Quantitative Microbiology in Food Processing

Modeling the Microbial Ecology

Edited by **Anderson de Souza Sant'Ana**

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PART I
Introductory section

CHAPTER 1
Introduction to the microbial ecology of foods

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1.1 Introduction

Food products become a microbial ecosystem when they are contaminated and colonized by microorganisms. Fresh foods allow rapid microbial growth due to a high content of nutrients whereas processed foods correspond to a harsher environment for growth, reducing the natural microbial population associated with raw food. In addition to natural microbiota related to its origin and environmental conditions, food may be contaminated from outside sources during production, processing, storage, transport, and distribution. Hence, growth and activities of microorganisms (bacteria, yeasts, and molds) are some of the major causes of food spoilage. However, few microorganisms are pathogens while many are useful in producing desirable changes during food fermentation. A large number of microorganisms can simultaneously grow in food if the abundance of nutrients is sufficient. As a consequence, the diversity and occurrence of microorganisms present depend on the composition of food, the extent of microbial contamination, and the treatments applied. Finally, intrinsic and extrinsic factors such as temperature, water content, and oxygen content have a considerable influence on the growth of microorganisms, depending on the properties of the microorganisms and on the interactions among them.

Microbial ecology of food concerns the study of the type of microorganisms present (diversity and structure), their rate of occurrence, activities (functionality), and interactions with each other (microbial communities) and their environment. Ecological studies also help to understand the transmission and dissemination of pathogens and toxins. Microbial ecology is intimately connected with microbial physiology as ecophysiological parameters determine the activities within individual cells and thus the responses of microbial populations to environmental influences. These combined effects control the type of microorganisms capable of growth in a particular food ecosystem (Leistner, 2000; McMeekin *et al.*, 2010).

Quantitative microbial ecology relies on predictive microbiology to forecast the quantitative evolution of microbial populations over time, using models that include the mechanisms governing population dynamics and the characteristics of food environments. In this respect, the diversity of the microbial community of a food ecosystem must be assessed, along with the identification of species and their comparative quantification. Traditional microbiological techniques (culture-dependent methods) have been used for decades for this purpose. However, these methods give a single viewpoint for describing a portion of the microbial dynamics and estimating microbial diversity. Culture-independent techniques based on direct analysis of genetic materials (DNA or RNA) are increasingly being used for characterization of microbial diversity structure and function. The

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development of these molecular methods and their applications in the field of microbial ecology of food has transformed our understanding of the nature and evolution of microbial populations and their metabolic activities (Ndoye *et al*., 2011).

This introductory chapter aims at providing some background in order to set the stage for further study of predictive microbiology, unit operations, processes, and the microbial ecology of specific categories of food products in the subsequent chapters.

1.2 Role of food characteristics and environment on microbial fate

Foods are classified as non-perishable for those that do not need time/temperature control, semi-perishable for those that remain unspoiled for a prolonged period and perishable for those that need time/temperature control to kill or prevent the growth and activities of microorganisms in order to extend their shelf life.

In 1971, Mossel defined four groups of ecophysiological parameters that influence the survival or growth of the microorganisms contaminating a raw or processed food: (i) intrinsic factors that are essentially chemical but with some important intrinsic factors that are physical and structural (e.g., pH, water activity, redox potential, available nutrients, presence of antimicrobial substances, food matrix); (ii) extrinsic factors that include the externally applied factors (e.g., temperature, relative humidity, etc.); (iii) implicit factors that are mostly dependent on the physiological properties of the microorganisms and microbial interactions; and (iv) processing factors (heat destruction, smoke, salts, organic acids, preservatives, and other additives) and conditions affecting foods (slicing, mixing, removing, washing, shredding,etc.) as well as influencing transfer of microorganisms (cross-contamination events) (Gould, 1992; ICFMS, 1980; Mossel, 1971; McMeekin and Ross, 1996).

In the context of quantitative microbial ecology, the growth of microorganisms could be modeled and then predicted as a function of only a few ecophysiological parameters such as temperature, pH, and water activity (a_w), sometimes with other factors such as the presence of preservatives and oxygen. Growth of a specific microorganism also depends on the initial microbial load, the sources of nitrogen and carbon, the processing method used in the food production, and the external environment of the food during storage, distribution, sale and handling. The physicochemical properties of foods in association with environmental conditions determine the selection of microorganisms capable of growing and multiplying at the expense of other less competitive species. As a result, the whole microbial ecology of the food system should be considered to accurately predict food spoilage (Braun and Sutherland, 2006). Such an integrated microbial model must take into consideration all these factors as input variables along with modeling parameters representing the processes applied during food manufacture and storage (Figure 1.1).

1.2.1 Temperature

The lag period and growth rate of a microorganism are affected by temperature as growth can be inhibited by decrease or increase of temperature below or above the optimum growth range. Indeed, every microorganism has a defined temperature range in which they grow, with a minimum, maximum, and optimum within the extended range of –5 to 90°C (Table 1.1). Organisms causing food spoilage can be grouped by temperature preference as (i) mesophiles (optimum temperature 30–45°C, minimum growth temperature ranging from 5 to 10°C and a maximum of 50°C); (ii) psychrophilic organisms (optimum growth range temperature of 12 to 15°C with a maximum range of 15 to 20°C); (iii) psychrotropes (formerly called psychrotrophs with an optimum temperature 25–30°C with a minimum of –0.4 to 5°C); and (iv) thermophiles (optimum temperature 55–75°C with a maximum as high as 90°C and a minimum of around 40°C).

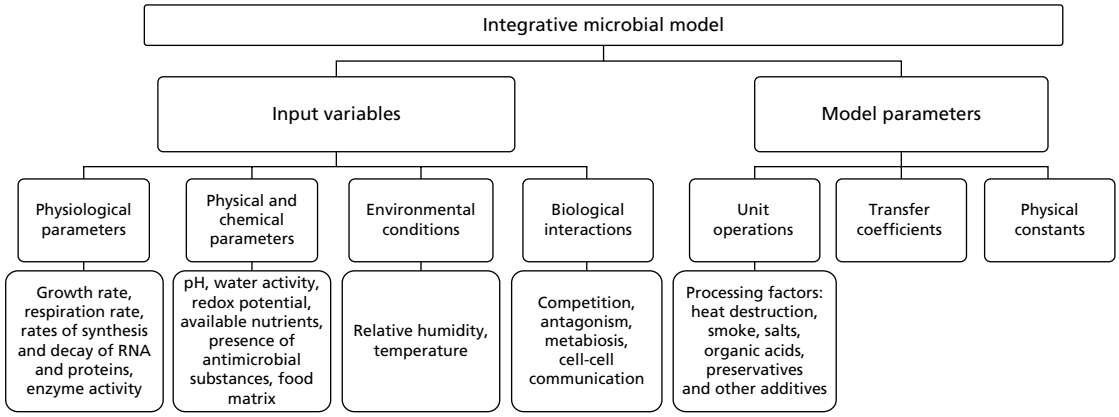

Figure 1.1 Integrative parameters affecting the development of microbial ecosystems in food.

