2

Factors That Influence Microbes in Foods*

LEARNING OBJECTIVES

The information in this chapter will enable the student to:

- distinguish among different methods of culturing foodborne microbes and choose the correct technique for a given application
- recognize how intrinsic and extrinsic factors are used in controlling microbial growth
- quantitatively predict the size of microbial populations using the equations that describe the kinetics of microbial growth
- qualitatively describe how altering the variables in the equations for the kinetics of microbial growth alters growth patterns
- relate biochemical pathways to energy generation and metabolic products of foodborne bacteria

INTRODUCTION

Never say that you are *just* a food microbiologist. Food microbiology is a specialty area in microbiology. Just as a heart surgeon is more than a surgeon, a food microbiologist is a microbiologist with a specialty. Food microbiologists must understand microbes. They must understand complex food systems. Finally, they must integrate the two to solve microbiological problems in complex food ecosystems. Even the question "How many bacteria are in that food?" has no simple answer. The first part of this chapter examines the question of how we detect and quantify bacteria in food. We present the concept of culturability and ask if it really reflects viability. Even though injured or otherwise nonculturable cells cannot form colonies on petri dishes, they do exist and can cause illness.

The second part of the chapter presents internal and environmental factors that control bacterial growth. Ecology teaches that the *interaction* of factors determines which organisms can or cannot grow in an environment. The use of multiple environmental factors (i.e., pH, salt concentration, temperature, etc.) to inhibit microbial growth is called *hurdle technology.* Hurdle technology is becoming an increasingly popular preservation strategy.

The kinetics of microbial growth are covered in the third part of the chapter. All four phases of the microbial growth curve are important to food microbiologists. If the lag phase can be extended beyond the normal shelf life, the food is microbially safe. If the growth rate can be slowed so

*This chapter is based on one written by Thomas J. Montville and Karl R. Matthews for *Food Microbiology: Fundamentals and Frontiers,* 2nd ed. It has been adapted and augmented with material on prions for use in an introductory text.

BOX 2.1

Mechanisms of energy generation

Anaerobes cannot liberate all of the energy in a substrate. They typically make one or two ATP molecules by burning a mole of glucose. This process is called *substrate level phosphorylation*. Strict anaerobes are killed by air.

Aerobes can burn glucose completely in the presence of oxygen. They generate 34 ATP molecules from a mole of glucose through a process called *respiration*. Strict aerobes cannot grow in the absence of oxygen.

Facultative anaerobes can generate energy by both substrate level phosphorylation and respiration. They can grow in a wider variety of environments than strict aerobes or strict anaerobes.

that the population size is too small to cause illness, the product is safe. If the rate of the death phase can be controlled, it can be used to ensure safety, as in the case of cheddaring cheese made from unpasteurized milk.

The fourth part of the chapter introduces the physiology and metabolism of foodborne microbes. Because this book does not assume a background in biochemistry, the biochemistry associated with foodborne microbes is introduced. The biochemical pathways used by lactic acid bacteria determine the characteristics of fermented foods. Spoilage is also caused by a product(s) of a specific pathway(s). Lactic acid makes milk sour; carbon dioxide causes cans to swell. The ability of bacteria to use different biochemical pathways that generate different amounts of energy (in the form of adenosine triphosphate [ATP]) governs their ability to grow under adverse conditions. Organisms grown in the presence of oxygen (aerobically) produce more energy (ATP) than those grown in the absence of oxygen (anaerobically) (Box 2.1). Thus, organisms such as *Staphylococcus aureus* can withstand hostile environments better under aerobic conditions than under anaerobic conditions.

FOOD ECOSYSTEMS, HOMEOSTASIS, AND HURDLE TECHNOLOGY

Foods are ecosystems composed of the environment and the organisms that live in it. The food environment is composed of intrinsic factors inherent to the food (i.e., pH, water activity, and nutrients) and extrinsic factors external to it (i.e., temperature, gaseous environment, and the presence of other bacteria). Intrinsic and extrinsic factors can be manipulated to preserve food. When applied to microbiology, *ecology* was defined by the International Commission on Microbial Specifications for Foods as "the study of the *interactions* between the chemical, physical, and structural aspects of a niche and the composition of its specific microbial population." "Interactions" emphasizes the dynamic complexity of food ecosystems.

Foods can be heterogeneous on a micrometer scale. Heterogeneity and gradients of pH, oxygen, nutrients, etc., are key ecological factors in foods. Foods may contain multiple microenvironments. This is well illustrated by the food poisoning outbreaks in aerobic foods caused by the strict anaerobe *Clostridium botulinum*. *C. botulinum* growth in potatoes, sautéed onions, and cole slaw has caused botulism outbreaks. The oxygen in these foods is driven out during cooking and diffuses back in so slowly that most of the product remains anaerobic.

CLASSICAL MICROBIOLOGY AND ITS LIMITATIONS

Limitations of Detection and Enumeration Methods

All methods based on the plate count and pure-culture microbiology have the same limitations. The plate count assumes that every cell forms one colony and that every colony originates from only one cell. The ability of a given cell to grow to the size of a macroscopic colony depends on many things. These include the cell's physiological state, the growth medium used (and the presence or absence of selective agents), and the incubation temperature.

Figure 2.1 illustrates the concepts of plate counting, qualitative detection, and most-probable-number (MPN) methodologies. Consider a 50-ml

