which poses no significant risk to human health.

Ozone

Another disinfection technology that is applied to water is ozone treatment. Although the mechanism of microbial inactivation by ozone is similar to that of chlorine, ozone is a more potent biocide than chlorine. In addition to reacting quickly with microbial and nonmicrobial substances in the water, another advantage of employing ozone as a disinfectant is that it is unaffected by the range of pH encountered in water treatments. However, ozone is applied to water as a gas, and volatilization losses make it difficult to maintain stable ozone levels under either laboratory or field conditions. Therefore, the performance of ozone operations relies on achieving the desired concentrations after a given contact period. Typically, a residual concentration of about 0.5 mg/liter after a contact time of up to 20 min is targeted. To achieve this target, application doses range from 2 to 5 mg/liter, with higher doses being needed for waters containing a high level of natural background organics (13). However, regardless of the applied dose, no residual ozone is present in the effluent, and therefore precautions must be taken to prevent recontamination.

Both bacteria and enteric viruses, including norovirus, are highly susceptible to ozone inactivation. For example, reductions of >3 log were achieved within 10 s when norovirus was exposed to 0.37 mg of ozone/liter at pH 7 and 5°C (35). More importantly, ozone is one of the few chemical oxidants capable of inactivating *C. parvum* under normal water treatment conditions, at least at warmer temperatures.

To exemplify the significance of temperature, the ozone inactivation rate constant for *C. parvum* decreased by a factor of 2.2 for every 10°C decrease in water temperature of laboratory waters, whereas in natural water, it decreased by a factor of 4.5 (36). Hence, the application of ozone to water by itself is not practical in cooler climates. Another factor that should be taken into account when the disinfection efficiency of ozone for protozoan parasites is estimated is the density of (oo)cysts. In a study conducted by Haas and Kaymak (37), when the initial microbial load was low, it was more difficult to achieve a given level of reduction than when it was high.

In addition to low temperatures, another obstacle to the use of ozone for disinfection purposes is the potential for the formation of bromate, a possible human carcinogen, when the raw water being treated contains bromide. Bromide concentrations greater than 50 g/liter may result in bromate formation at levels greater than the MCL of 10 g/liter. Bromate is particularly problematic because unlike many other organic by-products, it is not biodegraded in biological filters that usually follow an ozonation step. Treatment options to remedy this problem include ammonia addition, pH depression, hydrogen peroxide addition, hydroxyl radical scavenging, and scavenging or reduction of HOBr (38, 39). Ammonia interferes by reacting with HOBr, and even small ammonia concentrations show a positive effect. Unfortunately, the method is not efficient in waters that already contain medium to high levels of ammonia. Like ammonia, lowering the pH also acts to reduce the generation of bromate by shifting the $HOBr/OBr^-$ equilibrium towards HOBr. Once bromate is formed, however, its elimination is difficult, given that the most promising treatment, activated carbon filtration, had limited success in full-scale plants (38). Consequently, the potential for bromate generation and the diminished efficacy of ozone at low temperatures are likely factors that have contributed to the fact that only a limited number of commercial wastewater plants used ozone as their chemical disinfectant in the United States prior to 2010. However, it has been noted that there are several new ozone systems for wastewater disinfection under design, under construction, or recently put into operation as a result of improvements in ozone generation and dissolution technology (6).

Ferrate

Another oxidizing agent for disinfection processes that has generated a significant amount of interest is ferrate. Its chemistry results from the formation of iron in the plus-6 oxidation state (Fe^{6+}), but its key benefit is that by-product formation is minimized compared to that of chlorine. Although highly effective against bacteria and viruses, ferrate was projected to be less effective than ozone in the inactivation of protozoan parasites (40). Although recent advances have been made in on-site production of ferrate, making it more cost-effective, implementation of ferrate technology on a full scale has not occurred to date, due to difficulties associated with (i) the instability of ferrate, which is dependent on its method of preparation; (ii) the relatively low yield of ferrate; and (iii) the limited amount of research conducted to date demonstrating its capabilities and advantages over existing water and wastewater treatment methods (41).

UV Light

Since the early 1900s, UV light (wavelength between 180 and 320 nm) has been used to inactivate microorganisms in contaminated waters and is due to the absorption of UV photons by an organism's genomic DNA. While the damage does not kill the organism directly, it does result in the failure of the organism to reproduce and establish infection in the host.

There are several advantages to using UV as a disinfectant: (i) it is a physical process that does not rely on use of chemical additives, (ii) it requires relatively short contact times, and (iii) inactivation can occur without the formation of disinfection by-products. Although UV was believed to be an inadequate disinfectant against protozoa prior to 1998 (42), those initial findings were based on analytical methods, excystation and vital dye binding, that correlated poorly to animal infectivity assays. Since then, UV has proven to be highly effective in the inactivation of protozoa as it is for bacterial pathogens; however, adenoviruses, with their double-stranded DNA, are more resistant to UV disinfection, requiring ca.10 to 20 times the dose for the same degree of inactivation (Table 41.4) (43, 44). Given that UV doses commonly applied for water and wastewater treatment are between 30 and 40 mJ/cm2 (45), adenovirus would be projected to survive treatment.

Table 41.4. Comparison of effectiveness of UV disinfection for inactivation of several types of waterborne pathogens

Pathogen	UV dose (mJ/cm^2)	Log inactivation	Reference
C. parvum	10	2	48
	25	3	48
	2	1.5	167
	5	3	167
	3–9	3–3.5	168
Encephalitozoon intestinalis	6	>3.9	169
	9	4	169
Adenovirus type 40	103	2	170

UV disinfection performance and efficiency are impacted by two key water quality issues: the UV transmittance of the wastewater and the presence of particle-associated microorganisms. With both issues, particles (>10 μm) can shield microbes from UV light. Consequently, the effectiveness of UV light as a disinfectant is enhanced when water is filtered prior to disinfection by reducing the number of large particles with embedded and shielded microorganisms and by reducing the number of small particles that reduce UV light transmittance (6).

Another challenge with applying UV light as a disinfectant is the potential for photo repair of UV-inactivated microorganisms. Repair of UV-damaged cells has been observed when *Escherichia coli* was exposed to visible light (310 to 480 nm) (43). The

ability of bacteria to repair their damage may explain the ability of enterococci to survive the UV disinfection process and persist in biofilms of the distribution system in the absence of a chlorine residual (46). In contrast, when *C. parvum* was either exposed to fluorescent light or kept in darkness, UV-induced pyrimidine dimers in the DNA were continuously repaired; however, no recovery of animal infectivity was observed (47).

When compared on the basis of germicidal UV dose, there are no differences in inactivation of organisms treated with low- and medium-pressure UV radiation (48, 49). However, up to a decade ago, energy requirements and maintenance issues initially factored into their selection for small- and large-scale water systems, with small water systems favoring low-pressure lamps that had twice the germicidal efficiency (UV dose delivery per watt of input power) of medium-pressure lamps and large systems favoring the medium-pressure lamps, as less maintenance was involved because fewer lamps were required. Fortunately, in the past decade, substantial advances have been made in the UV industry with the development of low-pressure but high-output UV systems and microwave UV systems. These innovations allow installation of UV systems with reasonable energy use values, so that they are now more affordable than water systems employing sodium hypochlorite (6).

Another disinfection method of wastewater that has proven effective is exposure of the water to solar radiation, termed solar water disinfection. This method initially consisted of placing water in transparent plastic or glass containers which were then exposed to the sun from 6 to 48 h, depending on the intensity of the sunlight and pathogen sensitivity. In this case, the mechanism associated with germicidal activity was UV radiation, but this was supplemented by thermal heating of the water by solar light. As with exposure to UV radiation alone, waterborne pathogenic bacteria and protozoan parasites are inactivated extensively if not completely, whereas indications are that viral pathogens are more resistant to solar disinfection (50). Although its small scale limited its applications initially, significant efforts have been made to scale up the process using both static and flow-through systems. Consequently, as a result of its continued simplicity and low cost, the method is in daily use throughout the developing world (Asia, Latin America, and Africa) (50).

Multiple Disinfectants

In several disinfectant processes (i.e., ozone and UV light), no residual disinfectant is present in the treated water, and hence, regrowth of pathogens may occur. In those instances, many drinking water systems also apply chlorine or monochloramine to provide protection in the distribution system after primary disinfection has occurred. Surprisingly, the inactivation kinetics of *C. parvum* oocysts with these secondary agents after ozone pretreatment were shown to be significantly faster than the kinetics with free chlorine and monochloramine alone. For example, rates of secondary inactivation were 1.1 to 2.8 (chlorine) and 2.4 to 9.2 (monochloramine) times higher than the rates of corresponding primary inactivation with these agents at 30 and 10°C, respectively (51). Given that ozone is relatively ineffective for

inactivating *C. parvum* at low temperatures (see “Ozone” above), sequential disinfection with ozone plus monochloramine or ozone plus chlorine during winter in regions in which the water temperature is low may be a viable approach.

Other disinfectant treatments applied sequentially or in tandem have also proven to be synergistic in their inactivation of pathogens. For example, inactivation of *Giardia* was much greater when disinfection with chlorine and UV light was applied sequentially to wastewater (52). Similarly, enhanced inactivation of UV-resistant adenovirus was obtained when 10 mg/liter hydrogen peroxide was added to either ground or surface waters treated with UV light (53). Application of low concentrations of chlorine (1.5 mg/liter) was also beneficial in effectively inhibiting the photoreactivation of *E. coli* exposed to UV light (54).

Tertiary Treatments

Membrane Treatments Applied to Disinfected Water

Increased emphasis on improving treatments for reuse has led to major research efforts being undertaken and to the development of commercial membranes (thin films separating two phases and acting as selective barriers) for removal of microorganisms, trace organic substances, specific ions, and dissolved solids (9). Filter types used for removal of microbial contaminants along with pore sizes are listed in Table 41.5.

Table 41.5. Membrane filter type characteristics[a]

Filter type	Nominal pore size (μm)	Microbial contaminants targeted for removal
Microfiltration	0.05	Some protozoan (oo)cysts, bacteria, and viruses
Ultrafiltration	0.002–0.050	Most bacteria and some viruses
Nanofiltration	<0.002	Some viruses
Reverse osmosis	<0.002	Some viruses

[a] Adapted from Table 6-6 in reference 6.

To drive the separation and removal of microbial contaminants and other organic macromolecules/colloids, pressure is generally used, with the pressure and associated energy requirements increasing as the pore size decreases. Consequently, in striving to make these membranes more economical or provide better value, manufacturers of membranes are constantly developing new filtration technologies or modifying their existing technologies to improve filter performance by increasing the hydraulic loading rates or increasing water quality (55). Another issue that both manufacturers and users should be cognizant of is membrane integrity. Scanning electron microscopy showed that gaps or pores present in reverse osmosis membranes were

sufficiently large for MS2 viruses to pass through and were responsible for the observed variability in virus removal (56). Hence, to minimize the impact of any manufacturing defects or variability in membrane pore sizes, it is critical that water systems employ a multibarrier approach in their operations.

For water disinfection using membranes, it was long assumed that the most critical parameter is the pore size. More recently, it was shown that pore size can be greater than the particle size for significant retention, as both sieve retention and adsorption sequestration play a role in particulate removal (57). In the case of viruses, removal is greatest at pH values approaching the isoelectric point (pI) where their equal distribution of positive and negative sites leads to a maximum virus-virus coagulation and increases the size of the particle and potential for rejection. In natural environments, however, many different types of viruses are present and their pIs can vary over a broad range. Under these conditions, when viruses are in mixed environments, they tend to coagulate not only with the same virus but also with different viruses (58).

Fouling in membrane treatment systems represents one of the most significant barriers to their more widespread implementation in both municipal and industrial wastewater treatment facilities (55). Four key factors impact or contribute to fouling, most of which are interrelated: (i) process configuration; (ii) membrane material and configuration (or geometry); (iii) process operation, including system hydrodynamics; and (iv) biomass concentration and composition. Membrane fouling may be physical, inorganic, organic, or biological. To alleviate or minimize fouling by suspended solids, pretreating the source to reduce its particulate density is the first recommended course of action. The second course of action is to operate below the so-called “critical flux,” and this level may be increased by more vigorous aeration and/or by periodic backflushing. It is important to keep in mind that limited amounts of fouling may serve to also strain the aqueous solution and thus remove organisms that are smaller than the membrane’s pore size (59).

Application of Water to Systems (Wetlands, Lagoons, and Soil) for Biological Inactivation of Pathogens

Constructed wetlands and lagoons represent low-cost biological systems that have been documented to reduce microbial loads of wastewaters (60). The primary outlets for water reclaimed through such systems have been mainly agricultural and landscape irrigation, groundwater recharge, indirect reuse as potable water, and environmental reuse. In these systems, biodegradation of organic materials and pathogen reduction are intricately connected to the interactions of the various organisms making up the biological community. Biological factors influencing survival of pathogens include natural die-off due to starvation and competition with other microorganisms, predation by microinvertebrates, and the microbicidal effects of toxins, antibiotics, and enzymes produced by organisms in the biological community. Constructed wetlands also utilize the additional biological activity of plants. By providing additional surface area for microbial growth, plants increase

microbiological activity and thus improve the biological reaction hydraulics. In addition, the oxygen released into the subsurface by wetland plants is used for subsurface aerobic metabolism and nitrification-denitrification (61).

Constructed wetlands exhibit characteristics that are similar to those of natural wetlands in that they support similar vegetation and microbes. However, with both natural and constructed wetlands, design and operation may limit their effectiveness. In the first place, the water being treated must reside within the wetland long enough for the biological activities to have a noticeable effect. Thus, when a stabilization pond with a high retention time was used to treat water, up to 100% of *Giardia* cysts were removed when the initial concentrations ranged from 0.23 to 100,000 cysts/liter (62). In contrast, removal of pathogens (*Cryptosporidium* spp., *Giardia duodenalis*, and microsporidia) was significantly lower in constructed wetlands with free-surface flow than wetlands constructed with subsurface flow, as the former did not provide sufficient time for remediation (63). Another critical component for efficient operation of lagoons or stabilization pond systems is removal of sludge buildup. In the absence of this maintenance task, the removal of helminths was overestimated (64). The last two limitations to biological systems are that they operate in uncontrolled environments where seasonal effects have been shown to affect attenuation of *Cryptosporidium* oocysts in lagoon systems (65) and that wildlife has been shown to contribute a substantial load of pathogens to wetlands (63).

Soil aquifer treatment systems are another low-technology system; wastewater is applied to basins of soil consisting of layers of loam, sand, gravel, silt, and clay. Such systems are coarse enough to allow sufficient infiltration rates but fine enough to provide adequate filtration. During this process, resident pathogens may be removed from the water stream by straining or adsorption, with subsequent inactivation occurring via desiccation stress or indigenous microbial activity. In the case of viruses, adsorption is considered the major mechanism affecting their survival and transport, which in turn is affected by the soil's pH, organic matter content, aeration level, soil structure and texture, soil surface charge, soil porosity, and virus size and surface charge (66). For instance, viruses with higher isoelectric points consistently correspond with increased adsorption. Also, soils with high isoelectric points, such as most clay types, have been shown to be better virus adsorbers than those with low isoelectric points, such as most of the sands. In contrast, high levels of organic and surfactant in water will reduce virus adsorption by competing with the virus for binding sites (67). However, one drawback of virus adsorption to soil particles is that the latter protects the former from proteolytic enzymes or other virus-inactivating substances of the indigenous soil microflora (68).

While soil may effectively strain protozoan (oo)cysts, it is not an effective medium for inactivating them. In fact, depending on the soil type and temperature, *C. parvum* oocysts may survive from several months to years in agricultural soils. For example, significantly greater inactivation occurred in silt loam soil than in silt clay loam, whereas at temperatures between 4 and 30°C, rates of *C. parvum* oocyst inactivation increased with increasing soil temperatures. Freezing may also have lethal effects.