Table 1.1 Psychrophilic, psychrotropic, mesophilic, and thermophilic microorganisms of importance in food.

Group	Temperature (°C)	Examples of bacteria (genus name only)
Psychrophiles	–5 to 20	*Acinetobacter, Bacillus, Clostridium, Flavobacterium, Vibrio*
Psychrotropes	–5 to 35	*Pseudomonas, Enterococcus, Alcaligenes, Shewanella, Brochothrix, Corynebacterium, Lactobacillus, Listeria, Micrococcus, Moraxella, Pectobacterium, Psychrobacter*
Mesophiles	5 to 47	*Bacillus, Carnobacterium, Clostridium, Corynebacterium, Escherichia, Lactobacillus, Lactococcus, Leuconostoc, Listeria, Hafnia, Pseudomonas, Salmonella, Shigella, Staphylococcus, Vibrio, Yersinia*
Thermophiles	40 to 90	*Bacillus, Paenibacillus, Clostridium, Geobacillus, Alicylobacillus, Thermoanaerobacter*

Adapted from ICMFS (1980) and Jay (2005).

Changes in storage temperature as well as the time–temperature relationship have an impact on the evolution of these different groups. Refrigeration and chill temperatures promote growth of psychrophilic microorganisms, of which there are few that affect food spoilage, and psychrotrophic spoilage organisms such as pseudomonas, yeasts, and molds as well as pathogens such as *Listeria monocytogenes*. At high temperatures, spore-forming bacteria and lactic acid bacteria are able to multiply. Thus, temperature changes have an influence on the metabolic activities of some microorganisms and consequently on the biochemistry of the spoilage process. Time has an impact in relation to the storage temperature because it is a factor that influences the rate of growth of microorganisms: extended storage at low temperatures allows the growth of some psychrotrophic microorganisms. Further discussion on food safety and the role of quantification in microbial risk assessment will be given in Part IV of this book.

1.2.2 pH and acidity

The pH is a measure of acidity of a food that influences microbial growth and survival, as every microorganism possesses a minimum, an optimum, and a maximum pH for growth. Most bacteria exhibit an optimum pH near the neutral point (pH 7.0) although acetic and lactic acid bacteria are able to survive at reduced pH levels. Molds and yeasts are generally more

acid-tolerant than bacteria and therefore acidic foods are more susceptible to spoilage by these types of microorganisms.

Low pH values and associated high acid concentrations inhibit microbial growth and survival in foods due to the acid-induced denaturation of cell wall proteins. A decrease of pH also reduces the heat resistance of microorganisms. Moreover, the pH can interact with water activity, redox potential, salt, and preservatives to inhibit growth of food-borne pathogens and spoilage microorganisms. The undissociated form of weak acids shows antimicrobial activity because they pass freely through the cell membrane and then dissociate, as the cytoplasmic pH is usually higher than that of the growth medium. This leads to the release of protons, which in turn results in an acidification of the cytoplasm. Bacterial growth can be prevented by addition of weak organic acids alone or in combination with other preservatives as well as by production of lactic and acetic acids by fermentation.

Food products can essentially be divided into three types according to their pH: (i) low-acid foods where pH is greater than 4.6 and less than 7.0, (ii) acid foods that have a pH lower than 4.6, and (iii) acidified foods obtained by addition of acids into low-acid foods. This classification is based on the fact that pathogenic microorganisms generally cannot grow at pH values below 4.6. Low-acid foods can be fermented (fermented foods) by acid-producing bacteria that reduce the pH below 4.6. Foods can also be characterized by their buffering capacity, which is defined as the ability to resist changes in pH. The pH of foods with a low buffering capacity in the presence of acidic or alkaline compounds produced by microorganisms will change quickly, whereas foods with a high buffering capacity are more resistant to pH changes.

1.2.3 Water activity

Water is a requirement for growth and metabolic activities of microorganisms in a food product. However, microbes can only use water in an available form. Free water that is not in the bound state participates in many chemical and biochemical reactions, supports microbial growth, and acts as a transporting medium for compounds (sugars, salt, organic acids) in the food system. Water activity (a_w), defined as the free or available water in a food, is therefore a better indicator for microbial growth than the water content. In a food matrix, the requirements for moisture by microorganisms are expressed in terms of a_w (the a_w of pure water is 1.00 and the a_w of a completely dehydrated food is 0.00) and the lower limit for microbial growth in a food product will be determined by the a_w. Food products can thus be broadly classified by water activity into (i) high a_w (>0.92), (ii) intermediate a_w (0.85 to 0.92), and (iii) low a_w (<0.85). Fresh foods (meat, vegetables, and fruits) generally have a_w values higher than 0.97. By reducing water activity below 0.7, osmotic pressure is increased, thus inhibiting microbial growth and maximizing the shelf life of the food product. This reduction can be accomplished by adding sugars or salt, removing water by drying or baking, and binding the water to various macromolecular components such as cellulose, protein, or starch in the food.

Microorganisms exhibit optimum and minimum levels of a_w for growth, depending on a number of other ecophysiological factors (pH, temperature, oxido-reduction level, and nutrients). Bacteria are more sensitive than yeasts and molds, and Gram-positive bacteria are more resistant to lower values of a_w than Gram-negative bacteria. The growth of food-borne pathogens is inhibited below a_w 0.86, except that *Staphylococcus aureus* can grow down to a value of 0.83 and produces toxin below a_w 0.90. Growth of molds will be controlled at a_w 0.80 and mycotoxin production requires a higher a_w than that of growth.

1.2.4 Oxygen and redox potential

Based on their oxygen requirements and tolerance, microorganisms are classified into the following groups: (i) obligate aerobes are microorganisms that require oxygen for growth;

(ii) obligate anaerobes are microorganisms that do not need or use oxygen, which is toxic for them; and (iii) facultative aerobes (or facultative anaerobes) are microorganisms that can grow in the presence and absence of oxygen, switching to aerobic respiration in the presence of oxygen but under anaerobic conditions they grow by fermentation or anaerobic respiration.