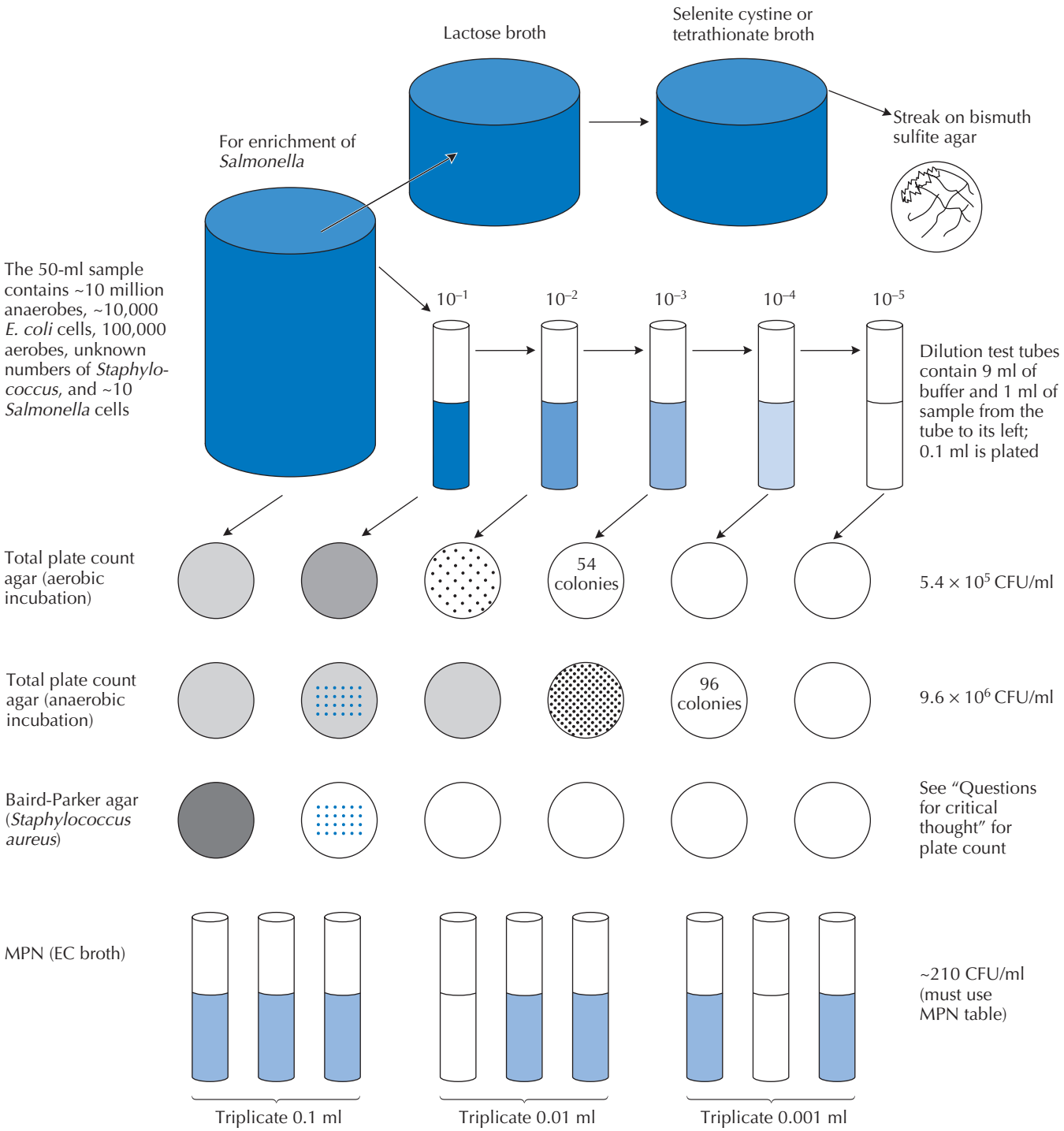


Figure 2.1 Methods of enumerating bacteria. The sample (which represents a 10^0 dilution) is diluted through a series of 10-fold dilutions prior to enumeration. To enumerate the bacteria, 0.1 ml from each dilution tube is plated on an agar plate with appropriate medium. After 24- to 48-h incubations, the colonies are counted and multiplied by the dilution factor to give the microbial load of the original sample on a per-milliliter basis. The color intensity indicates the culture density. If the number of cells present is expected to be low, the MPN technique is used. In this case, the sample is diluted into triplicate series of test tubes containing the media, and the pattern of positive tubes is used to determine the MPN from an MPN table (Table 2.2).

BOX 2.2

Powers of 10

Because microbial populations are so large, microbiologists usually speak in powers of 10. The answer to the question "How many bacteria are in that culture?" would probably be "10^8 or 10^9." This suggests that there is not much difference between 100 million and 1 billion microbes. Usually there is not. Try to remember these powers of 10:

100	$= 10^2$
1,000	$= 10^3$
100,000	$= 10^5$
1,000,000	$= 10^6$
1,000,000,000	$= 10^9$

When bacteria are being killed, a similar vocabulary is used. A 1-log-unit reduction is equal to a 10-fold reduction (see table): 90% of the population has been killed. This may sound effective, but if the initial number is 10^6, 10^5 are still left. That's a lot! For practical purposes, reductions of <1 log unit have no real significance. Regulatory agencies frequently require an "x log" reduction in microbial populations. "x" is product and process dependent, equaling 7 for pasteurized milk and 12 for low-acid canned food.

Log unit reduction	% Reduction
1	90
2	99
3	99.9
4	99.99
5	99.999
6	99.9999
7	99.99999
12	99.9999999999

sample of milk that contains 10^6 colony-forming units (CFU) of anaerobic bacteria per ml, 10^5 aerobic bacteria, a significant number of *S. aureus* (a pathogen) organisms, some *Escherichia coli* organisms, and a few salmonellae (Box 2.2). (A CFU ideally would be a single cell, but in reality it could be a chain of 10 or a clump of 100.) Since the bacteria are either too numerous to be counted directly or too few to be found easily, samples have to be diluted or enriched.

Plate Counts

Plate counts are used to quantify bacterial populations of >250 CFU/ml (for liquids) or >2,500 CFU/ml (for solids, which must be diluted 1:10 in a liquid to be pipettable). The food sample is first homogenized 1:10 (wt/vol) in a buffer to give a 10-fold dilution. This is further diluted through a series of 10-fold dilutions to give 10-, 100-, 1,000-, 10,000-fold, etc., dilutions and spread onto an agar medium. After incubation at 35°C, the average number of colonies per plate (on plates having between 25 and 250 colonies) is determined and multiplied by the dilution factor to give the number of bacteria in the original sample. This sounds simple, right? Wrong. At one time, the procedure described above was referred to as the *total plate count.* However, it counts only those bacteria that can grow on that agar medium at 35°C. The anaerobes, which are present in higher numbers, are not detected because they cannot grow in the presence of air. Bacteria that grow only below 15°C might be good predictors of refrigerated milk's shelf life. They are not detected either. *S. aureus,* which can make you sick, would be lost in the crowd of harmless bacteria. Variations of the standard aerobic plate count consider these issues. Plate counts can be incubated at different temperatures or under different atmospheres to increase their specificities for certain types of bacteria.

Table 2.1 Some selective media used in food microbiology

Bacterium	Medium	Selective agent(s)
S. aureus	Baird-Parker agar[a]	Lithium chloride, glycine, tellurite
Listeria species	LPM	Lithium chloride, phenylethanol, antibiotic
Lactic acid bacteria	deMann-Rogosa-Sharp (MRS)	Low pH, surfactant
Campylobacter species	Abeyta-Hunt-Bark agar	Microaerobic incubation, antibiotics

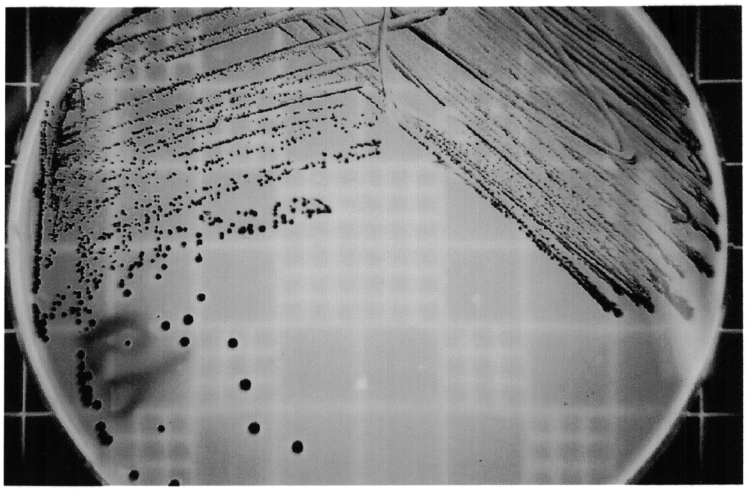
[a]The highest honor for a microbiologist is to have an organism named after oneself. Not far behind, and much easier to attain, is naming a medium after oneself.