Within 50 days, 99% of *C. parvum* oocysts exposed to soils that are frozen are inactivated (69).

REGULATIONS AND GUIDELINES FOR REUSE OF WATER IN FOOD SYSTEMS

Internationally, there are no comprehensive enforceable regulations or even guidelines (recommendations with limited enforceability) for water reuse; hence, different countries have developed different approaches to managing the process. These approaches basically follow one of two sets of guidelines, the World Health Organization (WHO) guidelines, first issued in 1989, or California's Title 22 effluent reuse guidelines, first issued in 1992. One of the major differences between these guidelines is that a much lower coliform density is specified in the California guidelines. The second major difference is that WHO specifies limits for intestinal nematodes, while California does not (70). In general, the WHO guidelines are less stringent than the California guidelines, and their requirements can be met by low-technology, lowcost, and controlled-risk treatments, such as holding in stabilization ponds. In contrast, the California criteria rely on more expensive but reliable wastewater treatment systems. Although these two approaches have served as the baseline for guidelines, countries that are actively engaged in evaluating the safety of their reused water systems using the more stringent approach have diverged in their recommendations for monitoring the treatment systems. For example, the U.S. Environmental Protection Agency (EPA) guideline suggests that MS2 coliphage (virus that infects coliform bacteria) be used for on-site validation (6). In contrast, the Australian guidelines recommend that indigenous *E. coli* be monitored mandatorily and coliphages or other pathogens such as adenovirus or enterovirus also be monitored as representative of viral contaminants (71).

As for federal oversight in the United States, the EPA guidelines, first published in 1992, were based on the California model and specify complex treatment processes as well as stringent water quality parameters (72). Since publication of that document, additional versions have been published that include itemizing regional variations of water reuse in the United States, providing new case studies from around the world highlighting how reuse application can and does work in the real world, and updates on advances in wastewater treatment technologies relevant to reuse (6). Coincident with these treatment advances has been the inclusion of guidelines directed at inclusion of appropriate disinfection processes that are capable of reducing or inactivating pathogens (e.g., enteroviruses) to low and acceptable levels (6).

Within the United States, federal regulations for water reuse do not exist; however, the federal regulations related to the Clean Water Act and the Safe Drinking Water Act must be followed (i.e., water quality standards must be no less stringent than federal standards). As a result, each state and city in the United States is responsible for its own reuse policy; this has led to dramatic diversity in those policies between states. However, among these variations is one common theme: a higher level of treatment is

advocated when the public has direct exposure to the reused water. Within this theme, though, states may differ as to whether they have specified different categories or grades of water quality based on the intended use.

Other major differences among the approaches taken by individual states are associated with the degree of specificity provided in the rules. For example, the states of California, Florida, Texas, and Washington have developed more specific water quality criteria for specific treatments, whereas other states, such as Hawaii and Virginia, have developed water quality criteria only on a case-by-case basis (73). More specifically, the Water Development Board of Texas proposed that water reclamation plants achieve a log reduction value (LRV) of 12 for viruses when the treated water is for direct reuse as potable water (74). Performance targets specified by California's Groundwater Replenishment Reuse Project, on the other hand, were more expansive, including not only the LRV of 12 for viruses but also an LRV of 10 for both *Giardia* and *Cryptosporidium* (oo)cysts when the effluent was to be used for groundwater recharge intended for indirect reuse as potable water (75). Regulations in different states also differ with regard to the standard methods being recommended for monitoring the level of efficacy achieved by the water treatment systems. For example, several states use total coliforms as the indicator organism, whereas other states use fecal coliforms. Given that performance targets for viruses and parasites are now also being included in the reused-water guidelines of some states, monitoring of viral indicators and protozoan parasites is implicit in those states (6).

Although the proportion of water used by the food processing industry is relatively small compared to the demands incurred by the community and other industries, high-quality water is typically used. In addition, food companies produce a significant amount of effluent that must be treated before it is released back into the environment. Consequently, many food businesses are looking for ways to conserve water through reuse and thus make their operations more profitable and models of good environmental stewardship. Towards that goal, 20 years ago, delegates from a number of countries sought to draft universal guidelines on water reuse for the food processing industry, but after multiple attempts, the consensus was that a general guidance for all operations was not possible. Instead, it was recommended that guidelines for water reuse should be developed for specific commodities, given the wide variation in water contamination that arises with different processing operations and the intended target purpose for reused water (76). Following that report, an International Life Sciences Institute task force concluded that instead of classifying only two categories of water (i.e., potable and nonpotable) for the food processing industry, the desired degree of water quality should be a reflection of its suitability or level of public health risk associated with its intended use. Therefore, to implement such a concept, a risk management framework in conjunction with application of a hazard analysis and critical control point (HACCP) system was considered an appropriate approach (77). In this manner, identification of acceptable levels of risk could be assigned to specific treatment options, and more stringent treatments that would meet stricter standards would not be necessary.

MONITORING OF PATHOGENS AND INDICATORS IN REUSED WATER

To assess risks associated with potential contamination of reused water, it is essential that an adequate monitoring and verification system be in place to demonstrate the level, if any, of contamination both in source water and after each treatment step. This precaution means that log reduction data provided by designers or manufacturers of treatment systems cannot be assumed to be valid; some objective empirical evidence of performance is required. An assortment of tools is available to assay specific pathogens; however, this is not typically a viable option for routine monitoring, as methods to detect low levels of pathogens are often costly and time-consuming. Alternatively, surrogates or indicator microorganisms (nonpathogenic organisms that are present in water at higher levels than pathogens and respond to stress and treatment conditions similarly to pathogens) are routinely used as measures of contamination and success of treatment processes. Examples of indicator organisms that have been investigated over the years include coliforms, fecal coliforms, *E. coli*, enterococci, bacteriophages, and *Clostridium* spores. However, a review of the literature that examined correlations between 14 potential indicator organisms and 18 waterborne pathogens (540 datasets published from 1970 to 2009) found that there was not a consistent correlation for any one indicatorpathogen pair, as some studies provided evidence of a correlation whereas others did not (78). Hence, in the absence of any one indicator being appropriate for all water conditions that could be encountered, investigators are now pursuing the potential for combinations of microbial indicators to be used as predictors of the quality of reused water (79).

Other low-cost measurement tools that are employed to provide early warning of failures in water treatment processes during operation are termed sentinel parameters, with the measurement employed depending upon the contaminant of interest and the treatment process being monitored. Examples of sentinel parameters include (ii) elevated particle counts or turbidity for filtration and UV treatments; (ii) conductivity, chloride, and ozone residual levels for chemical disinfectant treatments; and (iii) total organic carbon or transmembrane pressure for reverse osmosis treatments (80). However, inherent to the routine use of these process parameters or others that may be proposed is that they correlate with pathogen reductions that occur during the treatment(s). Hence, to accomplish that verification but circumvent the low and variable levels of pathogens that would be present in the water to be treated, challenge testing is often conducted using a “spike cocktail” added to the water system. Zimmerman et al. (81) identified the constituents which they believe should be present in such a cocktail and include both pathogens that are known to be resistant to some types of disinfection (adenovirus, enterovirus, norovirus, *Cryptosporidium* spp., and *Giardia* spp.) and indicators (enterococci, *E. coli*, somatic coliphage, F-RNA coliphage, anaerobic sporeformers such as *Clostridium* spp., and aerobic sporeformers such as *Bacillus* spp.) that have been used in previous research and survey studies. Employing such a large arsenal of targeted organisms is therefore

expected to help us develop a better predictive understanding of pathogen and process indicator attenuation in treatment systems.

Over the years, the field of pathogen and indicator detection has evolved dramatically. Traditionally, methods encompassed culture-based enumeration of bacterial pathogens and fecal indicators as well as concentration and detection of viruses and parasites by microscopy. Major limitations of these assays included an inability at times to discriminate between the targeted pathogen or indicator and other microbiota of the water sample, false-positive counts, laborious and time-consuming protocols, and occasional failure in the detection of viable but noncultivable bacterial pathogens cells (82). Then, pathogen detection shifted to molecular assays, where the organisms were disrupted and extracted nucleic acids were subjected to quantitative PCR (qPCR), fingerprinting, or sequencing (83). However, these assays are also fraught with limitations, one of the chief ones being that the presence of inhibitors in water (e.g., humic and fulvic acids) interferes in the amplification of the nucleic acids and reduces the sensitivity of the assay. To provide an alternative solution to this limitation, third-generation PCR technology, known as droplet digital PCR (ddPCR), has been developed to provide absolute quantification of the pathogen target genes with greater sensitivity and accuracy. As evidence of the success of this method, ddPCR was able to detect pathogen-specific genes (*Salmonella* spp., *Campylobacter jejuni*, and *Listeria monocytogenes*) in more pathogen–sampling-time combinations than either qPCR or culturing methods when poultry processing water samples with high organic loads were collected from final scalder and chiller tanks (84). Although water did not serve as the source of contamination, ddPCR has also been proven to be a promising method for detection of hepatitis E virus and *Cryptosporidium*, as ddPCR is less affected by inhibitors than qPCR (85, 86).

Prior to the arrival of ddPCR, preconcentration was developed to increase the sensitivity of qPCR for lowcost detection of waterborne pathogens. During preconcentration, sensitivity is enhanced by increasing the number of target organisms per unit volume. However, the drawbacks to this approach are the additional time it requires, and, depending on the method of preconcentration, there is the possibility of partial losses in the target organism (87). For example, highly turbid waters will clog filters and not only decrease the amount of water that can be effectively filtered but also lower the efficiency of the filter to capture the target organism. Hence, a method not affected by turbidity that has been applied to the collection and concentration of protozoa is flocculation. Using calcium carbonate as a coagulant, the (oo)cysts quickly settle to the bottom of the container, making this approach easy to perform and inexpensive (88). Unfortunately, viability of the *Cryptosporidium* oocysts recovered using this concentration method is suspect, as misshapen oocysts have been observed (89).

Alternatively, immunomagnetic separation is considered a more reliable method of isolation and concentration of pathogens. The method utilizes antibodies linked to the surface of magnetic particles to attach to (oo)cysts, bacterial cells, or viral particles, which are then selectively concentrated from surrounding matter using magnetic materials. Caution should be exercised with immunomagnetic separation, as lot-to-lot

variation in the specificity of commercially available fluorescent antibodies to *Cryptosporidium* oocysts has been documented (90). In addition, in the case of *Giardia*, a marked decrease in recoveries of cysts aged 20 months was noted and was attributed to declining amounts of surface-expressed antigens on aged cysts (91).

When the efficiency of wastewater treatment in reducing the pathogen populations is being evaluated, published results must be interpreted with caution. In the first place, the majority of studies have based their conclusions only on fecal indicators, and rarely has any study considered the infectivity of the pathogen. However, analyses directed at viability and infectivity of waterborne pathogens also have their limitations. For example, not only has high variability been noted in the 50% infective dose of *Cryptosporidium* for mice, but also the animal is not infected by the human-specific species *Cryptosporidium hominis* (92). Hence, infectivity assays based on *in vitro* culture were developed (92) and continue to serve as the primary assay for viability of protozoa. Over the years, other assays have also been promulgated as valid measures of viability for waterborne pathogens but have fallen short. For example, reverse transcriptase PCR targeting amplification of mRNA proved unreliable, as there were instances where the nucleic acid was not degraded soon after the inactivation treatment was applied (93, 94). In the meantime, new approaches to the detection of viable and infectious pathogens have been made, and many of those advancements have been covered in recently published reviews (82, 95–99). However, caution should be exercised in the adoption of any one of these novel approaches until it has been thoroughly vetted in multiple treatment systems and with various water qualities and conditions. To illustrate this point, the dyes ethidium monoazide and propidium monoazide, which intercalate into nucleic acids and inhibit PCR amplification, were able to differentiate viruses that were inactivated by chlorine but not inactivated by UV or thermal treatments (100). In any event, it is important to be cognizant of the method that was used in any study exploring the prevalence and fate of waterborne pathogens, as the results may be biased and not accurately portray the status of the system.

EXAMPLES WHERE TREATED WATER IS REUSED IN FOOD OPERATIONS

Production Systems

The generation of wastewater is expected to increase worldwide due to urbanization and increases in population. As such, treatment of wastewater to generate reclaimed water can be beneficial now and in the future for its capacity to be used as a source of water for irrigation of crops in the face of recurring drought and overwithdrawal of freshwater sources worldwide (6, 10). Furthermore, the use of reclaimed water for crop production offers several advantages. In the first case, the use of reclaimed water for irrigation can lessen the burden on wastewater treatment plants in their effort to minimize discharges to streams and rivers. Another advantage of using treated

wastewater for crop irrigation is the residual nutrients that are present, which allows the water source to be used as a liquid fertilizer by crops.

The quality of reclaimed water used for food production varies widely and depends on the intended use. In developed countries, regulations of reclaimed-water use for food crops are very stringent to protect public health and often require tertiary or secondary treatments with additional disinfection or filtration steps (6). However, in developing countries, the use of untreated or inadequately treated wastewater is common (101).

In the United States, California and Florida lead the way in using reclaimed water for production of crops, accounting for 90% of the total (6). One example of successful use of reclaimed water in California is in the Salinas Valley of northern Monterey. There, a water recycling project was initiated in response to excessive groundwater withdrawal, which was causing salt water intrusion in the valley. As part of this project, a reclamation facility and distribution system were built (102) that subsequently delivered reclaimed water to several thousand acres of land where farmers were growing raw and ready-to-eat food crops (lettuce, broccoli, cauliflower, fennel, celery, strawberries, and artichokes). Not only has the reclaimed water met California's highly restrictive microbial water quality criteria (<2.2 [most probable number] total coliforms/100 ml), but results from a multiyear pilot project have demonstrated that the crops are microbiologically safe for consumption.

Another successful example of a reclaimed-water system for crop irrigation is a system that was initiated in 1986 as a collaborative effort between the City of Orlando and Orange County, Florida (http://www.waterconservii.com/). In response to a need to expand wastewater treatment services and eliminate discharge to surface waters, the project has been operating free of charge by providing treated wastewater to irrigate 2,737 acres of citrus annually. The project collaborates with the University of Florida to monitor the potential impacts of reclaimed water on food safety and the environment. As a result, the city of Orlando and Orange County guarantee in a tour brochure (http://www.waterconservii.com/wp-content/themes/divi-child/dls/Tour_Pack.pdf) that their product is a "highly treated reclaimed water that is free of virus and fecal coliform and meets all the requirements of Citrus Irrigation Reclaimed Water Constituent Concentrations."

A large number of other examples of reclaimed water projects exist in the United States, beyond those in California and Florida, and several of these are detailed as case studies in the EPA document entitled "Guidelines for Water Reuse" (6). In addition, many countries, such as Israel, Spain, Italy, Cyprus, and Australia, that are found in arid and semiarid regions of the world use reclaimed water for irrigation of food crops extensively.