The oxidation–reduction or redox potential is an intrinsic factor that influences the growth of microorganisms in foods. The redox potential of the food varies according to the physicochemical characteristics, partial pressure of oxygen, and the presence of other gases in the storage atmosphere (water vapor, nitrogen, CO_2). The presence of substances that are highly hydrogenated, that contain SH radicals, reducing sugars, or other compounds such as ascorbic acid (vitamin C) and tocopherols (vitamin E) in a food creates a reducing environment. When the redox potential (Eh) is negative in terms of millivolts, this means a reduced state, while the presence of oxygen at the surface or in the bulk has an oxidizing effect (an oxidized state). Aerobic organisms require a food environment with a positive redox potential (+500 to +300 mV) whereas anaerobes require a negative potential (+100 to less than −250 mV) and facultative anaerobes tolerate a range in potential between +300 and −100 mV.

1.2.5 Nutrient content

The nutritional needs of microorganisms can usually be met in foods due to the presence of water, carbohydrates (sources of carbon and energy), fats, proteins, vitamins, and minerals. However, these nutrients must be available in an easily digestible form, such as simple sugars and amino acids, for many microorganisms. Some microorganisms possess specific enzymes that allow them to degrade more complex structures such as proteins and fibers. Most spoilage microorganisms have no fastidious nutritional requirements and possess essential metabolic activities such as glycolysis and proteolysis. In this way, a complex microbial community capable of degrading the nutrients present will colonize any type of food. Therefore, it is practically impossible to predict the microbial ecology of a food based on its nutrient composition.

1.2.6 Physical structure and microenvironments

The physical barriers to food spoilage by microorganisms are: (i) the skin of fish and meats, (ii) the shell of nuts and eggs, (iii) the external layers of seeds, and (iv) the outer covering of fruits and vegetables such as the husk or rind. These protective biological structures are usually composed of macromolecules that are relatively resistant to penetration or degradation. They constitute hostile microenvironments for the growth of microorganisms by having a low water activity, a lack of readily available nutrients, and a presence of antimicrobial compounds such as short-chain fatty acids (on animal skin) or essential oils (on plant surfaces). During the preparation of foods, processes such as cutting, grinding, and heating break down the biological barriers and change microenvironments, thus favoring contamination and proliferation of microbes inside the food product. The impact of these unit operations on specific microorganisms will be further detailed in Part II of this book. Most microorganisms will grow in the majority of foods, as individual free-floating (planktonic) cells in the aqueous phase or as an association of microbial cells with a solid substrate either through entrapment, constrained growth, attachment, or a combination of these factors (Skandamis and Nychas, 2012).

1.2.7 Food preservation processes (antimicrobials, preservatives)

Food preservation mainly involves a prevention or exclusion of microbial activity. This may be achieved: (i) by inhibiting the growth or shortening the survival of microorganisms, (ii) by excluding or removing microorganisms, and (iii) by killing the microorganisms. Some plant- and animal-based foods contain natural antimicrobial compounds such as essential oils and lysozyme, respectively, that inhibit the growth of spoilage microorganisms. Some chemical

food additives such as salts, sugars, and organic acids are commonly applied for creating a hostile environment in food products. The presence of gases (carbon dioxide, ozone, and oxygen) is also able to inhibit the growth and proliferation of microorganisms by direct toxic effects and by indirect inhibitory effects, by modifying the gas composition and thus altering the ecology of the microbial environment. Various types of food processing such as heating, smoking, and fermentation are also used for the formation of antimicrobial substances in food. Part II of this book contains more information on specific food preservation operations, including fermentation. Food fermentation is one of the oldest food processing technologies that can suppress the growth and survival of spoilage microorganisms in food products. This process depends on the biological activity of microorganisms that produce a large range of metabolites (acids, alcohols, and carbon dioxide) by fermentation or oxidation of carbohydrates or derivatives. For example, among members of competitive microbiota, lactic acid bacteria (LAB) exhibit unique metabolic activities and are employed as starters for the fermentation of milk, meats, cereals, and vegetables and are used as probiotic cultures (Champagne *et al.*, 2005). In addition to the production of lactic, acetic, and propionic acids leading to an acidic environment appropriate for controlling the growth and metabolic activity of many pathogenic and spoilage microorganisms, these beneficial bacteria are also able to produce ethanol, hydrogen peroxide (H_2O_2), diacetyl, and bacteriocins. Bacteriocins are protein or peptide antimicrobial substances that inactivate other bacteria through depolarization of the target cell membrane or through inhibition of cell wall synthesis. In addition, LAB can also produce antifungal compounds, including reuterin, carboxylic acids, cyclic dipeptides, and fatty acids (Crowley *et al.*, 2013).

Antimicrobial substances produced by microorganisms provide an additional hurdle for keeping the natural population of microorganisms under control. Indeed, traditional food preservation has often been achieved by the combination and interactions of pH, a_w, atmosphere, numerous preservatives, and other inhibitory factors, referred to as the "hurdle effect". These preservative factors (hurdles) temporarily or permanently disturb the homeostasis of microorganisms, defined as the tendency to uniformity and stability in the internal status of an organism; microorganisms remain in the lag phase or even die before homeostasis is re-established (Leistner, 2000).

1.3 Understanding microbial growth, death, persistence, competition, antagonism and survival in food

1.3.1 Principles of microbial growth

In a food environment where nutrients are not limiting, microbial cells increase in number in a characteristic manner and at a specific rate as determined by their genetic traits. It is well known by microbiologists that the growth curve exhibits four different phases: (i) the lag phase in which microorganisms, by a series of biochemical activities, acclimate to their environment and initiate cell reproduction and growth; (ii) the exponential or log phase, where cell components are synthesized in order to allow cell replication at a logarithmic rate determined by their generation time and ability to assimilate the substrate; (iii) the stationary phase, which begins when a microbial population tends to stabilize due to accumulation of metabolic end-products and limitations in substrates necessary for growth, leading to reduction of the growth rate; and (iv) the decline or death phase, when microorganisms die and lyse (autolysis) due to nutrient depletion and the toxic effects of metabolic end-products.