Selective, or Differential, Media

The concept of selective media is relatively simple. Some ingredient of the medium helps (in the case of an unusual sugar used as a carbon source) the growth of the target bacteria or inhibits (in the cases of antibiotics, salt, surfactants, etc.) the growth of bacteria other than the target bacteria. Some examples of selective media are given in Table 2.1. In the case of Baird-Parker agar (Fig. 2.2), lithium chloride, glycine, and tellurite allow *S. aureus* to grow while suppressing a larger number of other organisms. In addition, the *S. aureus* colonies are a distinctive black color. However, target organisms that are injured may be killed by the selective agent. In addition, the selective media are not entirely selective. For example, *Listeria monocytogenes* can grow on *Lactobacillus* deMann-Rogosa-Sharp (MRS) medium. Thus, the identities of suspect pathogens isolated on selective media must be confirmed by biochemical or genetic methods.

Differential media are those designed to make a colony of the target organism look different and stand out from the background of other organisms. For example, *S. aureus* colonies are black with a precipitation zone on Baird-Parker agar. *L. monocytogenes* has a blue sheen on McBride's agar and turns Fraiser broth black.

Figure 2.2 *S. aureus* forms black colonies when grown on Baird-Parker agar.



Most-Probable-Number Methods

MPN methods are used to estimate low numbers of organisms in a sample (Fig. 2.1 and Table 2.2). They are used most commonly for *E. coli* and coliform bacteria. Selective agents are added to broth medium. One milliliter of sample or diluted sample is transferred to each of three test tubes of medium for each of at least three 10-fold serial dilutions. The samples are incubated, and the pattern of positive tubes is recorded. This pattern is used to derive the most probable number of bacteria from a statistical chart. The lower sensitivity limit for a three-tube MPN is <3.0 bacteria per g. This can be lowered to <0.96 bacterium per g by using 10 tubes at each dilution instead of 3, but this is rarely cost-effective.

Enrichment Techniques

There is zero tolerance (actually, 0/25 g) for *Salmonella*, *L. monocytogenes*, and *E. coli* O157:H7 in ready-to-eat foods. The good news is that this makes it unnecessary to count these pathogens. The bad news is that one must be able to find one salmonella organism hiding among a million other bacteria. This is done by coupling preenrichment medium with selective enrichment, followed by plating the bacteria on selective medium and biochemical or genetic confirmation (Fig. 2.1). The preenrichment in a nonselective medium allows injured pathogens to repair themselves, recover, and regain resistance to selective agents. The enrichment step uses selective agents to promote pathogen growth to high levels while inhibiting growth of the background organisms. Then it is possible to isolate the pathogen on a selective agar and confirm the identity of the colony using other methods.

Table 2.2 Three-tube MPN table for quantifying low levels of bacteria[a]

No. of positive tubes			MPN/g	Confidence limits		No. of positive tubes			MPN/g	Confidence limits	
0.10	0.01	0.001		Low	High	0.10	0.01	0.001		Low	High
0	0	0	<3.0		9.5	2	2	0	21	4.5	42
0	0	1	3.0	0.15	9.6	2	2	1	28	8.7	94
0	1	0	3.0	0.15	11	2	2	2	35	8.7	94
0	1	1	6.1	1.2	18	2	3	0	29	8.7	94
0	2	0	6.2	1.2	18	2	3	1	36	8.7	94
0	3	0	9.4	3.6	38	3	0	0	23	4.6	94
1	0	0	3.6	0.17	18	3	0	1	38	8.7	110
1	0	1	7.2	1.3	18	3	0	2	64	17	180
1	0	2	11	3.6	38	3	1	0	43	9	180
1	1	0	7.4	1.3	20	3	1	1	75	17	200
1	1	1	11	3.6	38	3	1	2	120	37	420
1	2	0	11	3.6	42	3	1	3	160	40	420
1	2	1	15	4.5	42	3	2	0	93	18	420
1	3	0	16	4.5	42	3	2	1	150	37	420
2	0	0	9.2	1.4	38	3	2	2	210	40	430
2	0	1	14	3.6	42	3	2	3	290	90	1,000
2	0	2	20	4.5	42	3	3	0	240	42	1,000
2	1	0	15	3.7	42	3	3	1	460	90	2,000
2	1	1	20	4.5	42	3	3	2	1,100	180	4,100
2	1	2	27	8.7	94	3	3	3	>1,100	420	

[a]Source: http://www.cfsan.fda.gov/~ebam/bam-a2.html#tables. For three tubes with 0.1-, 0.01-, and 0.001-g inocula, the MPN per gram and 95% confidence intervals are shown.

Table 2.3 Influence of thermal history and enumeration protocols on experimentally determined $D_{55°C}$ values for *L. monocytogenes*

Atmosphere	$D_{55°C}$ value (min)			
	TSAY medium		McBride's medium	
	+ Heat shock	− Heat shock	+ Heat shock	− Heat shock
Aerobic	18.7	8.8	9.5	6.6
Anaerobic	26.4	12.0	No growth	No growth

[a] +, with; −, without.

Values derived from plate counts are also influenced by these factors. Table 2.3 illustrates these points by providing $D_{55°C}$ values (the time required to kill 90% of the population at 55°C) for *L. monocytogenes* with different thermal histories (heat shocked for 10 min at 80°C or not heat shocked) on selective (McBride's) or nonselective (tryptic soy agar with yeast extract [TSAY]) medium under an aerobic or anaerobic atmosphere. Cells that have been heat shocked and recovered on TSAY medium are about four times more heat resistant (as shown by the larger *D* values) than cells which have not been heat shocked and recovered on McBride's medium under aerobic conditions. Injured cells and cells that are viable but nonculturable (VNC) pose additional problems for food microbiologists. While they cannot form colonies and be detected, they can make people sick.

Injury

Microbes may be injured rather than killed by *sublethal* levels of heat, radiation, acid, or sanitizers. Injured cells are less resistant to selective agents or have increased nutritional requirements. Injury is a complex process influenced by time, temperature, the concentration of the injurious agent, the strain of the target pathogen, the experimental methodology, and other factors. For example, a standard sanitizer test may suggest that a sanitizer *kills* listeria cells. However, cells might be recovered by using listeria repair broth. Injured cells die as the exposure time and sanitizer concentration increase.