Despite the advantages promoted for reclaimed water, there are studies that suggest that irrigation of crops with reclaimed water could lead to contamination of those crops with pathogens. In those cases, some risk to human health from consumption could occur if the crop is not thermally treated. For example, in an Italian field study, both surface and subsurface irrigation with wastewater subjected to tertiary treatment

resulted in contamination of tomatoes and eggplants with fecal coliforms and fecal streptococci (103). The worst contamination occurred when the vegetables came into direct contact with the soil or plastic mulch. In a similar field study in Spain using wastewater subjected to secondary or tertiary treatment, lettuce, parsley, and tomato plants were positive for the opportunistic pathogen *Aeromonas* spp. following irrigation (104). Nevertheless, other studies of irrigation with reclaimed water have failed to detect pathogens in the harvested crop or have detected a substantial decrease in the prevalence of contamination of the crop. For example, solar disinfection reduced *E. coli* concentrations in wastewater effluents from $>10^3$ to 10^4 CFU/ml to <2 CFU/ml, and in turn, 26 of 28 lettuce samples were negative for the indicator after being irrigated with the treated water (105). In another study, Lopez-Galvez et al. (106) examined the prevalence of *E. coli* and enteric viruses in wastewater that had been subjected to secondary and tertiary treatments and was then used for zucchini production in a commercial hydroponic system in Spain. Despite the presence of *E. coli* and enteric viruses (norovirus and hepatitis A virus) in the reclaimed water, these contaminants were not detected on zucchini. Likewise, in a Cyprus field study, irrigation of tomato plants with water that had undergone tertiary treatment did not result in microbial contamination (107). Meanwhile, field studies in Israel also failed to identify irrigation of tomatoes with wastewater subjected to secondary or tertiary treatment as the source of produce contamination with several bacterial, viral, or protozoan pathogens (108).

The lack of detection of pathogens or indicator organisms on crops does not necessarily indicate an absence of contamination, since, as discussed above, different methods have different limits of detection. Moreover, many studies do not address the possibility that pathogens may be internalized either by uptake through roots or by migration into wounds (i.e., cut tissue), stomates, or hydathodes (109). These limitations are particularly troublesome when low levels of pathogens persist, as there is a danger of regrowth of those pathogens (104). Additional discussion on the issue of pathogen regrowth is found in “Pathogen Regrowth” below.

In general, surface or subsurface drip irrigation systems that minimize contact between reclaimed water and the edible part of the crop are assumed to be safer than spray or sprinkler irrigation systems (110). Greenhouse or hydroponics-based production systems might also be considered safer than open-field operations, as they afford better control to minimize cross-contamination (110). However, hydroponically grown crops have a greater propensity to internalize pathogens from contaminated water via their root system than plants grown in soil-based systems (109). Hence, due to this multitude of factors that could affect contamination of crops following application of treated wastewater, risk-based analysis is recommended to provide some perspective of the overall risk to public health. Additional discussion on this topic is found in “Risk Assessment” below, along with a table describing risk assessment outcomes when reclaimed water is used as the irrigation source.

Direct consumption of reclaimed water by livestock is not common in the United States (6). However, *de facto* (incidental and unintended) consumption is possible

when treated wastewater is introduced to surface waters upstream of areas where domestic animals drink (111). Indirect exposure also occurs when the animals graze on land irrigated with treated wastewater. Furthermore, the use of treated wastewater to grow animal feed is a common practice (112, 113). In this case, the EPA's guidelines still recommend that the wastewater undergo some form of secondary treatment and disinfection. However, the guidelines and regulations for use of reclaimed water on such crops are less strict than those for crops that are for human consumption. The main concern associated with use of reclaimed water for animal feed crops or pastures is the potential that farm workers or the public may have direct or indirect exposure. Hence, some setback distances between an irrigated field and either publicly accessible areas or potable-water supplies are also recommended. Higher microbial standards also exist for land irrigated with reclaimed water and used for grazing by milking animals than for non-milking animals. In those situations, there is often also a requirement that there be a 15-day delay between grazing and application of the reclaimed water.

Processing Systems

Water plays a valuable role in the food and beverage industry. For example, in addition to the potable water that may be incorporated into a product, other operations utilizing water include (i) cleaning and sanitation; (ii) steam and hot-water generation for processing, transport, and cleaning of food products; (iii) equipment cleaning; (iv) package container (bottles, cans, cartons, etc.) cleaning; (v) bottle conveyor belt lubrication; and (vi) processed-product warming and cooling. Different food processing sectors require different amounts of water to function, but in general, to minimize the amount of water used and wastewater generated, the first order of business should be to critically evaluate each operation as to whether water is required at the level at which it is being used. For example, plant complexity, scale of the factory, the age and type of processing, utilization of batch or continuous processes, the efficiency and simplicity of methods used for cleaning of equipment, and water management have all been cited as contributing to the amount of water used in the dairy industry (114). However, as cleaning operations are the major activity consuming water at milk processors (28%), water conservation efforts would have an impact. For example, instead of using water to dislodge materials from the floor, they could be vacuumed or scraped off the floor.

Once conservation measures have been implemented, reconditioning treatments are the next option for consideration to minimize levels of water used in a food processing operation. Fortunately, the knowledge of water reuse principles by both the public and industry has grown and in turn has precipitated the growth in the food and beverage processing industry's reuse of highly treated process water that meets water quality criteria for a specific operation and addresses public health concerns. Furthermore, implementation of reused water in food processing operations generates both social and economic benefits. More specifically, reuse of water leads to water conservation, and hence, a manufacturing site may be classified as "green." In addition, although there are costs associated with treatment, the overall operating costs are generally

reduced in that less potable water needs to be purchased from an outside source and there are reduced expenses incurred for wastewater disposal.

The quality of the water demanded for food processing operations will vary with each specific operation, and therefore, determining whether reused water is suitable for that operation will depend on the quality of the used water, the recovery and distribution method, and the treatments applied. Given that the wastewater from different operations likely has different levels of organic and microbial contamination, keeping these streams of wastewater separated is beneficial from the perspective of efficiency, in that each wastewater stream needs to be subjected only to treatments that would achieve the minimum standards for the use for which it is intended (115).

As expected, application of reused water in food processing facilities has primarily been directed at non-food related and cleaning uses, cooling functions, and fire extinguisher purposes, as these processes would not require that the applied treatments achieve potable-water quality. However, within the dairy, poultry, and vegetable/fruit industries, the use of reconditioned water in their manufacturing practices is now being allowed (116–118). In the case of poultry processing wastewater, regulations require that the treatment processes reduce microorganism concentrations (coliforms, *E. coli*, *Salmonella*, and total aerobic microbes) by at least 60%. The regulations also stipulate that the treated water have a light transmission at 500 nm of at least 60% of that of fresh water used in the process. After those standards are met or exceeded, reconditioned water is allowed to replace water in the chilling step in certain predetermined ratios based on the level of treatment efficacy. The reconditioned water may also be used for moving heavy solids in eviscerating troughs, scalders, tanks, feather flow-aways, and picker aprons and for washing the picking room floors (119). However, reused water that came into contact with raw product prior to treatment should not be allowed to come into contact with ready-to-eat product, on the assumption that pathogens residing in the used water survive the treatment.

As for published case studies investigating reused water in food processing operations, Casani et al. (120) present multiple examples in their review. More recent research studies are summarized in Table 41.6; however, only about half of the studies that are listed examined the efficacy of the treatment(s) with regard to the microbial safety of the water. In any event, regardless of the application of reused water, the principles of HACCP should be adhered to in order to control any potential hazards and prevent them from entering the food processing environment. In addition, continuous monitoring, audits, and frequent sampling of the reused water are required (3, 120).

LIMITATIONS TO REUSED/RECONDITIONED WATER BEING INTRODUCED INTO FOOD SYSTEMS

Aerosols

Another exposure mode by which farm workers and nearby residents can potentially be introduced to health hazards associated with irrigation of wastewater or reclaimed water is via inhalation of or skin contact with aerosolized pathogens (121). For the most part, incidences of diarrheal diseases and other health problems that are attributed to aerosols are associated with irrigation using untreated or inadequately treated wastewater, a practice that is common in many developing countries (101, 121). In those cases, exposure to aerosolized pathogens can be minimized by using nonspray irrigation systems (i.e., subsurface, drip, or furrow irrigation). However, several studies have demonstrated that when wastewater is properly treated, aerosolized pathogens do not appear to pose a significant health risk to farmers or nearby communities. For example, both dermal and nasal swabs from farm workers were negative for *Staphylococcus aureus* and *Enterococcus* after the workers were exposed to wastewater that had undergone tertiary treatment and had been spray-irrigated onto a field (122). Another observation from that study to indicate that there was minimal risk from aerosolized pathogens was that the farm workers exposed on those spray-irrigated properties had the same odds of infection with the two target pathogens as did office workers who had no exposure to the irrigation water.

The degree of risk associated with aerosolized pathogens is likely dependent on a pathogen's size and, hence, its ability to be aerosolized. For example, Courault et al. (123) detected low levels of enteric viruses in air above agricultural fields that received wastewater that had undergone tertiary treatment; however, higher levels were observed in cooler months. Despite these observations, the risk of norovirus exposure for the employees who were most exposed at the treatment plant generating the reclaimed water was judged to be acceptable. Based on this assessment, the risk of exposure at the farm that received the reclaimed water from the treatment plant would also have to be considered minimal.

Cropland Runoff

The largest non-farm-related use of reclaimed water in the United States is urban use (e.g., golf course and landscape irrigation) (6). In Florida and California, urban use accounts for 55% and 19% of the total share of reclaimed water use, respectively. This form of utilization can lead to cross-contamination of croplands through runoff after irrigation or rainfall. Part of the strategy to control urban runoff is the design and implementation of best management practices that address several factors, ranging from the selection of irrigation systems to better control water delivery to the use of physical barriers to slow and filter out pollutants (124, 125). The use of vegetative buffer strips, for example, has been shown to be effective in reducing microbial loadings from runoff to water bodies (126, 127).

Table 41.6. Selected research studies addressing processes and treatments applied to food processing wastewater for the purposes of water conservation

Water source	Treatment(s)	Targeted reused-water application	Results and observations	Reference
Poultry chiller water	Electroflotation (electrolytic gas generation)	Chiller water	Aerobic bacteria and coliforms were reduced by 6 and 5 log CFU/ml, respectively	171
	Membrane bioreactor (1st tank is an aerobic bioreactor, 2nd tank is an anoxic tank for settling particles, 3rd tank has tubular module membranes for ultrafiltration); emphasis on backflushing to decrease fouling	Chiller water	Aerobic microbes were reduced by 4 log CFU/ml	172
	Hollow fiber or spiral ultrafiltration using polymeric membranes	Chiller water	Aerobic microbial levels were reduced by more than 5 log CFU/ml	173
Beverage bottle-washing wastewater	Filtration and/or nanofiltration	Washing bottles	Reduction of 98% in chemical oxygen demand	77
Dairy whey	Combined ultrafiltration and reverse osmosis unit	Clean-in-place systems	47% of water can be recovered; generates protein and lactose by-products	3
	Combined ultrafiltration, reverse osmosis unit, and a reverse osmosis polisher unit	Combined ultrafiltration, reverse osmosis unit, and a reverse osmosis polisher unit	Near-infrared spectroscopy monitors urea in reused water so it may be stored for more than 24 h before application	174
Malting wastewater	Microfiltration	Reused in steeping	Treated water has acceptable	77

from barley soaking		operations	microbiological populations	
Produce wash water	Ultrasound (20 kHz, 0.28 kW/liter) in combination with peroxyacetic acid (3.2 mg/liter)	Washing produce	Log reductions of 6, 6, and 1.7 for *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* after 11, 11, and 20 min, respectively	175
	Ultrasound (26 kHz, 41.85 kW/liter) in combination with UV-C light (1.64 kJ/m2)	Washing produce	Log reductions of 3.6 for total aerobic bacteria and 30% and 79% reduction in suspended particles and chemical oxygen demand, respectively	176
Beet sugar wash water, condensate; barometric condenser discharge	Mechanical cleaning to remove soil organic matter	Beet washing, transport and cleaning, juice extraction, cooling towers, cleaning operations	Demand for potable water during production has decreased to nearly zero	77

Disruption to Indigenous Soil Microflora

Irrigation with treated wastewater can disrupt the indigenous soil microbial communities directly through introduction of microorganisms that are not common in the soil. In a comparison of diversity between treated wastewater and agricultural soil, bacteria in three phyla and 15 orders were found to be unique to treated wastewater (128). Application of this treated wastewater can therefore introduce exogenous microorganisms, including pathogens resistant to the treatment, to those soils. However, it is not clear how many of the microorganisms introduced in this manner persist in the soil system to impart long-term disruptions to the indigenous microorganism composition. To date, studies to address the fate of microorganisms present in the reclaimed water have primarily ignored nonpathogenic microorganisms and instead have focused on only pathogens because of their potential impact on food safety and human health. Based on those studies, pathogen survival in soils varies widely but is dependent on soil properties that include texture, pH, temperature, moisture, organic matter, and interactions with the indigenous microbial

communities (129, 130). Hence, it is likely that many of the same factors influence the survival of newly introduced nonpathogenic microorganisms, which in turn impacts the previous dynamic status of the soil microbial community.

Irrigation with treated wastewater can also disrupt the indigenous soil microbial communities indirectly through the addition of organic matter and nutrients. Such disruptions to the microbial community status occur when introduction of the supplements causes growth of copiotrophic microorganisms (131). For example, Bastida et al. (132) noted that the abundance of *Proteobacteria* and *Bacteroidetes* increased with application of treated wastewater. Similarly, in a study where an orchard soil was irrigated with treated wastewater for 6 years, the relative abundance of *Actinobacteria* decreased, with a corresponding increase in the relative abundance of *Gammaproteobacteria* (133). Interestingly, the change was temporary, as the relative abundance of the microorganisms reverted to the original proportion after the irrigation was stopped, highlighting the resilience of the indigenous microbial communities to the disruption. However, extended durations of irrigation with treated wastewater appear to continue to exert their effect on the microbial community. For instance, in Spain, irrigation with treated wastewater spanned 43 years and resulted throughout that time in a lower diversity of arbuscular mycorrhizal fungi (AMF) than irrigation with freshwater (134). This response was not unexpected, as enrichment of the soil environment with wastewater-derived nutrients and organic matter would be to the benefit of copiotrophic microorganisms. AMF, on the other hand, are adapted to living in environments where nutrient levels are relatively low. The proliferating copiotrophic group would therefore have competed with the AMF and reduced their diversity.

Disruption of the indigenous soil microflora may also occur upon long-term application of reclaimed water due to soil's accumulation of heavy metals (e.g., Cu, Zn, Pb, and Ni) and salt (128, 135) found in the water source. Buildup of either of these constituents has been shown to cause a decrease in microbial abundance, activity, and diversity (136, 137). Another disadvantage to the accumulation of heavy metals in soils is that they have been implicated in coselection of antibiotic-resistant microorganisms in soils (138). The drawbacks to that situation are discussed in more detail in the next section.

Antibiotic Resistance

The extensive use of antibiotics in humans has led to the prevalence of antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) in wastewater (139). As a result, this prevalence is a major concern due to the potential for the transfer in the environment of ARGs from nonpathogenic microbes to pathogenic microbes that can cause disease in humans. Unfortunately, treatment of wastewater does not eliminate ARB and ARGs, and significant amounts are still present. To illustrate this point, Carey et al. (140) reported the prevalence of *Enterococcus* spp. that were resistant to multiple antibiotics in treated wastewater that was being used for irrigation at three sites in the United States. Moreover, significant amounts of

tetracycline resistance genes were also present in the chlorinated effluent. In other cases, chlorination and UV light have been shown to exacerbate the problem by increasing the relative proportions of bacterial isolates that were resistant to multiple antibiotics (141, 142). Such exacerbation was also demonstrated in a laboratory study by Zhang et al. (143). In that study, sublethal levels of commonly used disinfectants (free chlorine, chloramine, and hydrogen peroxide) enhanced the conjugative transfer of ARGs between *E. coli* strains and between *E. coli* and *Salmonella enterica* serovar Typhimurium, highlighting the potential role of chemical disinfectants in the spread and prevalence of ARGs. The investigators in that study speculated that the residual amounts of disinfectants led to formation of reactive oxygen species that caused increased membrane permeability, thereby facilitating the conjugative transfer of ARGs. In any event, given that there is the potential for bacterial regrowth (see the next subsection) as well as continued transfer of ARGs during distribution and storage prior to irrigation, monitoring treated wastewater at the point of use is imperative (140).