1.3.2 Survival

Microbial populations in foods are subjected to stressful conditions such as low or high temperatures, acidity, low water activity, modified

atmospheres, or nutrient deprivation. By a variety of strategies, microorganisms attempt to resist and adapt to these hostile conditions, constantly switching between growth and merely surviving. The stress response, which results in a characteristic change in the pattern of gene expression, helps to restore cellular homeostasis and increase resistance to subsequent stressful conditions. Although death is an irreversible state, bacterial cells can be sublethally injured or enter a dormant state. These cells may repair the damage caused by the hostile environment and survive, even growing when conditions become favorable (Aertsen and Michiels, 2004; Wesche *et al*., 2009). The stress response in relation to microbial ecology of food will be discussed in Part IV of this book.

1.3.2.1 The viable but non-culturable state

Many stressed organisms may regain the characteristics of normal cells, but some severely injured cells remain metabolically active but cannot be resuscitated under routine laboratory conditions, entering a viable but non-culturable (VBNC) state (Wesche *et al.,* 2009). The VBNC state can be a significant means of survival if the cells have the ability to increase metabolic activity and become culturable once resuscitated (Oliver, 2005).

A large number of non-spore-forming bacteria are capable of entering the VBNC state. Although the exact role of this state in bacteria is yet to be elucidated, it can be induced by stressful conditions such as nutrient starvation, temperature, osmotic concentration, oxygen concentration, and food preservatives. Hence, the VBNC state might be an adaptive strategy for long-term survival of bacteria under unfavorable environmental conditions. In contrast to dead cells that have a damaged membrane, VBNC cells have an intact membrane retaining chromosomal and plasmid DNA and differ from "injured" bacteria that are unable to grow on selective media. VBNC cells do not grow on any medium, even if non-selective. However, VBNC bacteria have higher resistance to physical and chemical stresses than culturable cells and can resuscitate when environmental conditions become favorable (Li *et al*., 2014; Oliver, 2005, 2010).

1.3.3 Strategies for persistence

Many microorganisms associated with food survive treatments such as heat and disinfection, so they persist during storage and their numbers remain unchanged.

1.3.3.1 Sporulation

Some bacteria can form spores as a defense mechanism against unfavorable environmental conditions (e.g., Gram-positive bacteria such as *Bacillus* and *Clostridium*). Indeed, endospores are very resistant structures with no measurable metabolism but can confer a great advantage for these bacteria to persist for prolonged periods of time and endure extreme stress conditions (high temperatures and UV irradiation, extreme freezing, desiccation, chemical damage by disinfectants, and enzymatic destruction). Under favorable environmental conditions, the endospore can undergo activation and germination. Hence, metabolic activity is restored and the cell becomes vegetative.

1.3.3.2 Biofilm formation

Generally, bacteria do not live freely in suspension as planktonic cells and biofilms protect them from desiccation, bacteriophages, and sanitizing agents. Biofilm formation thus constitutes one of the survival strategies of microorganisms in hostile environments. The persistence of food-borne pathogens and spoilage microorganisms on foods and food contact surfaces often adversely affects the quality and safety of raw and minimally processed foods.

1.3.4 Competition

The composition of the microbial community of a food varies according to many ecophysiological factors that have been described so far. However, the ecosystem is also altered by the interactions among microbes themselves.

Mixed cultures in food fermentation processes represent some of the best examples of microbial interactions. Microbe–microbe interactions can be classified as positive (+), neutral (0), and negative (–). These interactions can be further subdivided into: (i) mutualism (+/+ interaction: both microbes involved benefit from the interaction, e.g., synergism or protocooperation among yogurt bacteria or mutualism between yeast and bacteria during sourdough fermentation); (ii) commensalism (+/0 interaction: one organism benefits from the interaction while the other is not affected, e.g., cultivation of propionic bacteria in the presence of LAB in Swiss-type cheese); (iii) amensalism (–/0 interaction: interspecies interaction in which one organism adversely affects the other without being affected itself, e.g., bacteriocin production by LAB and ethanol production by yeasts); (iv) parasitism (+/– interaction: one species benefits at the expense of another, e.g., bacteriophages in fermentations); and (v) competition (–/– interaction: two or more species, strains, or subpopulations of microbes compete for energy sources and nutrients) (Ivey *et al.,* 2013; Sieuwerts *et al.*, 2008; Smid and Lacroix, 2013).

Microorganisms compete for nutrients, adhesion/attachment sites, as well as by their ability to alter the environment by producing metabolites. Preservation methods combined with ecophysiological factors and the genetic characteristics of each microorganism (lag phase, growth rate, and total cell biomass yield) lead to selection of microbial associations of a particular food at any given point in time during production and storage. For example, psychrotrophic bacteria such as *Pseudomonas* spp. dominate proteinaceous foods (meat, poultry, milk, and fish) stored at refrigeration temperatures under aerobic conditions. In meat and fish products, a change in the atmosphere (e.g., vacuum packaging) promotes LAB at the expense of *Pseudomonas*. Microbial ecology of food provides a comprehensive overview of the dominance of an organism based on its origin, substrate composition, temperature, pH, a_w, and atmosphere, regardless of raw material and processing.

In addition to these conditions determining the association of microbiota in food, there are three aspects of microbial interaction that must be taken into consideration according to Gram *et al.* (2002), namely: (i) antagonism, (ii) metabiosis, and (iii) cell-to-cell communication.

1.3.4.1 Antagonism

In addition to changes in environmental conditions such as lowering pH by acid-producing microorganisms, antagonistic abilities include competition for nutrients. Scavenging growth-limiting compounds such as iron represents one type of nutritional competition. Microorganisms with higher metabolic activity may selectively consume required nutrients, resulting in growth inhibition of other organisms with lower activity. The growth rate of particular microorganisms may also be affected by an overgrowing microbiota in a phenomenon described as the "Jameson effect", which is essentially non-specific nutrient competition (Gram *et al.*, 2002).

1.3.4.2 Metabiosis

The microbial profile of a food evolves over time because of the changes in environmental conditions caused by the action of the community on the supply of nutrients from limiting metabolic compounds. This refers to the term "metabiosis", which describes the interrelationships among microorganisms to produce a given environment (Gram *et al.*, 2002).

1.3.4.3 Cell-to-cell communication

The role of quorum sensing (QS) or cell-to-cell communication in food microbial ecology is now considered a microbial behavioral pattern that is correlated with the density of the microbial population and with the ability to regulate gene expression as a function of cell density (Gram *et al.*, 2002; Skandamis and Nychas, 2012). In Part IV of this book, the quantitative aspects of quorum sensing will be applied to microbial ecology.