Data that illustrate injury are given in Fig. 2.3. Cells subjected to mild stress are plated on a rich nonselective medium and on a selective medium containing 6% salt. The difference between the populations of cells able to form colonies on the two media represents injured cells. (If 10^7 CFU of a population/ml are found on the selective medium and 10^4 CFU/ml can grow on the nonselective medium, then 10^3 CFU/ml are injured.) Media for recovery of injured cells are often specific for a given bacteria or stress condition.

Injury is important to food safety for several reasons. (i) If injured cells are classified as dead during heat resistance determinations, the effect of heating will be overestimated and the resulting heat process will be ineffective. (ii) Injured cells that escape detection at the time of postprocessing sampling may repair themselves before the food is eaten and cause illness. (iii) The selective agent may be a common food ingredient, such as salt or organic acids or even suboptimal temperature. For example, *S. aureus* cells injured by acid during sausage fermentation can grow on tryptic soy agar but not on tryptic soy agar +7.5% salt. These injured cells can repair themselves in sausage if held at 35°C (but not if held at 5°C) and then grow and produce enterotoxin.

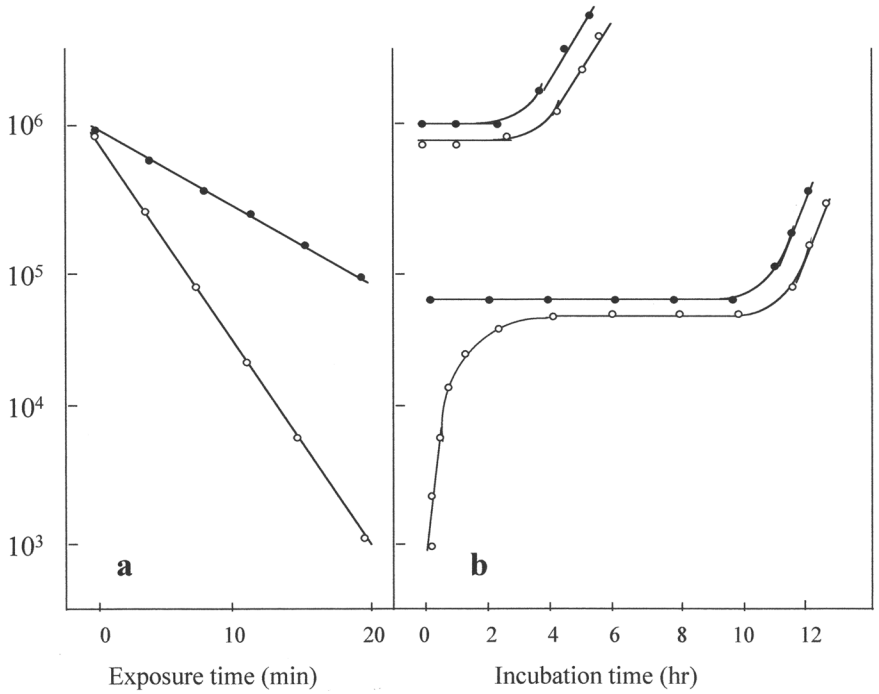


Figure 2.3 Data indicative of injury and repair. When bacteria are plated on selective (○) or nonselective (●) medium during exposure to some stressor (e.g., heat [a]), the decrease in CFU on a nonselective medium represents the true lethality, while the difference between the values obtained the two media is defined as injury. During repair (b), resistance to selective agents is regained, and the value obtained on the selective medium approaches that on the nonselective medium. Unstressed controls are shown at the top of panel b. (Source: M. P. Doyle, L. R. Beuchat, and T. J. Montville [ed.], *Food Microbiology: Fundamentals and Frontiers,* 2nd ed. [ASM Press, Washington, D.C., 2001].)

Repair is the process by which cells recover from injury. Repair requires RNA and protein synthesis and is often made evident by lengthening of the lag phase. Environmental factors influence the extent and rate of repair. The microbial stability of cured luncheon meat is due to the extended lag period required for the repair of spoilage organisms.

Viable but Nonculturable

Salmonella, Campylobacter, Escherichia, Shigella, and *Vibrio* species can exist in a state in which they are viable but cannot be cultured by normal methods. The differentiation of vegetative cells into a dormant VNC state is a survival strategy for many nonsporulating bacteria. During the transition to the VNC state, rod-shaped cells shrink and become small spherical bodies. These are totally different from bacterial spores. It takes from 2 days to several weeks for an entire population of vegetative cells to become VNC.

Although VNC cells cannot be cultured, their viability can be determined through microscopic methods. Fluorescent molecular probes are used to determine the integrity of the bacterial membrane. Bacteria with intact cell membranes stain fluorescent green, but bacteria with damaged membranes stain fluorescent red. Dyes can also be used to identify VNC cells, since respiring cells reduce the colorless soluble dye to a colored insoluble compound that can be seen under the microscope. Powerful new

methods for detecting VNC cells are being developed as our understanding of bacteria at the molecular level increases.

Because the VNC state is most often induced by nutrient limitation in aquatic environments, it might appear irrelevant to nutrient-rich food environments. However, changes in the salt concentration, exposure to sanitizers, and shifts in temperature can also cause the VNC state. When *Vibrio vulnificus* (10^5 CFU/ml) is shifted to refrigeration temperatures, it becomes nonculturable (<0.04 CFU/ml) but is still lethal to mice. The bacteria resuscitate in the mice and can be isolated postmortem using culture methods. Foodborne pathogens can become VNC when shifted to a cold temperature in nutritionally rich media. This has chilling implications for the safety of refrigerated foods.

Our increased awareness of the VNC state should lead to the reexamination of our concept of viability, our dependence on enrichment culture to isolate pathogens, and our reliance on culture methods to monitor microbes in the environment. We do not understand the mechanisms of VNC formation, the mechanisms that make VNC cells resistant to stressors, and what event signals the cell to resuscitate. The relationship between viability and culturability needs to be better understood.

Intrinsic Factors That Influence Microbial Growth

Characteristics of the food itself are called *intrinsic* factors. These include naturally occurring compounds that influence microbial growth, compounds added as preservatives, the oxidation-reduction potential, water activity, and pH. Most of these factors are covered separately in the chapters on physical and chemical methods of food preservation.

The influence of pH on gene expression is a relatively new area. The expression of genes governing proton transport, amino acid degradation, adaptation to acidic or basic conditions, and even virulence can be regulated by the external pH. Cells sense changes in acidity (pH) through several mechanisms:

1. Organic acids enter the cell only in the protonated (undissociated) form. Once inside the cell, they dissociate, releasing H^+ (the proton) into the cell cytoplasm. The cell may sense the accumulation of the resulting anions, which cannot pass back across the cell membrane. Additionally, they may sense the released protons, which cause the cytoplasm to become more acidic.
2. The change in pH results in a change in the transmembrane proton gradient (i.e., the difference in pH between the inside and outside of the cell), which can also serve as a sensor to start or stop energy-dependent reactions.
3. The changes in acidity inside the cell may result in the protonation or deprotonation of amino acids in proteins. This may alter the secondary or tertiary structures of the proteins, changing their functions and signaling the change in pH to the cell.