Based on the data demonstrating that treated wastewater can act as a reservoir of ARB and ARGs, it is reasonable to assume that they would be transferred to soils during irrigation. However, once transferred, selection of certain types of ARGs may occur, creating a soil with its own resistome (collection of ARGs). As an example, irrigation of six urban parks with treated wastewater in Australia significantly increased the abundance and diversity of selected ARGs in the soils (144). However, the creation of a different soil resistome upon application of reclaimed water is not certain, as was demonstrated when Israeli agricultural soils were irrigated for up to 16 years with freshwater or treated wastewater (145). During that time, no significant differences in ARB and ARGs were noted for soils irrigated with the two different water sources, and those results highlight the probability that the resistome is dependent on the ARB and ARGs that are introduced to the soil system and their stability in that system.

Pathogen Regrowth

When processes applied to wastewater fail to remove most or all of the organic material, such material can serve as a nutrient source for microbial and pathogen growth if the treated water is stored for any length of time. Hence, to measure the concentration of nutrients that could support bacterial growth, reused water samples are typically subjected to the assimilable organic carbon (AOC) bioassay. In the original assay, the water sample was heated to kill the indigenous bacterial population before inoculation of the sample with *Pseudomonas fluorescens* and then incubation of the mixture at 15°C to achieve maximum growth. Using an empirically derived yield coefficient, the results were then converted into acetate equivalents (146). Generally, water can be defined as biologically stable if its AOC value is less than 10 to 20 □g of acetate C/liter without disinfectant, or less than 50 to 100 □g of acetate C/liter if it was disinfected (147). When values are higher, bacterial regrowth is likely during distribution and storage. As confirmation that AOC supports microbial regrowth, AOC reductions were observed simultaneously with regrowth in reused water and distribution systems (148).

One of the drawbacks to the original AOC assay protocol was the long incubation periods that were necessary before maximum growth was achieved (1 week for high AOCs and up to 1 month for low AOCs) (146). Consequently, an ATP luciferin-luciferase method was adopted as a rapid AOC method which incorporated higher incubation temperatures and greater inoculation levels (149). Another recent extension to the assay has been to expand from evaluating the growth response of just one bacterial strain to inclusion of nine bacterial strains, as these strains exhibited unique substrate utilization patterns that offered a better assessment of differences in biodegradable organic matter that could be present in different reused water samples (150).

Although differences in the initial levels of nutrients present in the wastewater source may affect the subsequent AOC levels observed in treated water, the type of treatment also has an impact. For example, wastewater that had been treated with ozone resulted in reused water with AOC concentrations higher than those in reused water that had been treated with reverse osmosis (148). The potential for microorganisms to grow in nutrientfortified reused water systems also varies with the organism. For example, waterborne indicators such as *E. coli* and *Enterococcus* were infrequently detected and only at low concentrations, whereas opportunistic pathogens, such as *Aeromonas*, *Legionella*, *Mycobacterium*, and *Pseudomonas*, occurred more frequently (151, 152). Several variables may impact the regrowth potential of enteric and opportunistic pathogens, including (i) differential reactivation of viable but not culturable populations (153); (ii) differential response to residual disinfectant (i.e., chlorine) levels (154); (iii) selection of chlorine-resistant pathogenic bacteria, such as *Salmonella* (154); (iv) level of movement of reclaimed water during distribution or storage (high potential for generation of cyanobacteria blooms and subsequent generation of toxins); and (v) levels of indigenous competitive bacteria present in the water system. With regard to the latter variable, reactivation and regrowth of indigenous microflora were most likely to occur when the organisms were exposed to lower chlorine doses during treatment and decreased as the chlorine concentration increased (154).

Public Opinion

It is important to recognize that public acceptance of reused water is vital to utilization of this resource. More than 20 years ago, a survey of 500 respondents in Manama, Bahrain, was conducted and found strong opposition to using reused water regardless of the conditions. In fact, the respondents were willing to pay more to avoid using it (155). However, in that survey as well as ones conducted more recently with 400 participants in Arizona (156) and 2,800 participants across the United States (157), support increased as the intended application became less personal (i.e., less chance for bodily contact with the reused water). Acceptance of reused water was also greater with the segment of the population that had a higher level of education (155–157). Sex and age, on the other hand, showed no significant effect on the acceptance of water reuse (157). Surprisingly, drought conditions did not have a statistically significant effect on the number of reused-water supporters but did increase the

number of respondents who identified water shortages as an environmental problem (157).

In Australia, studies have found that the degree of acceptability is dependent on the type of water to be reclaimed. Rainwater harvested from one's household roof was considered more acceptable than gray water, which in turn was more acceptable than wastewater. Factoring into the acceptance of any one source, however, was the level of treatment that was to be applied to that source (158).

One of the most important factors that influence public acceptance is the perceived health risk of reused waters (159). In addition to concerns regarding the presence of pathogens and the unknown impact of chemicals used to treat the water, the public has also targeted the potential presence of endocrine-disrupting compounds in wastewater. Although these risks are real, the public's perception as to the degree of risk associated with each concern does not necessarily coincide with scientists' judgments on the matter. In fact, the public views the risks in more abstract terms, incorporating attributes such as uncertainty, dread, catastrophic potential, controllability, and equity into their risk equation (160). Moreover, when the public does not fully understand the situation, they also use their social and moral values, otherwise known as outrage factors, to evaluate the situation (161). In the end, water reuse surveys have documented that people are more trusting of their own intuition than of the authorities that monitor and regulate the process and product or the results of peer-reviewed scientific research (158, 162).

On many occasions, communities have acknowledged that there was a psychological barrier derived from the thought of using reclaimed water; hence, terminology is critical in the success in promotions of reused water. For example, survey respondents who totally rejected "reclaimed water" associated it with the terms "filthy" and "unclean," despite the fact that reclaimed water has been treated to high standards for removal of pathogens. People still perceive the water to be disgusting because it has been in contact with disgusting stimuli, in this case, human waste. Opponents of the reuse concept, particularly in relation to applications requiring potable water, have also capitalized on terminology to promote their viewpoint, using the phrase "toilet to tap" (156). Consequently, acknowledging the importance of terminology, projects that have investigated applications of reused water have attempt to avoid terms that imply treated wastewater. In Singapore, reused water was called NEWater, and in San Diego, it was referred to as repurified water. However, Rock et al. (156) reported that even the terms "recycled water" and "repurified water" were still judged negatively by a small percentage of those surveyed, as these terms still implied that the water had already been in use (163).

As noted above, the level of acceptance by the public varies and depends on the type of reuse advocated. Opposition is often fueled by unfounded fears, and to alleviate those fears, it has been advocated that an emphasis be placed on keeping the public fully informed and providing deliberative consultation (163). For example, a task force in Elsinore, CA, conducted 16 meetings with the public over a 7-month period before supplementing Lake Elsinore with recycled water. Similarly, the success of a water

recycling program at Irvine Ranch, CA, was attributed to a commitment to inform and educate the local community through public tours, in-school education, and outreach and community education that reused water is a means to protect the environment and save money and energy. However, public outreach and education have not always led to favorable outcomes; sometimes, they polarize the public even further (162). Furthermore, when implemented after the project's conception, education and outreach programs are generally considered inadequate. When reclaimed-water projects have been implemented in spite of unsuccessful education and outreach programs (e.g., Singapore's NEWater project, implemented in early 2003), the governmental systems tend to be more authoritarian (163).

Beyond efforts to educate the consumer about the technology used to reclaim used water, the extensive oversight of the industry, and the benefits of reused water, other implementation strategies have been presented to consumers for their views on whether the public would receive them favorably. In response to these inquiries, financial incentives (i.e., rebate programs and reduced water rates) were considered likely to increase the public's support for reused-water applications (156, 157). Increasing the costs to treat water to higher standards was also considered a strategy that municipalities should pursue (156). In contrast, there was divided support for mandatory programs (156), illustrating the common behavioral tendency that people will support and believe in what should be done, but opposition arises once the intended change is mandated (164). As support and acceptance of reused water varied with the intended use, it was suggested that implementation within communities should focus on applications with greater social acceptability (157). It is anticipated that when the public becomes more familiar with the technology and the general benefits of reused water, acceptance of reused water for other end uses (e.g., crop irrigation) will increase.

RISK ASSESSMENT

To estimate the likely burden of illness associated with ingestion of a specific waterborne pathogen in a specific reuse capacity, quantitative microbial risk assessments (QMRAs) are conducted, wherein experimental data are combined with mathematical models and statistical probabilities. As multiple hazards (pathogens or microbial toxins) may be present in a source water or wastewater system, one of the first considerations is to define representative organisms whose control is believed to represent control of all pathogens of concern. In some cases, consideration should also be given to the pathogen serotype present. For example, the risk of using water in a manner that creates aerosols (e.g., irrigation) is generally higher when the water is contaminated with adenovirus 2 than when adenovirus 41, a pathogen associated with gastroenteritis, is present (97).

Ideally, at least one bacterium, virus, protozoan, or helminth should be included in a QMRA to account for the wide array of potential pathogens that could be present in the water system. However, in actual practice, it has been more common to measure indicator organisms, as discussed in "Monitoring of Pathogens and Indicators in

Reused Water" above. This distinction in target organisms is important, as QMRAs using indicator organisms have been misleading due in large part to the differences encountered between the responses of indicator organisms and those of pathogens to treatments and environmental stresses before ingestion (165). Notwithstanding this limitation, some of the other parameters that serve as components in a QMRA include (i) pathogen populations within the water source, (ii) volume of water deposited on food, (iii) potential for regrowth in stored water systems, (iv) assessment of exposure levels by different segments of the population (quantities of food ingested at one time or over a period of time), and (v) susceptibility of the population segment to the waterborne pathogen (dose-response assessment). Additional information on these parameters as well as the systematic steps that should be taken in developing a QMRA are found in documents published by the USDA/EPA (166) and WHO (13).

Once QMRAs of water systems are conducted, collected data are translated into a probability of infection per year and then into an estimated disease burden per case, for which the metric disability-adjusted life year (DALY) is used. This metric takes into consideration not only the risk of illness but also the differential burden that illness entails in low-versus high-income regions. Therefore, to put everybody on the same footing, WHO has identified a health outcome target of 10^{-6} DALY per person per year (13). Another benefit of conducting QMRAs is the ability to understand the effects that various operational parameters may have on overall risk and thus enable the establishment of performance targets. For example, a risk-based goal of 10^{-4} infections per person per year is frequently used as the basis for developing pathogen removal targets. However, the determination as to whether a water quality intervention will be implemented will also likely depend on the relative level of improvement that could be expected (e.g., a decrease in disease burden of 5% or more would be considered extremely worthwhile) (13).

One scenario where QMRA is starting to be applied more routinely is for assessing the safety of irrigating vegetables with treated and untreated wastewater (Table 41.7). Comparison of the annualized risks of infection in the reported studies to the acceptable WHO target of 10^{-6} suggests that the public's risk of infection is dependent on the target pathogen as well as the level of treatment applied to the wastewater. Hence, in cases where the recommended risk level is exceeded, additional treatment of the water is advised to further reduce pathogen levels. Alternatively, management practices may also be modified such that interactions between the water and the edible portion of the crop would be minimized.

CONCLUDING REMARKS

Treatment of wastewater and applications of reclaimed/reused/reconditioned water in food operations on a global scale are relatively small. The main factor driving the adoption of reclaimed water has been water shortages in arid regions. Many other operations are attempting to implement the application of reused water to avoid the impending strain on our water resources. However, as discussed in this chapter,

microbiological safety risks are associated with the use of reclaimed waters treated to different extents. Future adoption of reclaimed water in food systems will be dependent on public opinion, which in turn will likely be dependent on the success or failure of education and marketing efforts to inform the population of the benefits and risks of this product. Assisting in those efforts will be the use of risk assessment tools (i.e., QMRA) that will ultimately relate specific treatments, costs, and applications to the risk of illness.

Table 41.7. Selected examples of QMRAs conducted to assess the microbial risk from consumption of ready-to-eat food crops that were overhead-spray irrigated with wastewater or reclaimed water

Location	Target crop(s)	Water treatment(s)	Target pathogen(s)	Annualized risk of infection (DALY/person/year)	Reference
California	Lettuce	Chlorine disinfection followed by filtration	Multiple	10^{-7} (enterovirus) 10^{-5} (*Giardia*) 10^{-4} (*Cryptosporidium*) 10^{-8} (*E. coli* O157)	177
Spain	Lettuce	Sedimentation and activated sludge (secondary); flocculation with iron chloride followed by filtration, UV treatment, and chlorination (tertiary)	Norovirus	10^{-4}(secondary) 10^{-4} (tertiary)	178
United ArabEmirates	Lettuce, cabbage, cucumbers	Chlorine disinfection	*Salmonella*	$>10^{-6}$	179
Beijing, China	Chinese chard, broccoli, flowering cabbage, lettuce	None	Rotavirus	$>10^{-6}$	180
Shepparton, Australia	Lettuce, broccoli, cabbage, Asian vegetables, cucumbers	Wastewater stabilization ponds	Norovirus	$>10^{-6}$	181

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Relevance of Food Microbiology Issues to Current Trends (2008–2018) in Food Production and Imported Foods

Tremendous strides have been made over the past 3 decades to improve the microbiological safety of the United States food supply. Through the efforts of our scientific, industrial, and regulatory communities, these improvements have occurred in part because of increased fundamental knowledge of foodborne pathogens and an increased ability to detect and control these pathogens. However, we live in a world that is never static, and as part of that dynamic, a number of current factors will undoubtedly influence the continuing evolution of the safety of foods. One such factor is the application of whole-genome sequencing (WGS) by the public health community for detection of foodborne-illness outbreaks and identification of the causative food and its source of contamination. Another consideration is the dynamic change of consumer attitudes for the types of foods that are preferred, which will influence the manufacture and marketing of foods, some of which may be at increased risk of pathogen contamination and growth. An additional contributor will be the recent regulatory changes that are providing rules on approaches needed to produce safe foods but with minimal adverse consequences in providing a sustainable and affordable food supply.

ADVANCES IN TOOLS FOR FOODBORNEDISEASE SURVEILLANCE AND RISK ASSESSMENT

Advances in WGS and Other Epidemiologic Tools on the Horizon

Approximately 2 decades ago, pulsed-field gel electrophoresis and multilocus sequence subtyping methods were introduced to identify foodborne-illness outbreaks and to link clinical isolates from outbreaks with sources of the implicated pathogens. However, these molecular profiling techniques are laborious, time-consuming, and difficult to standardize among laboratories. Moreover, they rely on pure cultures and are therefore challenged by the increasing use of culture-independent diagnostics in clinical laboratories (1). The adoption of WGS, which reveals the complete DNA composition of an organism, is revolutionizing the public health and food safety fields. This revolution is occurring because WGS, in contrast to traditional molecular typing methods, provides much greater resolution in differentiating temporal and spatial clusters of genetically related pathogens (2).

As more researchers and public health agencies have adopted the technology of WGS, sequencing speed has increased, and the acquisition and operational costs of high-throughput sequencing have decreased. Consequently, PulseNet at the U.S. Centers for Disease Control and Prevention (CDC) and public health agencies in other countries (e.g., Canada and Australia, as well as the continent of Europe) are transitioning from PFGE to WGS typing for foodborne-illness outbreak surveillance. Facilitating these efforts are multiple databases that house the gene sequences of thousands of foodborne bacterial pathogens. Examples of these databases include the U.S. GenomeTrakr database (3), the Canadian Syst-OMICS database (4), the European Microbial Identifier database (5), and the Advanced Research Infrastructure for Experimentation in Genomics database. Given that WGS has permeated major public health agencies throughout the world, these efforts are coordinated through steering committees that seek to harmonize data collection and develop policy decisions regarding how the data will be shared. Moreover, to facilitate the adoption of this technology by developing countries, such as Brazil, those countries are forming partnerships with the World Health Organization and the United Nations' Food and Agriculture Organization. In such collaborations, guidance is offered to those countries to enable them to use WGS technology and the available databases.