The food matrix is composed of interconnected microenvironments where the levels of intrinsic ecophysiological factors (oxygen, pH, a_w, nutrients, preservatives, and antimicrobial compounds) may change. A large variety of microorganisms proliferates as microcolonies or biofilms and reaches high densities (10^7–10^9 cfu/g) in these *in situ* environments. The growth and activity of any one species or strain will be determined by the presence of other species since we can assume that quorum or other sensing molecules are released (*in situ* cell-to-cell ecological interactions). Microorganisms interact and influence the growth of one another by synthesizing specific low-molecular-weight diffusible signaling molecules as a function of population density :(i) Gram-negative bacteria produce and utilize *N*-acyl homoserine lactones (AHLs) or autoinducer-1 (AI-1) for intraspecies communication; (ii) autoinducing peptides (AIPs) are produced and used by Gram-positive bacteria for intraspecies communication; and (iii) furanosyl borate diester derivatives or autoinducer-2 (AI-2) are produced by both Gram-positive and Gram-negative bacteria and seem to serve as a universal language for inter- and intraspecies communication (Bai and Rai, 2011).

1.4 Methods to study the microbial ecology of foods

The aims of microbial ecology studies are to determine changes in microbial populations by characterizing community structure, diversity, activity, and interactions in their natural environments. The three basic questions that detection methods must answer are: (i) "who is there?" by identifying the types of microorganisms such as food-borne pathogens, spoilage microorganisms, starter cultures, or potentially probiotic and beneficial microorganisms present in the specific food environment; (ii) "who is doing what?" by assigning functional roles to these microorganisms; and (iii) "how do the activities of these microorganisms contribute to specific ecosystem functions or processes" (Ndoye *et al.*, 2011; Ercolini, 2013).

Culture-independent methods can circumvent the limitations of traditional microbiological methods for the analysis of complex microbial ecosystems. These methods have been used in various types of foods, especially for cheese in recent years (Ndoye *et al.*, 2011). In contrast to conventional microbiological techniques based on cultivation of the microorganisms on media and phenotypic or genotypic characterization of a fraction of the community (culture-dependent methods), culture-independent techniques are based on direct analysis of DNA or RNA for efficient characterization of whole microbial communities, evaluation of *in situ* gene expression and determination of metabolic activities of microbial populations present in a particular food product. However, both culture-dependent and culture-independent methods have limitations and should be combined as much as possible through polyphasic approaches to undertake analysis of both the community and activity of natural microbiota and spoilage microorganisms (Cocolin *et al.*, 2013; Ercolini, 2013; Ndoye *et al.*, 2011).

1.4.1 Culture-independent analysis of microbial communities

The application of molecular techniques has modified our understanding of the microbial ecology of food, allowing significant insights into all aspects of microbial populations (identification of specific isolates, changes in microbial communities, nature of the functional groups). These techniques have been classified into two major categories: (i) partial community analysis approaches and (ii) whole community analysis approaches (Rastogi and Sani, 2011).

1.4.1.1 Partial community analysis approaches

These approaches are based on the direct extraction of total DNA or RNA from the food product. Then, the genetic materials extracted from food samples, either DNA or cDNA (after

reverse transcription of the total RNA), are amplified by polymerase chain reaction (PCR)-based methods. The application of the various molecular culture-independent tools allows: (i) direct identification of members of a community and assessment of their abundance; (ii) reliable fingerprinting of complex bacterial communities; (iii) analysis of the diversity and dynamics of the dominant microbial community; (iv) comparison of spatial and temporal changes in bacterial community structure; and (v) accurate quantification of target species (Ndoye *et al.*, 2011).

1.4.1.2 Whole community analysis approaches

Next-generation DNA sequencing (NGS) or high-throughput sequencing is a hundred times faster and cheaper than the conventional Sanger approach and is already considered as the most powerful culture-independent method for analysis of all the genetic information present in total DNA/RNA extracted from food samples or pure cultures. The NGS approach provides a more global perspective on food microbial communities, including molecular mechanisms of metabolically active microorganisms in food ecosystems. Metagenomics is defined as the investigation of the collective microbial genomes retrieved directly from environmental samples. When combined with other "omics" (functional genomics, transcriptomics, proteomics, and metabolomics), these data provide deeper insight into microbial diversity and the metabolic potential of microbial communities as well as predictive models of the contribution of individual microorganisms to the development of food quality and safety (Rastogi and Sani, 2011; Solieri *et al.*, 2012).

1.5 Perspectives on applying food ecosystem modeling

Predictive microbiology, which will be detailed in Chapter 2, was originally conceived for analyzing the behavior of pure cultures of food-borne pathogens, and then spoilage bacteria, in order to develop food processes that adequately control microbial growth throughout the shelf life of food products. For example, the growth boundary models for *L. monocytogenes* erroneously predict the growth of this pathogen, as it does not take into account the biofilm microbiota interactions (Guillier *et al.*, 2008). In addition, pH and a_w could not solely account for growth arrest in the stationary phase, without including non-specific competition for nutrients (Jameson effect). Considering factors that determine enzyme production has revealed crucial restraints on litter decomposition rates in soil (Allison *et al.*, 2012), so these metabolic factors have great potential for application to food products as well. Genome-scale metabolic models are becoming useful in analyzing interactions in multispecies microbial systems from a metabolic standpoint, requiring the integration of the ecological concept of trade-offs between individual and community fitness criteria (Zomorroddi *et al.*, 2012).