Cells must maintain their intracellular pH (pH_i) above some critical pH_i at which intracellular proteins become denatured. *Salmonella enterica* serovar Typhimurium has three progressively more stringent mechanisms to maintain a pH_i that supports life. These three mechanisms are the homeostatic response, the acid tolerance response (ATR), and the synthesis of acid shock proteins.

At a pH outside (pH_o) of >6.0, salmonella cells adjust their pH_i through the *homeostatic* response. The homeostatic response maintains the pH_i by increasing the activity of proton pumps to expel more protons from the cytoplasm. The homeostatic mechanism is always on and functions in the presence of protein synthesis inhibitors.

The ATR is triggered by a pH_o of 5.5 to 6.0. This mechanism is sensitive to protein synthesis inhibitors; there are at least 18 ATR-induced proteins. ATR appears to involve the membrane-bound ATPase proton pump and maintains the pH_i at >5.0 at pH_o values as low as 4.0. The loss of ATPase activity caused by gene disruption mutations or metabolic inhibitors abolishes the ATR but not the pH homeostatic mechanism.

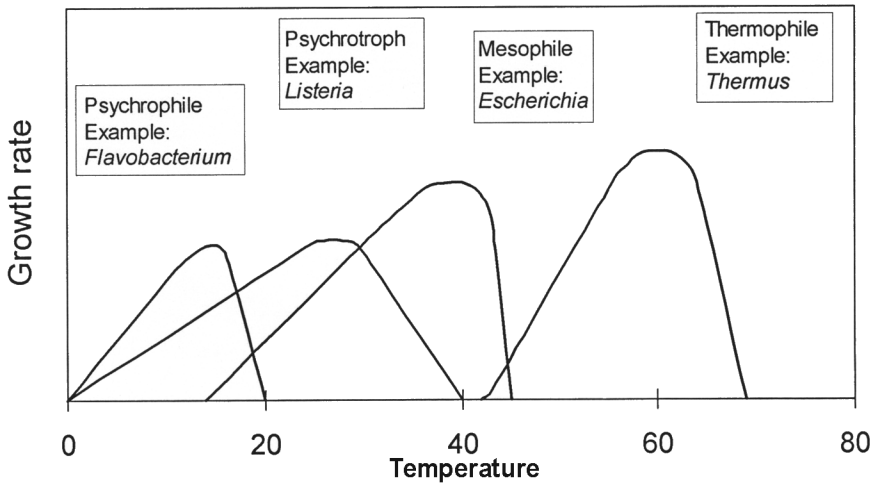
The synthesis of acid shock proteins is the third way that cells regulate pH_i. The synthesis of these proteins is triggered by a pH_o of 3.0 to 5.0. They constitute a set of regulatory proteins distinct from the ATR proteins.

Extrinsic Factors That Influence Microbial Growth

Extrinsic factors are *external* to the food. Temperature and gas composition are the main extrinsic factors influencing microbial growth. The influence of temperature on microbial growth and physiology is huge. While the influence of temperature on the growth rate is obvious and is covered in some detail here, the influence of temperature on gene expression is equally important. Cells grown at refrigeration temperature do not just grow more slowly than those grown at room temperature, they express different genes and are physiologically different. Later chapters provide details about how temperature regulates the expression of genes governing traits ranging from motility to virulence in specific organisms.

A rule of thumb in chemistry suggests that reaction rates double with every 10°C increase in temperature. This simplifying assumption is valid for bacterial growth rates only over a limited range of temperatures (Fig. 2.4). Above the optimal growth temperature, growth rates decrease rapidly. Below the optimum, growth rates also decrease, but more gradually. Bacteria can be classified as psychrophiles, psychrotrophs, mesophiles, and thermophiles according to how temperature influences their growth.

Figure 2.4 Relative growth rates of bacteria at different temperatures. (Source: M. P. Doyle, L. R. Beuchat, and T. J. Montville [ed.], *Food Microbiology: Fundamentals and Frontiers,* 2nd ed. [ASM Press, Washington, D.C., 2001].)



Both *psychrophiles* and *psychrotrophs* grow, albeit slowly, at 0°C. True psychrophiles have optimum growth rates at 15°C and cannot grow above 25°C. Psychrotrophs, such as *L. monocytogenes* and *C. botulinum* type E, have an optimum growth temperature of ~25°C and cannot grow at >40°C. Because these foodborne pathogens, and even some mesophilic *S. aureus* strains, can grow at <10°C, conventional refrigeration is inadequate to ensure the safety of a food.

The growth temperature influences a cell's thermal resistance. *L. monocytogenes* cells preheated at 48°C have increased heat resistance. Holding listeria cells at 48°C for 2 h in sausages doubles their heat resistance at 64°C. This thermotolerance is maintained for 24 h at 4°C. Subjecting *E. coli* O157:H7 to sublethal heating at 46°C increases its thermal resistance at 60°C by 50%. Shock proteins synthesized in response to one stress may provide cross-protection against other stresses. For example, cells exposed to acid may become more heat resistant.

Homeostasis and Hurdle Technology

Consumer demands have decreased the use of intrinsic factors, such as acidity and salt, as the sole means of inhibiting microbes. Many food products use multiple-hurdle technology to inhibit microbial growth. Instead of setting one environmental parameter to the extreme limit for growth, hurdle technology "deoptimizes" several factors. For example, limiting the amount of available water to a water activity (a_w, the equilibrium relative humidity of the product's atmosphere divided by 100) of <0.85 *or* a limiting pH of 4.6 prevents the growth of foodborne pathogens. Hurdle technology might obtain similar inhibition at pH 5.2 *and* an a_w of 0.92.

Hurdle technology assaults multiple homeostatic processes (Box 2.3). For example, the intracellular pH must be maintained within narrow limits. This is done by using energy to pump out protons, as described above. In low-a_w environments, cells must use energy to accumulate compatible solutes. Membrane fluidity must be maintained through homeoviscous adaptation, another energy-requiring process. The expenditure of energy to maintain homeostasis is fundamental to life. When cells channel the energy needed for growth into maintenance of homeostasis, their growth is inhibited. When the energy demands of homeostasis exceed the cell's energy-producing capacity, the cell dies.

Growth Kinetics

Growth curves showing the lag, exponential, stationary, and death phases of a culture are normally plotted as the number of cells on a log scale, or $\log_{10}$ cell number versus time. These graphs represent the state of microbial populations rather than individual microbes. Thus, both the lag phase and the stationary phase of growth represent periods when the growth rate equals the death rate to produce no net change in cell numbers. Food microbiology is the only area of microbiology where all four phases of the microbial growth curve are important. Microbial inhibitors can extend the lag phase, decrease the growth rate, decrease the size of the stationary phase population, and increase the death rate.