The U.S. Food and Drug Administration (FDA) has initiated a process (informally described as a "swab-athon") whereby, during its food facility

inspections, approximately 150 to 200 environmental samples are obtained and tested for pathogens such as *Listeria monocytogenes* and *Salmonella*. The pathogens that are obtained are then subjected to WGS, and their sequences are entered into the GenomeTrakr database. Not only can this information help to identify which preventive or sanitary controls may have failed and need to be corrected, but also, it may reveal the route by which contamination occurred. In addition, when cases or outbreaks of foodborne illness are detected, comparisons are made with sequences in the GenomeTrakr database to determine if there are matches that could be linked to the source, such as a food processing facility. Hence, WGS data are being used to aid in foodborne outbreak investigations (6).

Examples of active and retroactive epidemiologic investigations of illness outbreaks in which WGS has been useful are listed in Table 42.1. Additional examples of WGS's role in epidemiologic investigations of foodborne outbreaks involving atypical vehicles are described by Kase et al. (7). They describe how WGS was instrumental in identifying sources (e.g., pistachios and flour) of outbreaks that had long epidemic curves and likely would not have been detected if other molecular techniques had been used. In addition to determining the source of an outbreak of foodborne illness with greater speed and precision, WGS technology can help differentiate illnesses that are part of an outbreak from those that are not, identify which ingredient in a multi-ingredient food is the cause of the outbreak, and differentiate the sources of contamination within the same outbreak, and it can link small numbers of illnesses, including those distributed widely across several states, that would not likely be detected as an outbreak.

With the large amount of molecular data obtained from WGS analyses, additional uses of this information are being applied. For example, WGS genomic data may be able to predict whether a bacterium is resistant to antibiotics and, in so doing, help us to better understand how bacteria become resistant to antibiotics and how resistance spreads (8). However, Ellington et al. (9) noted that, to date, accurate inference of antimicrobial susceptibility from WGS data has been either poor or nonexistent and suggested that more research and data sets will be needed before it can replace phenotypic antimicrobial susceptibility testing.

Another application for WGS data is the detection of phylogenetic relationships between outbreak strains; such phylogenies may then be used to establish evolutionary relationships and spatial spread or geographic associations among different strains (10, 11). Furthermore, geographical information systems and geospatial mapping of illnesses could complement this application of WGS data (12–14). For example, Wang et al. (13) suggested that by automatically uploading WGS data for clinical isolates from hospitals and clinics throughout the world with "geotagged" metadata, there is the potential to revolutionize diagnostics and characterization of foodborne disease agents at the patient level. Another benefit of WGS is to use specific genomic regions of foodborne disease agents identified by WGS to develop rapid and cost-effective PCR assays (15).

WGS data may also be used to characterize bacteria beyond the species and/or subspecies level. For example, the White-Kauffmann-Le Minor scheme has been the gold standard for serotyping of *Salmonella* isolates and is based on serological detection of expressed O (somatic) and H (flagellar) antigens. Alternatively, *Salmonella* serovars may be identified through WGS by focusing on genetic differences in the somatic and flagellar genes and subjecting the data to analysis by one of several software programs (16, 17). This approach has been adopted by Public Health England, which uses multilocus sequence typing as a replacement for traditional serotyping of *Salmonella* (18). Moreover, this approach to serotyping is not limited to *Salmonella*, as serotyping of *Escherichia coli* using WGS data has been shown to be a superior alternative to the conventional typing method (19).

Table 42.1. Use of whole-genome sequencing to analyze foodborne-illness outbreaks[a]

Pathogen	Vehicle(s)	Results
E. coli STEC	Flour	WGS analysis conducted by FDA demonstrated that *E. coli* O121 isolates obtained from open flour product samples in multiple states were closely related to outbreak strains obtained in 2016. Subsequent testing by a third-party laboratory on nonrecalled flour samples at the behest of the implicated company revealed three STEC strains (O103, O26, and O111), of which one (O26) was subsequently identified as closely related genetically to a clinical isolate. WGS characterization of the remaining isolates obtained from the flour samples did not lead to identification of any additional clinical cases (119).
Salmonella Typhimurium	Multiple	Retrospective evaluation of five distinct foodborne-illness outbreaks in Australia. In four of the outbreaks, WGS confirmed that the human and environmental isolates epidemiologically linked by MLVA typing either were identical or differed by one or two SNPs. Moreover, additional isolates initially not considered to be linked with the outbreaks were determined to belong to the outbreaks, increasing the total outbreak size by 107%. In the fifth outbreak, isolates differed by up to 12 SNPs and thus suggested that the outbreak was caused by more than one strain (120).
L. monocytogenes	Caramel apples	Using WGS, environmental testing revealed contamination with *L. monocytogenes* at the implicated firm's apple-packing facility, and these isolates were highly related to the outbreak strains (121).

	Soft cheese	Five PFGE patterns of *L. monocytogenes* were initially included in the investigation; however, WGS revealed that these strains were closely related genetically (122).
	Ice cream	Outbreak-associated isolates with multiple PFGE profiles were determined to cluster together by WGS, and these were differentiated from epidemiologically unrelated isolates that exhibited outbreak PFGE profiles (123).

[a] Examples of recent outbreaks where whole-genome sequencing has been used as a tool to link an outbreak to a food source either during an investigation or to retrospectively evaluate its efficacy compared to traditional epidemiologic tools used in the investigation. STEC, Shiga toxin-producing *E. coli*; WGS, whole-genome sequencing; MLVA, multilocus variable-number tandem-repeat analysis; SNP, single nucleotide polymorphism; PFGE, pulsed-field gel electrophoresis.

Virulotyping is yet another opportunity to apply WGS data. Through the identification of a pathogen's genes that contribute to varying degrees of virulence and hence illness, virulotyping may provide a system to differentiate those isolates. Such a typing system would avoid the overly conservative approach that currently exists, in which all members of a particular bacterial genus (e.g., *Salmonella*) or species (e.g., *L. monocytogenes* and *Cronobacter sakazakii*) are considered equally pathogenic by public health and regulatory agencies.

Tools complementary to WGS are also being used to enhance the safety of foods. Examples include metagenomic and metatranscriptomic approaches, whereby either the entire DNA or the cDNA (created from RNA extracted from an entire community) is sequenced and used to identify and characterize baseline microbial communities along the food supply chain for the purposes of detecting safety and quality anomalies (20). It is also envisioned that meta-analysis (statistical combination of results from multiple studies, including those based on WSG) will be a common application in the near future (13).

Current Limitations of WGS and the Need for International Data Harmonization

Despite the wide appeal of employing WGS in research and epidemiologic food safety investigations, there are two fundamental challenges to

routine application of WGS: sensitivity and the determination of viability (21). Pathogen contamination of foods is often characterized by variability in the type of the target organism present, heterogeneity in distribution of the microorganisms within the matrix, and the presence of low levels of the target microbe. As a result, platforms developed for health care systems that rely on large numbers of pathogens often cannot be easily adopted for foods unless the microbial contaminants are grown to large populations to produce sufficient DNA for analysis (20).

Widespread adoption of WGS is also currently restricted by limited resources, e.g., financial resources and the availability of skilled personnel. These restrictions include not only the relatively high costs of WGS assays but also insufficient expertise in bioinformatics analysis as well as inadequate levels of computational power needed to process and analyze the large sets of sequenced data (22). Different groups employing different platforms, data analysis pipelines, and terminology of contextual data (23) have also contributed to the lack of harmonization and restrictions in the sharing of data. Hence, international efforts to address this issue are under way (2, 20, 23).

CONSUMER TRENDS THAT CAN AFFECT THE MICROBIOLOGICAL SAFETY OF FOODS

The scientific, industrial, and regulatory communities as well as consumers believe that under ideal conditions and given enough resources, the food supply could be made completely safe. However, available resources are not the only factor influencing the safety of foods. Rather, consumer preferences are another very important factor that can directly influence the microbiological safety of foods. From the perspective of food production, consumers through their food preferences largely dictate what foods are produced and manufactured, since the industry focuses much of its food manufacturing on the foods which most people prefer. However, there are niche markets in part due to fringe population groups interested in unique foods because of health, cultural, or religious heritage reasons or because they avoid certain traditional foods, such as meat. Some of the foods they desire, especially those with no antimicrobial preservatives or pathogen inhibitors, are challenging, if not impossible, to make microbiologically safe, as

extended shelf lives and some degree of consumer temperature abuse are factors that are likely to complicate safe food production.

Consumer Response to Food Recalls

Although everyone's goal is to always produce safe foods, the occurrence of human errors and the limitations of many food safety technologies make it impossible to produce a food supply that is completely safe. Hence, food recalls often occur and may arise in response to an outbreak or because of contamination in samples that are tested for pathogens. Food recalls result not only in extra costs to the food industry related to recovering, disposing of, or reconditioning the contaminated food product already in the market pipeline but also in additional economic repercussions when concerned consumers respond by avoiding the product. Pozo and Schroeder (24) determined that the degree of media coverage and whether a company has an orchestrated plan to promptly offset any negative publicity will often determine the magnitude by which consumers' responses affect sales. As an example, it was reported that the American Egg Board's $1 million media campaign designed to restore the image of eggs in consumers' minds was successful, as consumers' response by consuming eggs occurred more quickly than in other markets associated with similar food recalls (25). In addition to media coverage, other factors, including prior experiences and overall concern for food safety, influence how individuals respond to food recalls (26, 27).

A survey conducted 10 years ago revealed that 80% of responding consumers thought that food recalls were increasing, yet over 50% of the respondents greatly underestimated the actual number of recalls that were occurring annually (26). Despite these concerns, 12% of those responding also reported they knowingly ate a recalled food, with 57% of them indicating they ate the food because they did not believe it would harm them. An explanation for this behavior was that these consumers were suffering from optimistic bias and underestimated their own vulnerability.

Although consumers may be lackadaisical about recalled product they have already purchased (i.e., they do not want to throw it away or do not want to be bothered to return it to the store), consumers are more apt to not purchase the recalled branded product in the near future. For example, sales of recalled frankfurter brands declined roughly 22% 2 to 3

months after the recall and did not return to near pre-recall levels until 4 to 5 months later (28). Consumers are also known to adjust their purchase habits beyond the recalled brand or commodities to nonrecalled brands or other closely related commodities (27). This has occurred for both meat and poultry products (29). However, these trends in consumer responses to recalls may shift as different age groups become the dominant force in the marketplace. For example, it was observed in a survey that millennials remembered on average two or fewer recalls in the previous year than the older generation, which remembered two to five recalls (27). This disparity may relate to the different sources by which the two groups learn about that recall information (i.e., nonmillennials generally rely on television and newspapers, whereas millennials obtain their news primarily through social media networks, instant messaging, and texting). Moreover, even when presented with the same information, the different population groups can have different interpretations. For example, in the Peake et al. (27) survey, approximately 30% and 10% of millennials and nonmillennials, respectively, indicated that they would react in a way which would be considered unexpected given the warnings that they had just read regarding a peanut butter recall.

Consumers' Response to Foodborne Illness Outbreaks

Studies have revealed that consumers' purchasing response to outbreaks associated with contaminated food products is more negative than that for recalled product that was not associated with any illnesses. Using Nielsen Homescan data, Bakhtavoryan et al. (30) determined that there was a decrease in the demand for peanut butter following the 2007 *Salmonella* outbreak associated with peanut butter, but within 27 weeks following the crisis, peanut butter sales for the implicated company had recovered. The 2006 outbreak of *E. coli* O157:H7 in spinach had an even greater influence on consumer demand for spinach. In this case, consumers substituted lettuce for spinach but also avoided bagged salads that did not contain spinach. More specifically, over a period of 68 weeks, retail sales decreased by 20% for bagged spinach and by 1% for bulk spinach (31).

As shifts in consumer demand for products vary in response to outbreaks caused by different types of contaminated products, consumers'

purchasing habits also differ depending on the type of pathogen contaminating a food. For example, two outbreaks associated with cantaloupes occurred in successive years, 2011 and 2012. In the 2011 outbreak, the contaminating pathogen was *L. monocytogenes*, which caused 33 deaths (32). In contrast, in the 2012 outbreak, the contaminating pathogen was *Salmonella*, which caused only three deaths (33). When consumers learned of the 2011 cantaloupe outbreak with its high fatality rate, the demand for cantaloupes decreased and consumers substituted other melons. In contrast, the consumer response to the 2012 cantaloupe outbreak, with the lower fatality rate, exhibited no shift in demand for cantaloupes (34).

Foods Consumers Prefer That Pose a High Microbiological Food Safety Risk

Consumers' food preferences largely determine the types of foods that the industry produces and that are sold at retail. The food industry has relied in the past largely on product sales to influence its product development and production focus. However, in this digital age, industry is also listening to the conversations on social media where consumers voice their viewpoints and concerns. In response, the industry is making changes such as product reformulation by providing more "natural-like" foods that do not contain many of the food additives that have been commonly used to preserve freshness, flavor, and texture, enhance color, and reduce spoilage to extend shelf life. Consumers, especially the younger generation, want foods that not only look and taste good but are also healthful. In some cases, these choices reflect the advice given by nutritionists and the medical field. However, in other cases, consumers are basing their decisions on misguided, nonfactual information and can be increasing their risk of acquiring a foodborne illness. In the following sections, some of the food groups that have recently been surging in the marketplace due to consumer demand are discussed with regard to their microbiological safety risk.

Fresh Produce

Many public health organizations both in the United States and globally promote the consumption of fruits and vegetables as important components of a healthy diet to help prevent major diseases (35). As a

result of these advisements, the U.S. per-capita consumption of fruits and vegetables has had moderate growth. In the period between 1998 and 2008, the per-capita consumption increased 6% and 5% for fruits and vegetables, respectively (36). However, during that same period, produce accounted for 47% of reported foodborneillness outbreaks in the United States (37). Their percapita consumption has continued to increase since that period (increases of 8% and 1% occurred between 2008 and 2016 for fruits and vegetables, respectively), and outbreaks attributed to fresh produce have likewise continued to occur. Of concern is that many fruits and vegetables are ready to eat and do not receive an adequate antimicrobial intervention treatment to reduce or eliminate enteric pathogens.

A number of other challenges encountered by the produce industry that are of concern include the following: (i) the majority of fruits and vegetables are grown in fields, where they are exposed to the natural elements (i.e., precipitation runoff, wild animals, and bioaerosols) that could potentially introduce pathogens to the crops, and (ii) surveys monitoring the prevalence of pathogens on fresh produce have revealed very low levels of contamination, so detecting and removing contaminated fruits and vegetables through routine product testing are very difficult. The produce industry has attempted to address the issue of produce having a high microbiological risk of contamination by focusing on prevention, with water, soil amendments, and field workers' sanitation practices being the primary points of intervention for minimizing introduction of pathogens into the fields.