Advances in our understanding of microbial interactions will allow us to envisage more complex predictive models (Figure 1.2). Complex system science is a process of integrating a multiplicity of variables and knowledge from an array of disciplines (Perrot *et al.*, 2011). In the case of food, this means joining together the skills of mathematicians, physicists, and computer scientists with those of microbiologists to complete food science and engineering. First used in environmental ecosystem modeling, this approach is beginning to be applied in order to comprehend the Camembert cheese ripening process (Sicard *et al.*, 2012). Viability theory from complex science was employed to define an optimal trajectory for Camembert cheese ripening, which was validated through pilot studies by manipulating cheese size, relative humidity, and temperature controls. As a result, the cheese ripening process was shortened by four days without significant changes in the microorganism kinetics. The quality target was reached and the sensory properties of the cheeses produced were similar to those obtained under

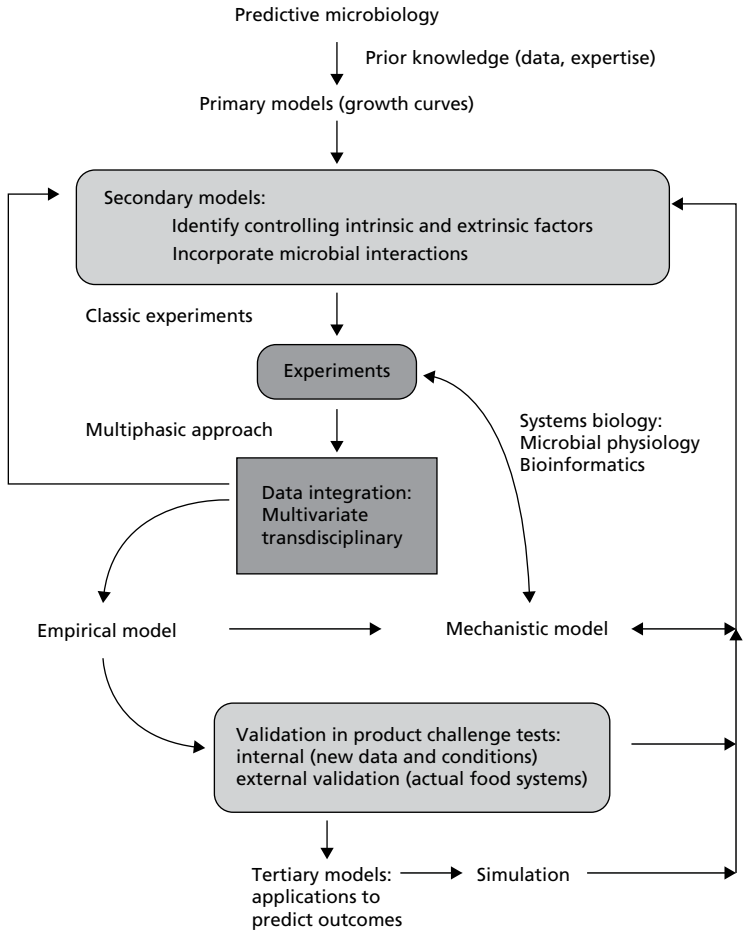

Figure 1.2 Towards developing models from predictive microbiology to quantitative microbial ecology and systems biology.

standard conditions (Sicard *et al.*, 2012). This is one example of how microbial community modeling can have a concrete impact on developing more efficient and less costly food processes.

Multiple species community modeling is still in its early stages and faces many challenges, especially applied to food. The next step, microbial community engineering, has even greater challenges and potential rewards in ensuring food quality. Continual innovation in analytical methods will contribute to improve the prospects of microbial community modeling in the future.

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CHAPTER 2

Predictive microbiology: mathematics towards understanding the fate of food-borne microorganisms in food processing

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2.1 Introduction

Predictive food microbiology is a subdiscipline of food microbiology that uses models (i.e., mathematical equations) to describe the growth, survival, or inactivation of microbes in food systems. Mathematical growth and inactivation models are increasingly used in the context of the hazard analysis and critical control point (HACCP) system for food safety and as a means to demonstrate compliance of a product with microbiological criteria enforced by food safety and hygiene legislation.

Three ideal uses of predictive models are: (i) to narrow the choices for processing steps to be considered in an HACCP plan as having a major impact of final product safety, (ii) to assist in choosing the most resistant biological hazards (i.e., microorganisms) controlled at a specific processing step, and (iii) to define a safe operational design space without the need for additional data generation (e.g. when a model has been appropriately validated in representative food–model systems and deemed to provide 'fail-safe' predictions). When using predictive models in HACCP plan development the intrinsic and extrinsic factors (pH, a_w, temperature, etc.) used as inputs for the model should be chosen with care. If the conditions modeled suggest that growth could occur or that there is limited lethality for the product/process, then additional studies, product reformulation, or modification of the target shelf life would be warranted. If there is less confidence in the model, then limited challenge studies may be warranted to verify the prediction from the model.

The long roadmap of predictive modeling over the last four decades, along with the advances towards understanding and quantifying microbial responses down to the single cell level, have led to the appointment of predictive modeling as one of the most promising decision–support methodologies for food safety assessment by the Food Industry and competent authorities. Predictive modeling has been greatly benefitted by the technological and scientific evolution in both collection and processing of data, through the introduction of –omics, the deployment of advanced microscopy techniques (e.g., confocal laser microscopy and fluorophores fused in the genome), the application of chemometrics, data mining, and the emergence of advanced data modeling techniques (e.g., artificial neural networks). As a next step, the rising trend for application of predictive modeling in daily practice has intensified the need to systematically exploit the vast number of available predictive models so far. Meeting

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this demand is being markedly achieved through the development of collective predictive modeling repositories (e.g., ComBase, Pathogen Modelling Program, iRisk, Food Spoilage and Safety Predictor, Sym'Previus, etc.) equipped with search engines for guided retrieval of the appropriate food-specific or generic models (i.e., not food-specific) associated with particular hazards and built-in fitting or simulation modules, in order to visualize and numerically express the model outputs in comprehensive and ready-to-use formats. Such a variety of predictive model and risk assessment/risk ranking software tools may indeed help the food producers, researchers, and food safety inspectors to apply the concepts of predictive modeling in quality-by-design, identification of safe product formulations, and evaluation of product compliance with safety standards and microbiological criteria. A comprehensive and quite extensive review of the available software tools can be found in the study by Tenenhaus-Aziza and Ellouze (2014) and thus the underpinning principles and validity range of these tools will not be further analyzed in the present chapter.

Models alone should be applied with caution and with proper disclaimers in the context of decision-making during HACCP plan development. Use of models requires experience and judgment, both in modeling and food microbiology. Therefore, it is of vital importance to be clearly perceived that the predictive models and associated software tools should not replace the expert opinion, but rather assist the experts (and sometimes even the non-experts) to elicit a food safety plan. When models alone are used to make a decision, those models must be shown to be valid for the food in question and should take into consideration lot-to-lot variation. Validation may be based on published or unpublished data for very similar or identical foods. Nonetheless, even in cases when the available predictions are obtained from lab–media-based models, which may potentially overlook some significant food-specific impacts on microbial behavior, such predictions are still very useful in guiding more focused and targeted challenge testing.