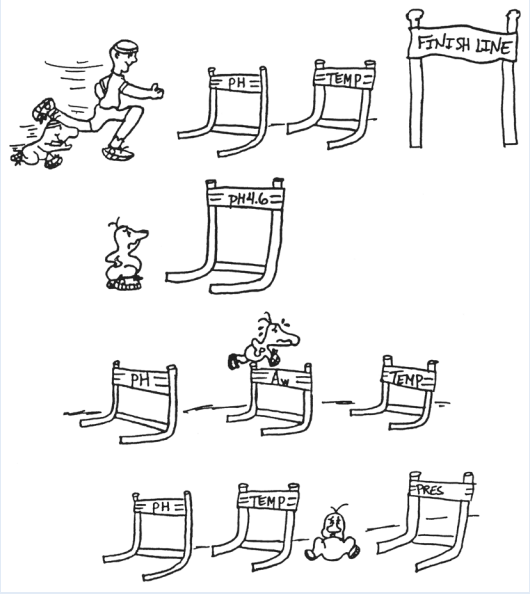
During the lag phase, cells adjust to their new environment by turning genes on or off, replicating their genes, and in the case of spores, differentiating into vegetative cells (see chapter 3). The lag phase duration depends on the temperature, the inoculum size (larger inocula usually have shorter

BOX 2.3

Hurdle theory and interactions of inhibitors

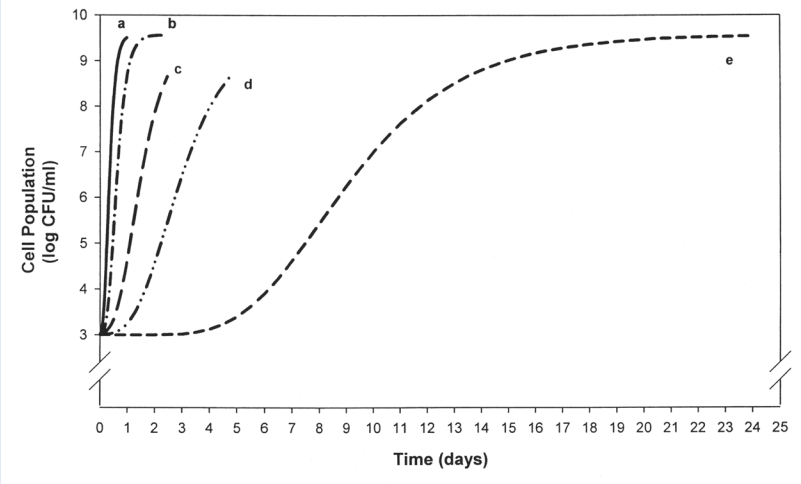
Food preservation can be thought of as a race between the human and the microbe to see who gets to eat first (see cartoon). Some of the preservative hurdles, such as pH 4.6 for *C. botulinum*, are just too big. They can stop a microbe when used alone. However, more often, combinations of preservatives are used. These may take so much of the microbe's energy that it slows down or even stops.

Food microbiologists agree that the interactions among preservatives are of utmost importance. These interactions are difficult to envision and hard to teach. The interaction of temperature, pH, and water activity on the growth of *L. monocytogenes* is illustrated more quantitatively in the graph. Under optimal conditions of 30°C, pH 6.5, and an a_w of 0.99 (plot a), the population grows rapidly to high levels. Dropping the pH to 5.5 (plot b) slows down the organism a little. Dropping the temperature by 15°C (plot c) has a greater, but still not very large, effect. When the pH and the temperature are both dropped (plot d), there is a much greater effect. At this point, the addition of a third hurdle (a_w = 0.95) (plot e) makes a radical improvement, and it takes the population 15 rather than 5 days to reach maximum size.



Courtesy of Donna Curran.

The data for this figure were taken from the U.S. Department of Agriculture pathogen-modeling program. This interactive program is wonderful for demonstrating interactions against a variety of foodborne pathogens, and it can be downloaded from http://www.ars.errc/mfs/PATHOGEN.htm.



lag phases), and the physiological history of the organism. If actively growing cells are transferred into an identical fresh medium at the same temperature, the lag phase may vanish. These factors can also be manipulated to extend the lag phase to the point where some other quality attribute of the food (such as proteolysis or browning) becomes unacceptable. Foods are considered microbially safe if the food spoils before pathogens become an issue. However, "spoiled" is a subjective and culturally biased concept. It is safer to produce conditions that prevent cell growth regardless of time (such as reduction of the pH to <4.6 to inhibit botulinal growth).

Table 2.4 Representative specific growth rates and doubling times of microorganisms

Organism	Conditions	$\mu(h^{-1})$	t_d(h)
Bacteria	Optimal	2.3	0.3
	Limited nutrients	0.20	3.46
	Psychrotroph, 5°C	0.023	30
Molds	Optimal	0.1–0.3	6.9–20

During the log, or exponential, phase of growth, bacteria reproduce by binary fission. One cell divides into two cells that divide into four cells that divide into eight cells, etc. Food microbiologists often use doubling times (t_d) as the constant to describe the rate of logarithmic growth. Doubling times, which are also referred to as generation times (t_{gen}), are inversely related to the specific growth rate (μ), as shown in Table 2.4.

Equations can be used to calculate the influence of different variables on a food's final microbial load. The number of organisms (N) at any time (t) is proportional to the initial number of organisms (N_0).

$$N = N_0 e^{\mu t} \quad \textbf{(1)}$$

Thus, decreasing the initial microbial load 10-fold will reduce the number of cells at any time by 10-fold. Because μ and t are in the power function of the equation, they have a greater effect on N than does N_0. Consider a food where N_0 is 1×10^4 CFU/g and μ is 0.2 h^{-1} at 37°C. After 24 h, N would be 1.2×10^6 CFU/g. Reducing the initial number 10-fold reduces the number after 24 h 10-fold to 1.2×10^5 CFU/g. However, reducing the temperature from 37 to 7°C has a more profound effect. If the growth rate decreases twofold with every 10°C decrease in temperature, then μ will be decreased eightfold to 0.025 h^{-1} at 7°C. When equation 1 is solved using these values (i.e., $N = 10^4 e^{0.025 \times 24}$), N at 24 h is 1.8×10^4 CFU/g. Both time and temperature have much greater influence over the final number of cells than does the initial microbial load.

How long it will take a microbial population to reach a certain level can be determined from the following equation:

$$2.3 \log(N/N_0) = \mu \Delta t \quad \textbf{(2)}$$

Consider the case of ground meat manufactured with an N_0 of 10^4 CFU/g. How long can it be held at 7°C before reaching a level of 10^8 CFU/g? According to equation 2, $t = \{2.3(\log 10^8/10^4)\}/0.025$, or 368 h.

Food microbiologists frequently use doubling times to describe growth rates of foodborne microbes. The relationship between t_d and μ is more obvious if equation 2 is written using natural logarithms (i.e., $\ln[N/N_0] = \mu \Delta t$) and solved for the condition where $t = t_d$ and $N = 2N_0$. Since the natural logarithm of 2 is 0.693, the solution is

$$0.693/\mu = t_d \quad \textbf{(3)}$$

Some typical specific growth rates and doubling times are given in Table 2.4.