Initial prevention efforts were guidelines known as good agricultural practices, but these were not mandatory in the United States. Then, as part of the Food Safety Modernization Act (FSMA), the produce safety rule, finalized in 2015, established for the first time science based minimum standards for the safe growing, harvesting, packing, and holding of fruits and vegetables for human consumption. However, to avoid the excessive economic burden that implementation of these measures would impose on small farmers, exemptions based on the farm's income from its produce sales were specified in the rule. Therefore, a large portion of the locally grown produce purchased at farmer's markets is not likely to be subjected to the standards established in the produce safety rule. These exemptions do not mean that on-farm

food safety measures are completely absent from these market sources. In many cases, they are subject to local and state requirements, but the degree of rigor of those requirements varies from location to location (38). To date, studies comparing the prevalence of pathogen contamination in produce from farmers' markets versus other retail sources have been inconclusive. More specifically, Young et al. (39) identified two studies in their scoping review where a higher prevalence of either *Campylobacter* or *Salmonella* was found in produce from farmers' markets than in produce from other sources, whereas in another two studies that they identified, no difference in the prevalence of *E. coli* was found in produce from these different sources. However, these results may change if the food safety standards applied by most of the produce industry are more effective in reducing the levels of contamination. Consumers should be advised that when purchasing or consuming locally grown produce, they should determine that the vendor is both knowledgeable and diligent in applying safe food production and handling practices in their operations.

Ethnic Foods

Immigration brings cultural diversity to countries, and as part of the immigrants' heritage, traditional foods from their homeland are often desired and are imported to meet the demand (40). Moreover, many of these new residents open restaurants featuring their unique cuisine. The popularity and growth of ethnic foods worldwide are well documented (41, 42). However, along with the increased popularity of ethnic cuisines, there has been an increasing number of foodborne illnesses in the United States (41, 42). At least one factor contributing to this finding is that food code violations for ethnic restaurants are substantially more numerous than those for nonethnic restaurants (43). Several reasons have been proposed to explain the higher microbiological risk of eating ethnic foods, including the following: (i) a large proportion of ethnic restaurants use unfamiliar ethnic ingredients (44); (ii) some types of ethnic restaurants use a large portion of ingredients that are considered to have a higher microbiological risk, such as fresh vegetables in Mexican food or raw fish in Japanese food (41); (iii) employees may lack the knowledge of how to prepare safe ethnic foods (e.g., fried rice prepared from kernels contaminated with *Bacillus cereus*) (44); and (iv) language barriers may

prevent effective communication of safe food preparation practices to owners and employees of ethnic restaurants (44, 45).

Raw Milk and Products Made from Raw Milk

Raw milk contains a diverse microflora which can include pathogens transmissible to humans. In the United States, interstate retail sales and distribution are not allowed, and within many states, raw milk retail sales of any kind are prohibited. Despite these strict regulations, a perception has persisted for many years that a heat treatment used to pasteurize milk destroys some of its nutritional and health benefits (46) and has likely been a major factor contributing to the fact that 3.4% of Americans drink unpasteurized milk (47). A closer examination of the basis for these nutritional claims reveals that they are largely based on anecdotal evidence, and when scientific studies have been conducted, the experimental design was either weak or questionable (48). In fact, many of the beneficial properties attributed to drinking raw milk, such as having a greater nutritional value than pasteurized milk, have been determined to be minimal when milk is stored at refrigeration temperature.

The nutritional benefits of drinking raw milk stored above refrigeration temperatures, when growth of lactic acid bacteria produces health-benefiting antimicrobials, is negated by the simultaneous acidification and coagulation of milk, reactions which render the product spoiled and unsuitable for consumption. Moreover, there is in fact an increased risk associated with consumption of unpasteurized milk products compared to that of pasteurized milk products, as evidenced by the nearly 3-fold-higher incidences of outbreaks with the former than the latter during the period from 1993 to 2006 (49). A comparison of more recent data shows that the number of U.S. foodborne-illness outbreaks caused by unpasteurized milk increased from 30 during 2007–2009 to 51 during 2010–2012 (50). Considering all of these factors, most food safety professionals consider drinking raw milk to be a substantially greater public health risk than drinking pasteurized milk (51). However, some consumer groups, not trusting health officials' recommendations regarding consumption of raw milk (46), continue to promote raw milk as a product that is superior to pasteurized milk. Moreover, these raw-milk proponents have used the Internet and social media channels to

disseminate their message, resulting in a surge in the popularity of drinking raw milk.

As with raw milk, consumer interest in consuming dairy products made from raw milk (cheese, butter, cream, and buttermilk) has also increased. One of the main drivers for the popularity of raw-milk cheese is that it is considered to have more intense and varied flavors than cheese made from pasteurized milk. Several recently published review articles have addressed the microbial benefits and risks of dairy products made from raw milk (52, 53). The overall consensus is that the microbiological risks associated with cheese made from raw milk are reduced when the cheese is aged (cured) for sufficient time at temperatures that minimize the growth of pathogens (53). Thus, U.S. federal guidelines mandate that raw-milk cheese be held at a temperature of not less than 35°F (1.7°C) for at least 60 days (54) based on information indicating that pathogenic microbes should be inactivated by the low pH, low water activity, high salt, and competitive bacteria dominant in the ripening cheese (55). Several studies have revealed that when cheese is manufactured under such conditions, there is a very low prevalence of pathogens in these products (55, 56). Furthermore, outbreak data from the United States between 1993 and 2006 suggest that raw-milk cheeses do not appear to present a greater microbiological safety risk than cheeses made from pasteurized milk, as 38 (42%) and 44 (49%) of the 90 foodborne outbreaks were attributed to these dairy products, respectively (57). However, closer examination revealed that the outbreaks involving cheese made from pasteurized milk occurred in venues where cross-contamination was a likely vehicle (57). In addition, studies have revealed that a 60-day aging period did not guarantee complete control of *E. coli* O157:H7 in cheeses manufactured from raw milk contaminated with initial population levels as low as 33 CFU/ml (58, 59). Moreover, in light of the 2013 *E. coli* O157:H7 outbreak in Canada that was linked to aged (60-day) raw-milk Gouda cheese (60), the contribution of other potential factors (e.g., cheese type, composition of the raw-milk microbiota, and type and level of the pathogen isolate) needs to be more thoroughly investigated. Thus, aging of raw-milk cheese may not be regarded as a fail-safe solution to produce a risk-free product (53), and appropriate warnings should be provided to consumers.

Antimicrobial Preservative-Free Foods

A reason that consumers desire raw milk is that they perceive it as "natural." Also included in this natural food group are non-genetically modified foods (61) and foods where chemical additives are absent or processing of the foods has been minimized (62). Many surveys have confirmed the popularity of this movement (63). In reality, naturalness is difficult to quantify or measure, especially since the term is not regulated and the aspects that are considered essential in perceiving a food item as natural vary among consumers and different stakeholder groups (63). However, three categories have emerged as components of a food to be classified as natural: (i) the origin of the food or the way the food has been grown; (ii) the extent to which technology and ingredients have been applied to the food (in other words, how the food has been produced); and (iii) the properties that constitute the final product (63).

The food industry has responded to consumers' food preferences as they have evolved. Three major approaches have been taken to address consumers' concerns regarding the inclusion of synthetic food additives, specifically antimicrobials, in their products. The first was to change on the package label the terminology used to describe the antimicrobial (62). For example, in the European Union, an added chemical is listed by its name rather than by its "E" number, given that consumers perceive E numbers as being artificial. Another labeling change has been to use a "clean label" version of the chemical being added (i.e., the label contains recognizable ingredients) rather than identifying the active agent itself (e.g., using the term "vinegar" or "egg white" instead of "acetic acid" or "lysozyme," respectively) (62).

The second approach to addressing consumers' concerns regarding synthetic additives in foods involves removing antimicrobials from product formulations; however, most consumers are not aware of the functions of these ingredients (62). More specifically, antimicrobials serve a useful purpose not only in controlling harmful microbes that may be in foods but also in preventing the growth and metabolic activity of spoilage organisms. The repercussions of eliminating antimicrobials from food product formulations include (i) shorter shelf lives resulting from spoilage occurring sooner, (ii) increased risk of survival and growth of pathogens, (iii) increased risk for mycotoxin production, (iv) more consumer complaints as consumers fail to recognize changes to expiration dates, (v) higher prices as changes in formulations and

distribution practices need to be implemented, and (vi) increased food waste due to microbial spoilage (62).

A third approach to addressing consumers' concerns regarding synthetic additives in foods is to replace the synthetic chemical with natural sources of antimicrobials (e.g., essential oils) or bioprotective cultures (e.g., lactic acid bacteria). However, there are shortcomings to using this approach (62). First, these natural sources of antimicrobials can contain multiple antimicrobials, some of which are more effective than others (64). Furthermore, given that these natural sources are derived from biological systems and may be subject to variations in environmental conditions and other factors affecting biological variability, the proportion of the active antimicrobial constituents within the natural source may vary considerably, leading to variable antimicrobial efficacies. Another challenge is that many of these natural antimicrobial sources often have low concentrations of the antimicrobials and have potent aromatic or flavor compounds associated with them. Hence, to achieve antimicrobial activities comparable to those of the synthetic chemicals, large amounts of the natural preservative must be added but may simultaneously introduce undesirable aromatics or flavors into the product. An example is the use of cranberry concentrate to serve as a natural source of benzoate to control mold and pathogen growth. The relatively large amount of cranberry concentrate needed to provide the level of benzoate to control the microbes would add undesirable flavor to the finished product. Another example is cured meats. Instead of adding nitrites directly to cured meats to control pathogens such as *Clostridium botulinum* and *Clostridium perfringens*, celery juice concentrate, which is a natural source of nitrate that is converted to nitrite in the meat by microbial activity, is added. However, to avoid compromising the flavor with the celery concentrates, lower levels of nitrite were added from the celery concentrates. As a result, there was a greater potential for *C. perfringens* to grow when the meats were cooled, thereby increasing the microbiological risk in these cured meats relative to the risk in products that had synthetic nitrite added (65).

Innovative Approaches Needed To Provide Consumers Credible Food Safety Information

Over the past 2 decades, social media communication has evolved such that consumers often obtain unreliable information regarding the safety of their foods. To enable consumers to make informed decisions, food safety professionals need to challenge unsubstantiated claims and provide credible, science-based information. However, an underlying complication is that safe food handling behaviors and perceptions occur as part of a complex interaction of everyday consumer practices and habits. Moreover, most consumers are not concerned about food safety practices in the home and are generally not motivated to change their behavior based on new knowledge about food safety risks (66). Hence, to overcome these barriers, innovative approaches are needed to educate the younger generation, although traditional approaches may be sufficient for educating the older generation. Both stakeholders and experts agree that social media could be an effective tool in crisis communication and issue awareness, as they offer speed, accessibility, and the potential for changing food habits through social pressures (66–69). However, to minimize information overload and to overcome the low levels of trust associated with many Internet and social media sites (70), it is important for educators to not only capture the initial attention of visitors to the site but also instill trust and confidence with information presented within that site. An example is an approach used by Sillence et al. (71), focused on unpasteurized raw milk, which revealed that good website design was only one component to an effective Internet site. Another important element is its content, which should be of personal and social relevance in describing the risks and benefits of the food product being addressed.

IMPORTED FOODS

Globalization of the world's food supply has been beneficial to both importing and exporting countries, with a variety of supply- and demand-side factors driving the marketplace. Many of the importing countries in this global marketplace are developed countries, whereas many of the exporting countries are developing nations. Consumers in developed countries desire a year-round supply of seasonal fruits, vegetables, and nuts, as well as ethnic foods. Hence, these items are largely supplied by many developing countries where these foods are available. Developing nations are also able to provide these foods at reasonable costs, as they have low land values, water availability where needed, appropriate

climate conditions, and cheap labor. The book *Imported Foods: Microbiological Issues and Challenges* (72) describes the trends of foods imported into the United States during the early 2000s, as well as the weaknesses inherent in the food safety net and programs designed to mitigate those deficiencies (72). The remainder of this section updates the trends relating to food imported into the United States and the continued deficiencies that exist with this part of the U.S. food supply.

Food Groups Representing the Greatest Microbiological Risk

Thirty-two food groups are used by the FDA's OASIS (Operational and Administrative System for Import Support) to categorize foods imported into the United States. The food groups that represent the greatest microbiological safety risk are often those that have the greatest number of total shipments refused. On that premise and based upon the recent summary prepared by Bovay (73), the top four groups and the percentages of the total food shipments refused by the FDA between 2005 and 2013 that they constituted were (i) fishery and seafood products (20.5%), (ii) vegetables and vegetable products (16.1%), (iii) fruit and fruit products (10.5%), and (iv) spices (7.7%). However, refusals may not always be attributed to the microbiological violation codes, which include the following: (i) *Salmonella*, (ii) filthy, (iii) insanitary, (iv) *Listeria*, (v) histamine, and (vi) "imptrhaccp" (described as failure to provide verification of compliance of sanitary conditions during preparation, packing, or holding). In any case, among these four food groups, 83% of the total violations were attributed to a pathogen or toxin (73). Despite this association, refusal statistics may not be used as conclusive evidence that these food groups have a greater microbiological safety risk due to the preferential sampling that is assigned to specific food groups. The FDA has not had the resources to physically inspect all food import shipments, and in fact, they physically inspect less than 1% of all regulated food imports into the United States (74). Hence, to obtain the maximum benefit from the inspections that are conducted, the FDA uses a risk-based prediction algorithm to target shipments posing what is considered a greater risk to human health or that are more likely to be in violation of U.S. laws.

Another approach to identifying specific imported food groups that are of greater microbiological risk than other food groups is to consider foodborne-illness outbreak data collected by the CDC between 1996 and 2014 (75). The food groups that accounted for the greatest percentage of outbreaks and illnesses within the United States using this data set are listed in Table 42.2.

Magnitude of U.S. Imports of Selected Food Groups

Focusing on the food group that accounts for more than half of the outbreaks listed in Table 42.2 (i.e., seafood and fish), it is noteworthy that more than 90% of the seafood and fish consumed in the United States in 2017 was imported (76). Moreover, the major countries of origin for most farmed seafood and fish products, such as shrimp and tilapia, are developing countries (Table 42.3), with the U.S. per-capita consumption of shrimp and tilapia being 2.0 and 0.5 kg/year, respectively, in 2017 (77). Of concern is that these countries are known to employ sanitary practices in production and processing that are inferior to those employed in developed countries (78). Food safety hazards that may be potentially encountered in these countries are addressed in greater detail in the following section.

Table 42.2. Imported food groups that have accounted for the greatest percentage of foodborne outbreaks and illnesses within the United States between 1996 and 2014[a]

	% of imported food-related:	
Imported food group	Outbreaks	Illnesses
Fish, mollusks, crustaceans, other seafood	55	11
Fruit	12	32
Vegetables (row and seeded)	10	29
Spices	2	5
Nuts/seeds	3	1
Other	18	22

[a] Adapted from table in reference 75.

Three other major food groups from which one-quarter to over one-half of the U.S. imported food supply came in 2016 were fruit, fruit juices, and vegetables. However, within each group, the import share of individual commodities varied greatly. For example, in 2016, the import share (percentage of the total) of head and leafy lettuce was only 6% and 4%, respectively, whereas for the vegetables listed in Table 42.4, they ranged from 14% to 90% (36). Per-capita consumption of the commodities is also provided in a column in Table 42.4 to illustrate the amount of each imported commodity that Americans consume. Of the major countries exporting these fruits and vegetables to the United States, more than 65% of the total imports are from developing countries (Table 42.5).

Table 42.3. Source countries of U.S. shrimp and tilapia in 2018 based on volume[a]

Shrimp		Tilapia	
Country	% of total imported[b]	Country	% of total imported[c]
India	36	China (mainland)	75
Indonesia	20	China (Taiwan)	6
Vietnam	10	Honduras	4
Thailand	9	Indonesia	4
Ecuador	8	Colombia	4
China (mainland)	5	Costa Rica	2
Mexico	4	Ecuador	1
Other	8	Other	4

[a] Information extracted from spreadsheet in reference 124.