Based on microbial responses, expressed as a change in numbers and stress tolerance, the combinations of intrinsic and extrinsic environmental determinants to which microorganisms may be exposed are divided into the following major domains: the growth era and the domain including the combinations that allow survival or cause death of microorganisms (Booth, 2002). The conditions that lie between these two domains refer to a zone where microbial responses are uncertain and characterized by the growth/no growth interface (Le Marc *et al.*, 2005). This zone is strongly associated with the so-called cardinal values (T, pH, a_w, etc.) for growth and outlines the biokinetic range of microbial proliferation. Such values are species- or even strain-dependent and thus introduce significant variability in the assessment of the impact of marginal growth conditions on microbial growth, an issue commonly encountered in quantitative microbial risk assessment. To remedy that, models have been proposed that embed the theoretical growth-limiting values for critical hurdles, such as temperature, a_w, pH, $\%CO_2$, and preservatives, as biological meaningful parameters in the model structure. Notably, a theoretical interface also exists between survival and inactivation separating combinations that cause growth cessation but not cellular death from those that are lethal (McKellar *et al.*, 2002).

Different processes and product formulations determine the dominant phenotype (i.e., growth or inactivation) and proper mathematical models should be retrieved to accurately (or, better to say, realistically) describe microbial dynamics. In this chapter, the available classical and modern modeling approaches are categorized according to the existing food processes and associated microbial responses, starting from below the growth boundaries and extending to the superoptimal growth limits. The available model structures are detailed in each category.

2.2 Probability and kinetic models for food processing and HACCP

The probability of growth models constitutes the cornerstone of predicting microbial dynamics, acting as the filter of the primary microbial response (growth or inactivation) and guiding the selection of the subsequent modeling tool, i.e., growth or inactivation model, for predicting the change in microbial numbers along processing or distribution. Such models together with cardinal growth models may be of great assistance to HACCP, by offering science-based numerical evidence for setting critical limits, establishing process or product criteria, and assessing the compliance of a given process to these limits.

2.2.1 Probability of growth models

Probability models can be used to predict the likelihood of the occurrence of a microbial response as a function of intrinsic and extrinsic factors of foods and processing environment (Ross and Dalgaard, 2004). Microbial responses that have been modeled using this approach include spore germination, toxin formation by *Clostridium botulinum*, growth initiation, and survival or death of bacteria as a result of lethal pH and organic acid combinations. The logistic model was the first to be used for fitting data on the probability (%; 0–100) of one of the above responses over time at a given set of conditions. Common dependent variables that were monitored over time include the fraction of positive (turbid) tubes, the probability of a single spore to give growth, or the probability of toxigenesis (Table 2.1). Parameters of the logistic model, such as P_{max}, probability rate (time^{-1}; i.e., the slope of the tangent through the inflection point, commonly symbolized as k), and mid-time point τ, were then expressed as a quadratic expression of the explanatory variables, such as T, pH, organic acid salt, preservatives, and CO_2 concentration (Table 2.1). The evolution of these models has led to the non-linear and ordinary logistic regression models, which describe the growth/no growth (G/NG) interface of microbial growth or death (McKellar *et al.*, 2002) as a function of intrinsic and extrinsic factors of foods (Table 2.2). These models are fitted to dichotomous (binary) datasets generated by storage of inoculated media or foods for a long period of time, e.g., 60 days. Microbial responses are then recorded by visible inspection of biomass precipitation and measurement of optical density (OD) (in liquid cultures), or by comparing the final and the initial counts of the microorganism (e.g., in foods). Probability values of 1 and 0 are assigned to cases that gave growth and no growth, respectively. Logistic regression models may assist the Food Industry in the establishment of critical limits and product criteria, as part of HACCP. Furthermore, they may be used as a reliable proof of whether a product complies with microbiological criteria and also contributes to the achievement of a Food Safety Objective (FSO) (van Schothorst *et al.*, 2009).

G/NG models were first developed for pathogenic bacteria, such as *Escherichia coli* O157:H7 (Presser *et al.*, 1998; McKellar and Lu, 2001), pathogenic *E. coli* (Salter *et al.*, 2000), and *Listeria monocytogenes* (Tienungoon *et al.*, 2000), describing the interface of these pathogens as a function of pH, T, a_w, and organic acids. The concept of interface in microbial responses has also been extended to fungi, spoilage yeasts, and lactic acid bacteria (LAB) in shelf-stable acidified sauces and fruit juices (Vermeulen *et al.*, 2007a, 2008). In the special case of mycotoxigenic fungi, the prediction of growth initiation eventually represents the risk of toxigenesis (Garcia *et al.*, 2011). Similarly, the assessment of growth of spoilage molds and yeasts or LAB in acetic acid-based sauces and fruit juices characterizes the microbial stability of their formulation (Vermeulen *et al.*, 2007a, 2008; Dang *et al.*, 2010; Panagou *et al.*, 2010). Therefore, proper models may be used for optimization of product development, considering that once spoilage organisms initiate growth, then spoilage is inevitable. For this reason, the initial G/NG models for shelf-stable acidified

Table 2.1 Probability models for *Cl. botulinum* growth from spores and toxigenesis.