MICROBIAL PHYSIOLOGY AND METABOLISM

Think about your room. Its natural state is disordered. It takes energy to combat the disorder. This is as it should be—it is a law of the universe. (Tell that to your mother, roommate, or partner the next time she or he

Authors' note

We have written this book assuming no coursework in biochemistry, but microbes cannot be understood without understanding some biochemistry. Consider this section your minimal primer in biochemistry.

complains about your messy room.) The second law of thermodynamics dictates that *all* things go to a state of maximum disorder in the absence of energy input. Since life is a fundamentally ordered process, all living things must generate energy to maintain their ordered state. Foodborne bacteria do this by oxidizing reduced compounds. Oxidation occurs only when the oxidation of one compound is coupled to the reduction of another. In the case of aerobic bacteria, the initial carbon source, for example, glucose, is oxidized to carbon dioxide, oxygen is reduced to water, and 34 ATP molecules are generated. Most of the ATP is generated through oxidative phosphorylation in the electron transport chain. In *oxidative phosphorylation*, energy is generated (in the same sense that money is made to be spent later; think of ATP as the cell's energy currency) when oxygen is used as the terminal electron acceptor. This drives the formation of a high-energy bond between free phosphate and adenosine diphosphate (ADP) to form ATP. Anaerobic bacteria, which do not use oxygen, must use an internal organic compound as an electron acceptor in a process of fermentation. They generate only 1 or 2 moles (mol) of ATP per mol of glucose used. The ATP is formed by *substrate-level phosphorylation*, and the phosphate group is transferred from a phosphorylated organic compound to ADP to make ATP.

Carbon Flow and Substrate-Level Phosphorylation

The Embden-Meyerhoff-Parnas Pathway Forms Two ATP Molecules from Six-Carbon Sugars

The Embden-Meyerhoff-Parnas (EMP) pathway (Fig. 2.5) is the most common pathway for glucose catabolism (glycolysis). The overall rate of glycolysis is regulated by the activity of phosphofructokinase. This enzyme converts fructose-6-phosphate to fructose-1,6-bisphosphate. Phosphofructokinase activity is regulated by the binding of adenosine monophosphate (AMP) or ATP to inhibit or stimulate (respectively) the phosphorylation of fructose-6-phosphate. Fructose-1,6-bisphosphate activates lactate dehydrogenase (see below) so that the flow of carbon to pyruvate is tightly linked to nicotinamide adenine dinucleotide (NAD) regeneration. This occurs when pyruvate is reduced to lactic acid, the only product of this homolactic fermentation. Two ATP molecules are formed in this pathway.

The Entner-Doudoroff Pathway Allows the Use of Five-Carbon Sugars and Makes One ATP Molecule

The Entner-Doudoroff pathway is an alternate glycolytic pathway that yields one ATP molecule per molecule of glucose and diverts one three-carbon unit to biosynthesis. In aerobes that use this pathway, such as *Pseudomonas* species, the difference between forming one ATP molecule by this pathway versus the two ATP molecules formed by the EMP pathway is inconsequential compared to the 34 ATP molecules formed from oxidative phosphorylation. In the Entner-Doudoroff pathway, glucose is converted to 2-keto-3-deoxy-6-phosphogluconate. The enzyme keto-deoxy phosphogluconate aldolase cleaves this to one molecule of pyruvate (directly, without the generation of an ATP molecule) and one molecule of 3-phosphoglyceraldehyde. The 3-phosphoglyceraldehyde then follows the same reactions of the EMP pathway. One ATP molecule is made by substrate-level phosphorylation using phosphoenol pyruvate to donate the phosphoryl group.

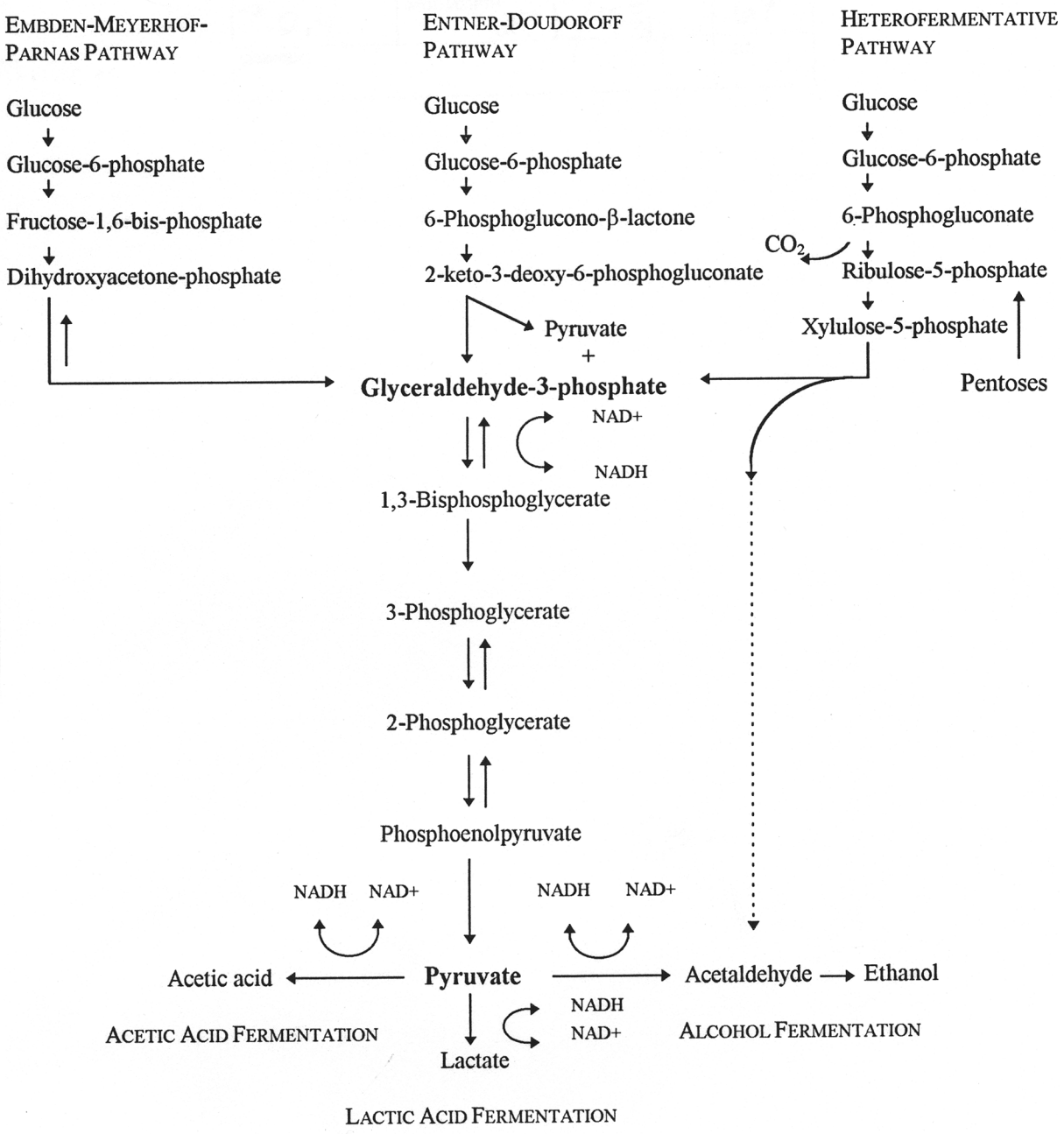


Figure 2.5 Major catabolic pathways used by foodborne bacteria. (Source: M. P. Doyle, L. R. Beuchat, and T. J. Montville [ed.], *Food Microbiology: Fundamentals and Frontiers,* 2nd ed. [ASM Press, Washington, D.C., 2001].)