[b] Frozen and prepared shrimp represent 77% and 23% of total shrimp imports, respectively.

[c] Frozen (whole and fillets) and fresh (fillets) tilapia represent 88% and 12% of total tilapia imports, respectively.

Table 42.4. Selected fruit and vegetable commodities and their U.S. per-capita consumption for which a major portion are imported into the U.S.[a]

	% imported of total supply		Per-capita consumption (kg or liter per year) in 2016	
Food commodity	2006	2016	Total[b]	Imported[c]
Noncitrus fruit, fresh	47	53	21.6	11.4
Avocados	73	86	3.2	2.8
Watermelons	17	30	7.5	2.2
Grapes	57	50	3.7	1.8
Cantaloupes	33	43	3.2	1.4
Mangoes	100	100	1.4	1.4
Papayas	94	98	0.6	0.6
Strawberries	8	14	3.6	0.5
Raspberries	25	48	0.4	0.2
Fruit juices	44	63	21.2	13.4
Apple	82	84	7.2	6.0
Orange	22	46	9.1	4.2
Vegetables	16	28	65.8	18.4
Tomatoes	35	56	9.7	5.4
Cucumbers	52	73	3.6	2.6
Squash	43	64	2.3	1.5
Onions	8	14	8.5	1.2
Garlic	52	63	1.2	0.8
Asparagus	74	90	0.7	0.6
Broccoli	9	18	3.4	0.6
Carrots	9	18	3.5	0.6

[a] Information extracted from spreadsheets in reference 36.

[b] Domestic and imported.

[c] Values obtained by multiplying total per capita consumption by the % imported of total supply.

Spices are another food group that has had many shipment refusals. In addition, several outbreaks of foodborne illness have been traced to imported spice contamination (Table 42.2). As with the food groups described above, a large percentage of spices consumed in the United States are imported (79), with developing countries being the primary sources (Table 42.5). Since the U.S. per-capita consumption of spices in 2012 of 1.5 kg/year (80) is likely to increase as consumers demand more preservative-free food, pathogen contamination could be amplified because of the many additional foods that will contain spices as an ingredient. An additional complication is that spices are often a "stealth ingredient" in foods, in that they are not known to be added to a food associated with an outbreak and, hence, are not identified as the source of an outbreak. Therefore, spices are more likely to be frequent sources of foodborne illnesses than is being recognized.

Table 42.5. Top six sources and their percentage of total U.S. imports (based on U.S. dollar value) for selected food groups in 2017[a]

	Fruit and preparations[b]		Vegetables (fresh)		Fish/shellfish		Spices	
Rank	Country	% of total	Country	% of total	Country	% of total	Country	% of total
1	Mexico	40	Mexico	68	Canada	15	Madagascar	22
2	Chile	11	Canada	18	China	13	Vietnam	15
3	Guatemala	7	Peru	4	India	11	Indonesia	14
4	Costa Rica	6	China	2	Chile	9	India	13
3	China	5	Guatemala	2	Indonesia	9	China	9
6	Peru	5	Costa Rica	1	Thailand	7	Brazil	4

[a] Information extracted from spreadsheets in reference 36.

[b] Includes fresh, frozen, dried, preserved, and prepared fruit and fruit juices.

Production and Sanitation Practices within Developing Countries

Developing countries represent most of the major exporters to the United States of the types of imported foods most often associated with foodborne illnesses and outbreaks (Tables 42.2 and 42.5). Similarly, across all food groups imported into the United States and using value (in U.S. dollars) as the measure of ranking, 11 of the top 15 sources were from developing countries in 2016 (Table 42.6). However, developed countries may export more highly valued items than developing countries. For example, one of the major foods exported into the United States from Canada is salmon, whereas tilapia, which is a less expensive fish, originates mainly from China. Hence, although Canada ranks higher than China for value of imported fish and shellfish (Table 42.5), China must have a larger proportion of the import volume, which translates to greater product exposure and potential adverse public health risks.

Several factors contribute to concerns regarding the microbiological safety of imported foods from developing countries. These concerns are based on two major sources of information: (i) outbreaks that have been epidemiologically linked to foods imported from these countries and (ii) surveys of both imported and domestically produced foods from these countries as well as surveys of practices and knowledge associated with producers of these foods. Selected examples of various microbiological food safety risks associated with specific imported food groups are listed in Table 42.7.

Table 42.6. Major source countries of U.S. food imports in 2017 based on U.S. dollar value[a]

Country	% of total food imports
Mexico	23.0
Canada	18.4
European Union	18.2
China	3.7
Indonesia	3.1
Brazil	2.7
Australia	2.7
Chile	2.4
Colombia	2.2
India	2.2
New Zealand	2.1
Thailand	2.0
Vietnam	2.0
Guatemala	1.7
Peru	1.6

[a] Information extracted from spreadsheets in reference 36.

One of the common deficiencies contributing to an increased likelihood that the food produced or processed in developing countries will be contaminated is the absence of adequate regulatory oversight and enforcement of good hygienic practices (81). Under such conditions, food producers and processors often do not have an adequate incentive to obtain the necessary knowledge and resources to implement sanitation and good agricultural practices that would reduce the likelihood of contamination.

Another major limitation for many developing countries is the use during production of water that is contaminated with pathogens, which is a result of those countries lacking the basic infrastructure to treat the wastewater used in food production and processing (82–84). In other cases, the use of animal waste-contaminated water in agriculture is

recommended by some sustainability-conscious advocates as beneficial from a sustainability perspective, while the potential associated food safety risks are not recognized (85). For example, in one type of integrated multitrophic aquaculture system, animal wastes are dumped into ponds, where the farmed fish may directly feed on the feces or the feces may serve as a nutrient source for algae and plankton, which the fish then consume. In either case, those fecal wastes may introduce enteric pathogens into the pond environment if the animals are carriers of human pathogens such as *Salmonella* and enterohemorrhagic *E. coli*. The extent to which the addition of fecal wastes leads to an increased prevalence of contaminated fish appears to be dependent on the type of pathogen or indicator microbe and the potential for the pathogens to survive in the environment or be introduced from other environmental sources (Table 42.7). The increased interest in integrated multitrophic aquaculture systems among countries within the European Union has led to the realization that in order to utilize products produced in these systems, substantial regulatory reform and oversight are needed (86).

Another common practice of many aquaculture producers in developing countries is the indiscriminate use of antibiotics to prevent disease in fish and shrimp. For example, Vietnamese shrimp farming has been adversely affected by several diseases, which has led to extensive use of antibiotics as a prophylactic. In addition to the potential for antibiotic residues to be present in the exported seafood (87), incomplete assimilation of the antibiotics by fish leads to high levels remaining in ponds, sediments, and the surrounding environment (88). Such residues, whether in fish or the environment, at subtherapeutic levels pose a major risk for the proliferation of antibiotic-resistant bacteria, including foodborne pathogens such as *Salmonella* (89, 90).

Table 42.7. Reported instances of microbial contamination of imported foods or food raised or grown in developing countries being documented or being a subject of concern

Food item(s)	Key points of survey, incident, or study
Fish, aquacultured	Similar levels of *E. coli* were found in the muscle of fish raised in integrated pig-fish ponds and nonintegrated fish ponds. In contrast, *E. coli* levels in the intestines were 100 times higher in fish from integrated than nonintegrated ponds (125).
Tilapia	No significant difference in the occurrence of *Salmonella* spp. was found in fish raised in traditional nonintegrated and integrated tilapia-pig aquaculture systems (manure from pigs serves as fertilizer in the ponds). In the integrated systems, *Salmonella* spp. were found in 20.0%, 40.0%, and 11.1% of fish mucus, fish intestine, and pig feces, respectively, whereas in nonintegrated systems, the pathogen was found in 40.0% and 40.0% of fish mucus and intestines, respectively. Commercial feed could not be linked to the common genotype of *Salmonella* found in both systems and suggested that *Salmonella* may be able to survive and possibly multiply in tropical aquatic environments (126).
Shrimp	Use of antimicrobials to treat diseases, most commonly due to *Vibrio parahaemolyticus*, can lead to development of antimicrobial-resistant microbes. In addition, when the antimicrobial treatment is not effective to stop the outbreak, many shrimp farmers harvest the shrimp early (127).
Seafood	Intermediate resistance to ampicillin was exhibited in 63% of *V. parahaemolyticus* isolates recovered from 21% of shrimp samples (n = 38) imported into the U.S. Resistance to nitrofuran was found in 75% of *L. monocytogenes* isolates recovered from 42% of salmon, shrimp, and tilapia samples (n = 171) imported into the U.S. (128).
Lettuce	Prevalence of norovirus, rotavirus species A, or human adenovirus collected from irrigation waters and lettuce at

	three to five sites in Brazil ranged from 17 to 50% and 25 to 42%, respectively (84).
Snap peas	An outbreak in 2005 of *Cyclospora cayetanensis* in Ontario, Canada, was linked to the consumption of snap peas imported from Guatemala (129).
Berries, frozen	A prolonged outbreak (2013-2014) occurring primarily in Italy was epidemiologically linked to imported frozen berries (130).
Produce, fresh	A review documents the numerous outbreaks associated with the consumption of fresh produce that was imported from developing regions and contaminated with the protozoan parasites *Cryptosporidium*, *Giardia*, and *Cyclospora*. The possible sources of contamination and control measures are discussed (131).
Spices	The U.S. FDA conducted a survey on the prevalence of *Salmonella* in 11 spices with □1% found in product offered for sale at retail establishments but ranging from 1.7 to 18% in products entering the U.S. (132).

Efforts To Improve the Safety of Foods Exported from Developing Countries

Although food produced and processed in developing countries and exported to global markets is advantageous for many factors driving the supply chain (e.g., low land values, water availability for some locations, appropriate climate conditions, and cheap labor), unsuitable production and processing practices that do not comply with the food safety standards established by an importing country can prevent an industry from exporting products to other countries. Multiple factors contribute to this situation, including outdated laws, lack of knowledge in the industry due to limited or inadequate coordination and communication with organizations handling food safety issues, underfunding of national research institutes that can provide practical solutions to food safety-related deficiencies, and lack of awareness of food standards and quality criteria (91).

Many approaches are being taken to improve the microbiological safety of foods being exported by developing countries. Historically, private

enterprise has been an external driving force that has successfully incorporated safe food production and handling practices into the food production and processing operations of developing countries, but the level of involvement and enforcement of internal policies varies (92). At one end of the spectrum, there can be complete involvement at all phases of the food continuum (production, processing, and transportation) in applying preventive controls to prevent hazards from occurring, but at the other end of the spectrum, where the smallest amount of control over safety of imported foods occurs, the private enterprise selects their suppliers and routinely monitors by inspecting and testing the safety of the incoming foods. Because only a small percentage of the imported food can be adequately inspected and tested, unacceptable products can enter the food supply.

Another approach to improving a developing country's food safety practices is for the country to develop and implement regulations that specifically address food safety practices. Examples of countries where this approach has been successively applied are Malaysia (93) and Thailand (94).

Governments and private enterprises are not the only organizations involved in setting food safety-related criteria for food exports from developing countries. Many nongovernmental organizations (NGOs) have developed certification criteria (95, 96). An example is the Global Good Agricultural Practices (GLOBALG.A.P.) program in Germany that is a certification process for many agricultural products, including those from aquaculture. Another NGO, the Global Aquaculture Alliance (a U.S. organization representing aquaculture producers, seafood traders, and major restaurant chains) created the Aquaculture Certification Council to certify that aquaculture facilities throughout the world were applying responsible and established social, environmental, and food safety standards. However, it is important to recognize that not all NGOs address food safety issues. For example, the Aquaculture Stewardship Council's certification standards primarily address environmental stewardship and working conditions for producers (96). Nevertheless, to encourage food retail corporations to address food safety as well as social and environmental responsibility, the criteria of many NGOs include required third-party audits and certification rather than self-reporting or certification by business partners (96). However, to meet these

requirements, access to these certification bodies is critical. For example, Mohammed and Zheng (97) determined that distance between the food producer and the certifying organization negatively affected the number of certified sites in a country. Thus, developing countries in Africa and Asia would be at a disadvantage in adopting food safety standards, as they would likely be faced with higher certification costs due to this disparity.

In many developing countries, it is recognized that advances to their food safety system will require a multifaceted approach (98). For example, to encourage farmers in China to comply with higher product safety and quality standards, a survey revealed that a small price increase would be a sufficient incentive to change their practices (99). In other cases, countries may need to obtain sufficient capital before they can provide the basic infrastructure to establish and maintain basic clean water and sanitation practices (98). In countries such as Vietnam, where more than 330,000 small-scale shrimp producers contribute to a very fragmented supply chain (96), it is currently difficult to provide adequate tracking documentation regarding compliance with certification, and addressing this deficiency will require an innovative approach.

For foreign suppliers of food products to the United States, restrictions that are included within the Foreign Supplier Verification Program (FSVP) of the FSMA are now mandated. The compliance date for this rule was 30 May 2017, and it requires that importers perform certain risk-based activities to verify that food imported into the United States has been produced in a manner that meets applicable U.S. safety standards. Moreover, to reflect modern supply and distribution chains, the rule offers importers flexibility in meeting key FSVP obligations in that they may rely on certified third parties to provide the required analyses, evaluations, and activities as long as those importers review and assess the corresponding documentation (100). Additional information on the FSVP is provided in Table 42.8 and in “Current Outlook and Impact” below.

RECENTLY ENACTED REGULATORY AND POLICY CHANGES TO IMPROVE FOOD SAFETY

U.S. Food Safety Modernization Act

In 2011, the FSMA was signed into law with the purpose of improving public health within the United States by strengthening the country's food safety system. In particular, it sought to provide the FDA, who has oversight of more than 80% of the U.S. food supply, with new enforcement authorities. With these new powers, the FDA would then be able to proactively protect the food supply rather than continuing to react to problems after they occur. In response to the FSMA's mandate, seven foundational rules have been written that encompass different aspects of the food supply chain. They are (i) current good manufacturing practices and hazard analysis and risk-based preventive controls for food for animals; (ii) current good manufacturing practices and hazard analysis and risk-based preventive controls for human food; (iii) standards for the growing, harvesting, packing, and holding of produce for human consumption (produce safety rule); (iv) foreign supplier verification programs; (v) sanitary transportation of human and animal food; (vi) accredited third-party certification; and (vii) mitigation strategies to protect food against intentional adulteration (101). Specific areas covered within several of these rules are provided in Table 42.8, whereas summaries and access to the complete documents published in the *Federal Register* can be found on the FDA FSMA website (101).

