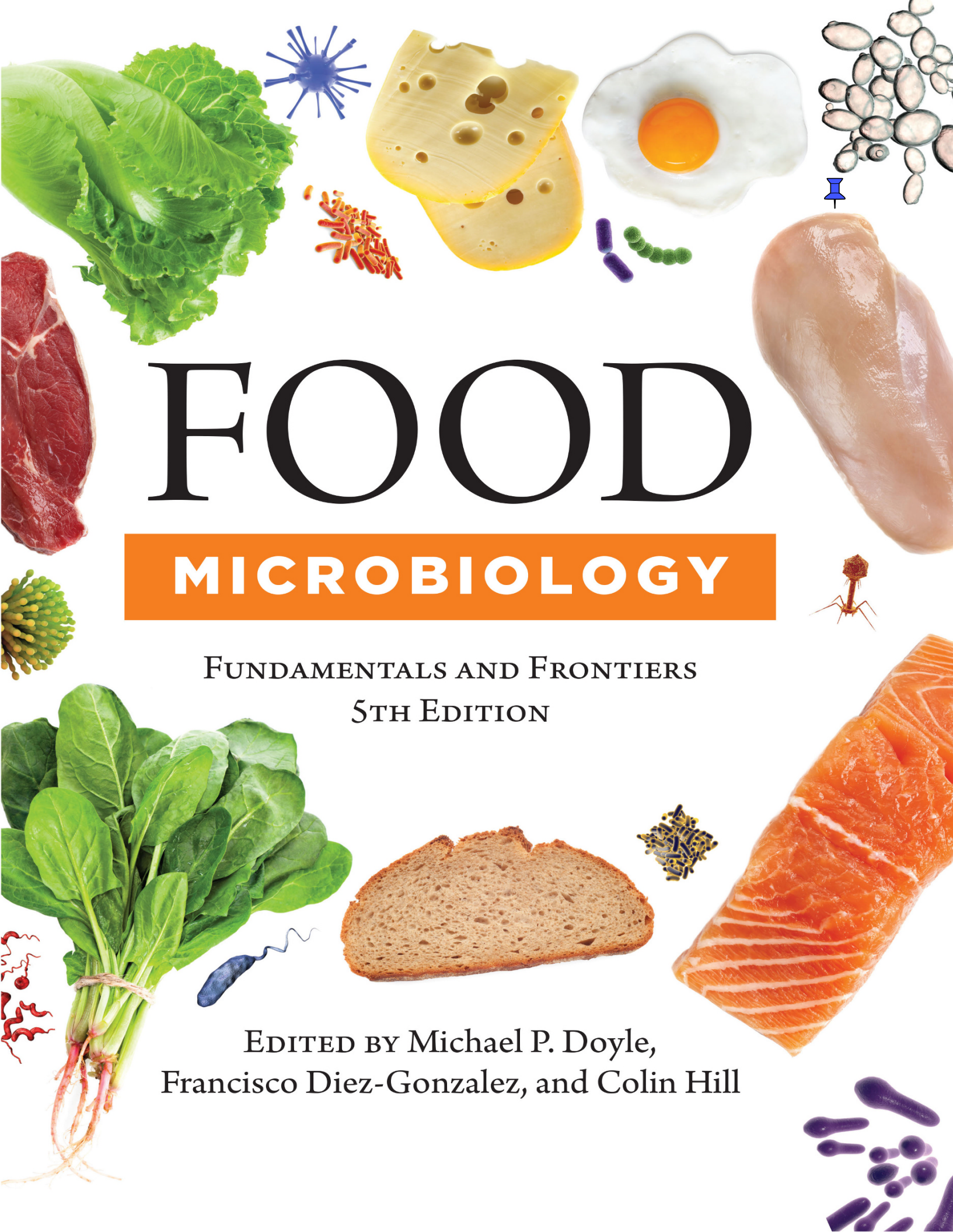

FOOD

MICROBIOLOGY

Fundamentals and Frontiers
5th Edition

Edited by Michael P. Doyle,
Francisco Diez-Gonzalez, and Colin Hill

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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.

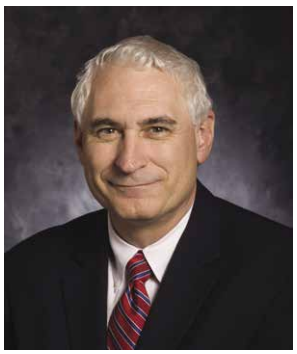
The sixth section, "Fermentations and Beneficial Microbes," addresses microbes and microbial substrates and end products that promote human health. Studies of the human intestinal microbiome have revealed exciting information regarding the significance of diet to the gut microbiota and the effects of 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.

Michael P. Doyle
Francisco Diez-Gonzalez
Colin Hill

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.

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Factors of Special Significance to Food Microbiology

I

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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

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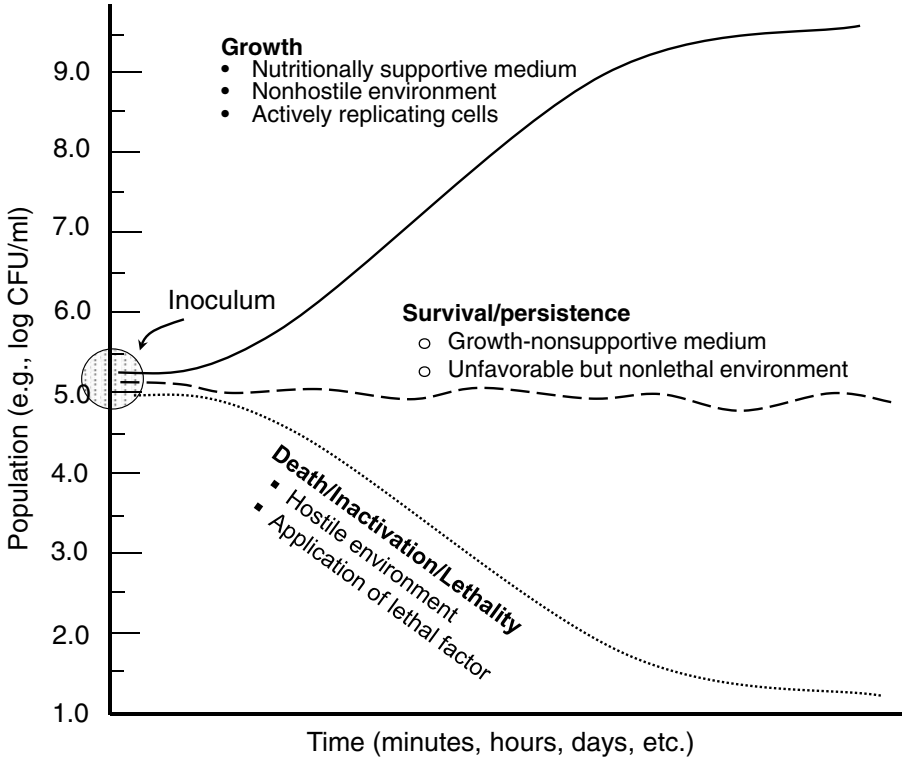

Figure 1.1 Conceptual behavior of microorganisms in various environments. The scale of the *y* axis symbolizes populations of single-celled foodborne microorganisms.

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, easy-to-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.

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 exponential-growth-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