Homolactic Catabolism Makes Only Lactic Acid

Homolactic bacteria in the genera *Lactococcus* and *Pediococcus,* and some *Lactobacillus* species, produce lactic acid as the sole fermentation product. The EMP pathway is used to produce pyruvate, which is then reduced by lactate dehydogenase, forming lactic acid and regenerating NAD.

Heterofermentative Catabolism Gives Several End Products

Heterofermentative bacteria, such as leuconostocs and some lactobacilli, have neither aldolases nor keto-deoxy phosphogluconate aldolase. The heterofermentative pathway is based on the catabolism of five-carbon sugars (pentoses). The pentose can be transported into the cell or made by decarboxylating hexoses. In either case, the pentose is converted to xylulose-5-phosphate, with ribulose-5-phosphate as an intermediate. The xylulose-5-phosphate is split into glyceraldehyde-3-phosphate and a two-carbon unit. The two-carbon unit can be converted to acetaldehyde, acetate, or ethanol. Although this pathway yields only one ATP molecule, it offers cells a competitive advantage by allowing them to utilize pentoses that homolactic organisms cannot use.

The Tricarboxylic Acid Cycle Links Glycolysis to Aerobic Respiration

The tricarboxylic acid (TCA) cycle links gylcolytic pathways to respiration. It generates reduced NAD (NADH) and reduced flavin adenine dinucleotide (FADH) as substrates for oxidative phosphorylation and makes some ATP through substrate level phosphorylation. With each turn of the TCA cycle, the reaction 2 pyruvate + 2 ADP + 2 FAD + 8 NAD $\rightarrow$ $6CO_2$ + 2 ATP + 2 FADH + 8 NADH occurs, and oxygen is used as the terminal electron acceptor to form water. The TCA cycle is used by all aerobes. Anaerobes may have some of the enzymes in the TCA cycle, but not enough to complete the full cycle.

The TCA cycle is also the basis for two industrial fermentations important to the food industry. The industrial fermentations for the acidulant citric acid and the flavor enhancer glutamic acid have similar biochemical bases. Both fermentations take advantage of impaired TCA cycles.

CONCLUSION

Microbial growth in foods is complex. It is governed by genetic, biochemical, and environmental factors. Developments in molecular biology and microbial ecology will change or deepen our perspective on the growth of microbes in foods. Some of these developments are detailed in this book. Other developments will unfold over the coming decades, perhaps accomplished by readers whose journey begins here.

Summary

- Food microbiology is a highly specialized subfield of microbiology.
- The amount of energy a microbe makes depends on the metabolic pathways it can use.
- The plate count determines how many of a specific organism can grow on a given medium under the incubation conditions used.
- Selective media allow low numbers of specific pathogens to be enumerated when they are present in a larger population of other bacteria.
- Enrichment allows very low numbers of specific pathogens to be detected in the presence of large numbers of other bacteria.
- The MPN method allows very low numbers of bacteria to be estimated from statistical tables.
- Injured cells may escape detection by culture methods but still cause illness when consumed.

- Viable but nonculturable cells are exactly that.
- Intrinsic factors, such as pH and water activity, and extrinsic factors, such as time and temperature, can be manipulated to control microbial growth.
- Hurdle technology challenges bacteria by several mechanisms to inhibit growth.
- The microbial growth curve consists of lag, log, stationary, and death phases of growth. Each phase is important to microbial food safety and can be manipulated.
- The equation $N = N_0 e^{\mu t}$ describes the exponential phase of microbial growth.
- Bacteria use different metabolic pathways to generate the energy they need to maintain an ordered state.

Suggested reading

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Kell, D. B., A. S. Kaprelyants, D. H. Weichart, C. R. Harwood, and M. R. Barer. 1998. Viability and activity in readily culturable bacteria: a review and discussion of the practical issues. *Antonie Leeuwenhoek* **73:**169–187.

Montville, T. J., and K. R. Matthews. 2001. Principles which influence microbial growth, survival, and death in foods, p. 13–32. *In* M. P. Doyle, L. R. Beuchat, and T. J. Montville (ed.), *Food Microbiology: Fundamentals and Frontiers,* 2nd ed. ASM Press, Washington, D.C.

Questions for critical thought

1. The plate count is based on the assumptions that one cell forms one colony and that every colony is derived from only one cell. For each assumption, list two situations that would violate the premise.
2. Given the data in Fig. 2.1, calculate the number of *S. aureus* organisms in the sample using the proper units.
3. Why *can't* you count, say, 3.2×10^4 CFU of *S. aureus*/ml in a population of 5.3×10^7 other bacteria using a standard plate count?
4. If the solid circles in the diagram below represent test tubes positive for growth, what would the MPN of the sample be?

0.1 ml	0.01 ml	0.001 ml	0.0001 ml	0.00001 ml
●●●	●●●	○○●	○○●	○○○

5. What is the difference between intrinsic and extrinsic factors in food? Name one intrinsic factor and one extrinsic factor that you think are very important for controlling microbes in food. Why do you think each is important? How does it work?
6. What three mechanisms do cells use to maintain pH homeostasis? Why do they need three? Loss of which mechanism would be worst for the cell? Why?

7. Consider equation 1. Your food product has an initial aerobic plate count of 3.8×10^4 CFU/g. After 1 week of refrigerated storage, the count reaches an unacceptable 3.8×10^6 CFU/g. You decide that an acceptable level of 3.8×10^5 CFU/g could be achieved if the initial number were lower. What starting level of bacteria would you need to achieve this goal? (Hint: put your calculator away and think about it.) In real life, how might a food manufacturer achieve this lower initial count?
8. Make a chart listing the three main catabolic pathways and, for each, the key regulatory enzyme, carbohydrate-splitting enzyme, amount of ATP generated, and final metabolic product.
9. Both anaerobic lactic acid bacteria and aerobic pseudomonas are naturally present in ground beef. In beef packed in oxygen-permeable cellophane, pseudomonas is the main spoilage organism. In beef packed in oxygen-impermeable plastic film, lactic acid bacteria predominate. Why?