Table 42.8. Major sections and provisions specified in three of the seven rules that were mandated in the FSMA (100, 102, 133)

- HARPC for human food rule (reassessment every three years)
  - Updates to the current good manufacturing practices
    - Employee hygiene training required
    - Confirmation that employees qualified for assigned duties
  - Food safety plan (prepared by qualified individual)
    - Hazard analysis
    - Written preventive controls (process, allergen, sanitation, and other controls)
    - Oversight and management of preventive controls (monitoring, corrections, documented corrective actions, documented verification of controls—e.g., scientifically validating preventive process controls)
    - Supply chain program to address hazards
    - Written recall plan if hazard identified
- Produce safety rule
  - Agricultural water for produce (other than sprouts)
    - Microbial water quality (target organism and numerical criteria)
    - Indicate options for corrective actions
    - Microbiological testing
  - Biological soil amendments
    - Raw manure
    - Stabilized compost
  - Domesticated and wild animals
  - Worker training and health and hygiene
  - Equipment, tools, and buildings

    - Sprouts
        - Prevention of microbial seed contamination
        - Testing of spent irrigation water
        - Testing sprout environment for *Listeria monocytogenes*
        - Corrective actions if out of compliance
- Foreign supplier verification program rule
    - Scope
        - Overview of who is covered
        - General responsibilities of food importers
    - Hazard analysis (identify potential hazards [biological, chemical, physical] based on experience and database of food item)
    - Evaluation of food risk and supplier performance by importer or certified third party
    - Supplier verification either by importer or certified third party (e.g., on-site audits, review of supplier records, sampling and testing)
    - Corrective actions
    - Exemptions and modified standards
    - Unique facility identifier

Before the FSMA foundational rules were drafted, extensive input was obtained from the industry, the public, and other stakeholders through public meetings, webinars, and listening sessions with the intent that the final rule be practical and flexible but effective. As a result, dates of implementation have been staggered depending on when the final rule was published and to give sufficient time for different segments of the industry to implement changes (i.e., smaller companies were given more time to adapt, as it would likely impose a greater financial burden on them). However, one of the overarching themes encompassed within these rules is that whenever possible, science-based preventive controls

should be used to minimize hazards in the production, processing, transport, and storage of food. However, in some cases, such as the produce safety rule, insufficient data were available to make a risk-based recommendation on application times of raw manure relative to harvest of the produce. Hence, it was decided that adherence to the USDA's National Organic Program standards could be used until sufficient data were collected to evaluate whether to continue with those metrics or adopt more stringent metrics (102).

Other common components within the seven foundational rules are delineation of the tools that will be used by the FDA to ensure compliance by commercial entities within the food chain. Examples include mandated inspections by the FDA of food facilities, expanded requirements for industry to have available written documentation of their preventive plans and activities, and authorization for the FDA to issue mandatory recalls when unsafe food is not recalled by companies voluntarily as well as to suspend a company's registration, prohibiting it from distributing food.

Although all FSMA foundational rules are anticipated to lead to substantial improvements in the safety of the U.S. food supply, the use of hazard and risk-based preventive controls (HARPC) for human food is among the most important of the FSMA rules. This rule is a successor to the hazard analysis and critical control points (HACCP) system that the U.S. food industry had been observing and emphasizes prevention of hazards before they occur rather than their remediation after they occur. To illustrate this point, some of the differences that exist between HACCP and FSMA-HARPC include the following: (i) FSMA-HARPC places a much greater emphasis on science, research, and prior occurrences of outbreaks than does HACCP; (ii) an FSMA-compliant food safety plan must be prepared and overseen by a qualified employee at each facility; (iii) good manufacturing practices, allergen controls, integrated pest management, and vetting suppliers are expected to be integral components in FSMA-compliant food safety plans to a greater extent than they were in HACCP plans; (iv) in place of corrective actions, corrections that involve minor improvements and require less documentation to the food safety plan are allowed; (v) a food defense component must be a part of the plan and must address both terrorism and economically motivated adulteration; (vi) in addition to chemical, biological, and physical hazards

that were covered in HACCP systems, radiological hazards must also be addressed within the food safety plan; and (vii) preventive controls include all control points appropriate for food safety but do not require critical limits, as they did in HACCP systems.

Another FMSA foundational rule that is anticipated to have a major impact on reducing food safety risk is for the produce industry. More specifically, the produce safety rule provides enforceable standards for the production of minimally processed fresh produce, whereas, prior to the adoption of this rule, such criteria did not exist. Instead, only nonenforceable good agricultural practices and good manufacturing practices guidelines and guidelines developed by trade associations were being followed by much, but not all, of the produce industry. Within the specifications of the new produce safety rule, there are several concerns. One concern is the costs of complying with the new rule, as it could disproportionately burden small farms and drive them out of business. Hence, multiple studies have been conducted to address the cost that has been or will be incurred as a result of implementing either the food safety criteria of the California Leafy Greens Marketing Agreement (LGMA) or the standards of the produce safety rule. Major findings of those studies are listed in Table 42.9, but in general, compliance costs were determined to be marginal, although there were economies of scale (i.e., expenditures per acre decreased with increased farm size).

For this reason, the FDA chose to exclude from compliance farms whose annual produce sales were less than $25,000 for the three preceding years. Included in this category would be many local operations that engage in sustainable practices, community engagement, and direct farm-to-table networks (farmers' markets and roadside stands). Although these farms represent a small portion of total acres devoted to growing produce, they are growing in popularity each year (103). Unfortunately, many of these growers do not have a food safety plan, not because of financial constraints, but because they are not required to do so under the FSMA (104). Moreover, the growers selling their produce in direct market channels may not have the requisite knowledge to engage in practices that would ensure the safety of their products (39). Although the number of individuals made ill from consuming contaminated produce from these sources may be small compared to the number affected by outbreaks associated with commercial retail brands, negative publicity from a

produce-associated outbreak from a small farm may spread to include other types of produce grown in the area or even to other areas of the country, thereby adversely affecting produce sales in general.

Table 42.9. Costs associated with implementation of on-farm food safety standards within the LGMA or the FSMA produce safety rule

Location	Produce item(s)	Major findings	Reference
California	Muskmelon, spinach, tomatoes	Costs incurred by produce due to food safety outbreaks are much greater than LGMA compliance costs. Third-party audits, staffing, and water testing were the most significant compliance costs.	134
Florida	Cabbage	When attempting to meet LGMA standards, the probability of operating at a net loss over a 2-year period (based on values on year in which study was conducted) increased by 17%.	135
California, Florida, and Texas	Cabbage, cantaloupe, citrus, onion, spinach, tomato, watermelon	The cost of complying with the FSMA produce rule was not size neutral and could have negative impacts on the profitability of small farms.	136
Mid-Atlantic	Leafy greens, tomatoes	Based on a survey, adoption of food safety practices similar to those required under the FSMA would impose a fairly modest cost burden on farms of all sizes, but there would be substantial economies of scale.	137
California	Leafy greens	Distribution of costs associated with food safety practices being conducted to meet the requirements of the LGMA were 38% for food safety and clerical staff involved in food safety tasks,	138

		32% for time expended by harvest foreman, 17% for time and other expenditures involved with audits, 11% for product unharvested due to animal intrusion, and 2% for testing of water.	
National, but more farms representing the South and Northeast than West	Vegetables, berries, fruits, and tree nuts	Expenditures per acre decreased with farm size as a result of complying with FSMA produce rule. In addition, growers using sustainable farming practices spent more than conventional growers on many food safety practices.	139

Another concern associated with the produce safety rule is the metrics and frequency of sampling required for the microbial quality of agricultural surface water used for irrigation. These criteria are based on the EPA standards for recreational water, as there are otherwise insufficient data available for establishing standards that would be relevant to surface water, streams, and ponds used for irrigation. Therefore, to address this deficiency, Havelaar et al. (105) sampled six agricultural ponds in west central Florida to evaluate the empirical and theoretical basis of the microbial water quality parameters within the produce safety rule. Their findings revealed that 20 samples were insufficient to determine the bacteriological quality of irrigation ponds and the specified rolling data set included in the rule led to delays in detecting a shift in water quality. Although increasing the number of samples was postulated as a possible solution to this problem, it increased the costs of monitoring. Hence, these researchers advocated that further data collection and analysis be conducted to better understand the factors influencing the variability of the bacteriological quality in irrigation ponds.

Projected Evolution of Regulatory and Policy Initiatives

Current Outlook and Impact

Currently, the FDA does not recognize any food safety program as an alternative to FSMA's produce safety rule. However, in the future, partnerships between industry and government are expected and could pave the way for recognition of alternative compliance and verification programs that will be accepted by the FDA (106). One of the strengths of the FSMA is that it aligns with many regulatory programs adopted in developed nations. In addition, it is compatible with binding international trade rules as well as nonbinding internationally recognized standards set forth by institutions such as the World Health Organization, the World Trade Organization, the Codex Alimentarius Commission, and the Global Food Safety Initiative (GFSI). However, in many cases, industry-driven criteria may be more stringent than governmental regulations (106). Under those circumstances, individual food manufacturers often select a third-party food safety auditing scheme (e.g., GLOBALG.A.P., Primus GFS, Global Aquaculture Alliance, Global Red Meat Standard, etc.) that is recognized by the GFSI. Once the company has passed the audit, the company is then certified against the benchmarked scheme.

There are many advantages to companies going through a GFSI certification process, including the following: (i) it is currently required by many of their current retail customers; (ii) it is an asset that can be used for bringing in new customers; (iii) it reduces the number of third-party food safety audits; and (iv) it facilitates continuing improvement of the company's food safety program (107). However, to date, most companies do not use these third-party certification programs as a marketing strategy to convince consumers that their products are safe. Moreover, in contrast to the newly required foreign supplier verification program and the accompanying program established for accreditation of third-party auditors, the United States does not currently provide any oversight for audits performed on domestic food operations (106). However, if such a system were to be implemented in the future, it would be one more layer of bureaucracy but it would ensure that when deficiencies are identified, corrections are automatically implemented. Another outcome of incorporating into the U.S. federal regulations a mechanism to oversee audits or auditors would be the opportunity to include this on the package label, similar to the USDA label of inspection. Support for implementing such a strategy is based on studies conducted in China and Thailand, where safety claims were viewed as an important

factor by consumers with regard to purchasing the product or a willingness to pay more for the product (108, 109).

One of the challenges confronting the implementation of FSMA concerns its FSVP rule. In contrast to the previous policy regarding imported foods that relied on testing product as it came into the country, the current rule places the responsibility for safety of the food products on the importers who are introducing food products into the United States, and it is those entities that will be held responsible should any food safety problems arise. The justification for the FDA's major departure in their approach to ensuring the safety of imported foods was the increasing volume of food products being imported from foreign countries, limited official capacity to inspect foreign suppliers' sites and imported food at ports of entry, complexity of the food supply chain, and the alarming number of cases of food adulteration by foreign countries supplying food products to U.S. markets, for which China has been the most notorious.

The number of serious incidents of compromised food safety that China has experienced has had an economic ripple effect on its international trade. Hence, in addition to executing violators, China has been engaged in its own changes to its regulatory system to alleviate concerns by countries receiving their food products (110). More specifically, it revamped in 2015 its 2009 Food Safety Law (111). Unfortunately, expectations for several of its goals have not been met. For example, the 2015 amendment aimed to establish a national traceability mechanism for food safety; however, Lin (110) reported that a supply chain approach to food safety regulations, not to mention full traceability, had yet to be adopted. Furthermore, most loopholes present in the 2009 law (i.e., a thin rule of law [discrepancy between laws and enforcement], coordination between central and local governments, and a lack of supply chain approach) continue to be present in the 2015 amendment (110). However, two other mechanisms have played an increasingly important role in improving the regulatory effectiveness of China's food export system: (i) public-private partnerships such as those adopted by the European Union's food safety regulatory system (112) and (ii) the adoption of bilateral agreements between China and multiple World Trade Organization countries, including the United States (110).

In the case of the U.S.-China Food Safety Agreement, and in line with the mandates of the FSMA's FSVP rule, the FDA is now theoretically able to

expand its regulatory boundaries to suppliers in China. More specifically, the U.S.-China Food Safety Agreement incorporates three major components: (i) it provides for confidential exchange of foodborne-illness information as a means to contain and remedy the problem and to minimize the harm that could be inflicted on companies not involved in the incident; (ii) companies within China that export foods to the United States must be registered and be inspected on an annual basis by the Chinese government; and (iii) through offices established in Beijing, Shanghai, and Guangzhou, the FDA is empowered to conduct their own on-site inspections of Chinese companies exporting food to the United States (110). However, criticisms of the agreement are that the framework is based more on economic interests than on public health and that it applies only to specific products rather than all foods (110). Moreover, because of the decision to incorporate outsourcing of its regulatory authority to third parties as a major component of its policy, there is uncertainty as to its effectiveness and accountability. At issue is the structural mismatch between the broad scope of power granted to the FDA and the long chain of power out-sourcing to governments or private companies (i.e., FDA first delegates regulatory authority to accreditation bodies, which in turn accredit third-party auditors, who then certify that producers' food products are safe) (110). Hence, if these concerns materialize, they will likely be addressed in the future as food safety regulations and policies evolve.

Evolution of Food Safety Regulations and Policies

Quantitative microbiological risk assessment (QMRA), a structured probabilistic process for assigning a public health risk to a foodborne pathogen in a food product, has been and will continue to be an integral component in the development of regulations and policies addressing the safety of the world's food supply. Although the individual components within a QMRA have been identified, the degree of the contribution of some of the components may only be estimated. For example, current models are still only in their infancy in addressing the considerable biological variability and uncertainty that exist in food systems. Recent developments in "-omics" technologies (genomics and transcriptomics) may fill the knowledge gaps on pathogen strain diversity, virulence, and physiological variability. As these data gaps are filled and statistical techniques are refined to translate genome-level multidimensional

information into the single dimension associated with the measure of risk (113), QMRAs will become more robust. In turn, there will be greater assurance that the projected outcomes are realistic and could serve as the impetus to change policies if current management strategies are considered either of negligible effect or insufficient to enhance the safety of foods.

At present, the designated standards for the presence of foodborne pathogens within a food are often not consistent between countries, and this highlights the lack of harmonization that exists worldwide. For example, one standard that is not consistently applied between countries, because it is being used in many cases as a trade barrier, is zero tolerance (i.e., the pathogen must be absent from the food). Primarily intended to convey a level of concern in safeguarding the public from consumption of foods contaminated with pathogens, zero tolerance emerged as a target goal in the absence of being able to establish specific thresholds for highly infectious and toxico-infectious pathogens (114). Moreover, in instances when zero tolerance is being used, it is important to understand that it is an operational standard wherein the zero is being defined relative to a sampling plan and testing protocol (114). Otherwise, in the absence of these defining criteria, zero could be a moving target as different groups use different assays with different sensitivities. Zero tolerance will likely continue to be used as a target standard for the immediate future, despite some critics' argument that it is more realistic to reduce to a level that is as low as reasonably possible the prevalence of pathogens in ready-to-eat foods that do not receive a treatment to kill pathogens (115).

Regardless of the current or future criteria that are used to determine which food safety regulations and policies are developed and applied, there must be some measure(s) by which the standards contained within them can be judged to be effective. Presently, there are two main measures: (i) the prevalence of foodborne pathogens in foods or in the environments in which the foods are grown or processed and (ii) the prevalence of reported foodborne illnesses and outbreaks. In the absence of improvements in these measures, critics of the policies may have a valid argument that the costs for implementation of the standards do not provide sufficient public health benefits. As an example of the vulnerability of food safety regulations to such scrutiny, the current U.S.

administration, in line with the view that regulations stifle the economic growth of the country, indefinitely delayed implementation of several key provisions within the FSMA rules (116).

Economic considerations are not the only external force that may determine the type of regulations and their degree of stringency. Other competing priorities, including nutritional and sustainability issues (117), could also influence the type of regulations that would be enacted to protect the food supply. To assist in making decisions as to practices that could affect multiple outcomes (i.e., food safety, operational costs, environmental sustainability, and nutritional food value), a valuable new tool known as multidimensional criterion analysis was recently developed to address such multidimensional issues (118).

CONCLUDING REMARKS

Approaches to enhancing the safety of foods have been evolving over the past decade. As part of that evolution, the implementation of the FSMA is likely to lead to reductions in the microbiological risk associated with foods; however, consumers' desire for foods that carry a high microbiological risk has made it more difficult to achieve that goal. The adoption of WGS as a tool to identify and track pathogens through the food chain has led to the identification of outbreaks that would not otherwise have been detected; however, it is envisioned that over the long term, this tool will ultimately decrease both the number and size of outbreaks. Further advances in food safety are coming, but the degree to which they will be adopted will likely have to be weighed against how they affect other factors that the consumer or industry considers important (e.g., sustainability, nutritional components of the food, costs, convenience, and taste).

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