Response variable	pH	T°C	a_w/NaCl	Other	Inoculum size	Product	Reference
Spore germination %	6.9–7.0	35°C	0.5%	N_2 $CO_2 + H_2$ H_2 $N_2 + H_2$	10^6 spores/ml	Peptone yeast extract broth	Foegeding and Busta (1983)
Time to swelling	Not determined	27°C	2.3–2.4%	0, 0.02% sodium isoascorbate	100 spores/can	Perishable canned cured pork	Tompkin *et al.* (1978)
Time to turbidity ($\ln T_m$)	5.5 6.5	5–12°C	0.5–2.5%	0–90% CO_2 CO_2 : H_2 N_2	10^6 spores/ml	PYGS	Fernandez *et al.* (2001)
Lag phase: longest incubation showing no toxic samples		4–30°C	0–2%	0–3% NaCl	10^{-2}–10^4 (7) spores/ml	Cooked turkey meat Chicken beast	Meng and Genigeorgis (1993)
Log *P*: probability of toxinogenesis LP: lag phase		5–25°C	Natural NaCl content of the product	Irradiation 0.5–1.0 kGy 0–20% O_2 15–60% CO_2	Undefined $p\% = 1/(1 + e^{-\mu})$	Pork chops μ = polynomial	Dodds (1993)
Log *P*: probability of a single spore giving growth LP: lag phase		1–30°C	Natural NaCl content of the product	Vacuum 70% CO_2–30% Air 100% CO_2	10^{-1}–10^4/50 g Fillet(6)	Salmon fillets with 3 levels of microflora	Garcia and Genigeorgis (1987)
LP: lag times		4–30°C	Natural NaCl content of the product	Vacuum 70% CO_2–30% N_2 100% CO_2	10^{-2}–10^4 (7) spores/sample 0 log–4 log	*Fresh fish* Rockfish Salmon Sole	Baker and Genigeorgis (1990)
Log *P* (%) of a single cell of LM to initiate growth CN: cells needed for one cell to initiate growth	>5.9	4–30°C	0.5–12.5%	Methyl paraben (0–0.2%) Na Propionate (0.3%) Na Benzoate (0.1%) K Sorbate (0.3%)	$>10^5$–10^{-2} / 2.4 ml (8)	Broth	Razavilar and Genigeorgis (1998)
Fraction of positive tubes P ($P = P_{max}/(1 + e^{k(r-t)})$)	5–7.0	4–28°C	0–4%		10–10^5 spores/tube	BAM broth	Whiting and Oriente (1997)
Time to turbidity	5.5–7.2	15–34°C	0–2.5%	Expansion of time to turbidity	10^0–10^4 spores/tube	RCM broth	Whiting and Strobaugh (1998)
Time to germinate under the microscope	7.0	20–37°C	0.5%	Microscopic examination Slide-agar-cover slip sealed with paraffin wax	Single spores	Nutrient agar	Billon *et al.* (1997)

Table 2.2 Representative growth/no growth models. When inoculation levels are not specified, high population densities, e.g., >4.5 $\log_{10}$ cfu/ml were used.

Micoorganism	Substrate	Model type	Factors/ranges	Total data	Measured by	Reference
Listeria monocytogenes	Cooked meat product	Ordinary	a_w: 0.88–0.98 T: 0–10 °C Inoc:10 and 10^4 cfu/cm^2	4 replicates per conditions 36 combinations	Viable count data per 60 days	Mataragas *et al.* (2006)
Listeria monocytogenes (inoculum) 1 strain	TSB	Ordinary	a_w: 0.888–0.997 pH: 3.76–6.44(HCl) T: 4–30 °C Inoc:0.90,2.58,4.20,6.01 log cfu/well	4 replicates per conditions 500 combinations	OD for 60 days	Koutsoumanis and Sofos (2005)
Listeria monocytogenes 5 strains	TSB TSB + 1.5% agar	Ordinary	a_w: 0.900–0.993 pH: 4.24–6.58 T: 4–30 °C Inoc: broth on agar surface	4 replicates per combination 180 combinations	OD for 30 days	Koutsoumanis *et al.* (2004a)
Salmonella typhimoriom 5 strains			a_w: 0.913–0.990 pH: 3.76–6.44 T: 10–35 °C	4 replicates per combination 350 combinations	OD for 62 days	Koutsoumanis *et al.* (2004b)
Listeria monocytogenes 5 strains	Nutrient broth	Ordinary	a_w: 0.960–0.990 pH: 5.0–6.0 T: 7 °C Acetic acid (AA): 0–0.8%	20 replicates per combination 180 combinations	OD for 30 days	Vermeulen *et al.* (2007b)
Listeria monocytogenes 5 strains	Nutrient broth	Square root cardinal model with ordinary terms for inoculum level (n, $\sqrt{n}$)	a_w: 0.960–0.990 pH: 5.0–6.0 T: 7 °C AA: 0, 0.2 and 0.4% Inoculum size: 2×10^5 to 1 cfu/well	66 combinations and 20 to 40 replicates per inoculum size and condition (total of 47.808 cases)	OD and plating for 90 days	Gysemans *et al.* (2007) Vermeulen *et al.* (2009)
Zygosaccharomyces bailii	Sabouraud and broth with glucose (7.5%) and fructose (7,5%)	Ordinary using undissociate and dissociate acid terms	a_w: 0.950 pH: 3.0–5.0 T: 30 °C LA: 0–3% AA: 0–3.5%	12 replicates per combination 336 combinations	OD for 30 days	Vermeulen *et al.* (2008)

Zygosaccharomyces bailii	Sabouraud and broth with glucose (7.5%) and fructose (7.5%)	Ordinary using total AA (%) terms	a_w: 0.93–0.97 pH: 3.0–5.0 T: 22 or 30 °C AA: 0–2.5%	20 replicates per combination 150 combinations per temperature	OD for 45 or 60 days	Dang *et al.* (2010)
Lactobacillus plantarum Lb. *fructivorans*	MRS broth with glucose (7.5%) and fructose (7,5%)	Ordinary	a_w: 0.950 pH: 3.0–5.0 T: 30 °C LA: 0–3% AA: 0–3%	12 replicates per combination 180 combinations	OD for 30 days	Vermeulen *et al.* (2007[a])
Listeria monocytogenes 10 strains	TSBYE	Ordinary	*Experiment I* pH: 3.82–7.42 T: 10 and 30 °C SL: 0–10% SD: 0–0.5%	8 replicates per combination 220 combinations	OD for 40 days	Skandamis *et al.* (2007c)
			Experiment II SL: 0–6% SD: 0–0.5% T: 4–30 °C NaCl: 0.5 and 2.5% Atmosphere: anaerobic and aerobic	4 replicates per combination 346 combinations		
Escherichia coli 0157:H7 4 strains	TSB	Ordinary	*Experiment I* pH: 3.52–7.32 with lactic acid a_w: 0.955–0.995 T: 10–35 °C Inoculation levels: 3.2×10^1, 3.6×10^3, 3.4×10^5 cfu/ml	4 replicates per combination 160 combinations per inoculum	OD for 60 days	Skandamis *et al.* (2007b)
			Experiment II pH: 3.52–7.32 with lactic acid a_w: 0.938–0.995 T: 10–35 °C Inoculum preparations: 1. TSB-G 2. TSB-G 3. Water washings (pH6.87) 4. LA-washings (pH4.92)	4 replicates per combination 360 combinations per inoculum preparation		

(*Continued*)

Table 2.2 (Continued)

Micoorganism	Substrate	Model type	Factors/ranges	Total data	Measured by	Reference
Listeria monocytogenes 4 strains	Lightly preserved seafood	Square-root cardinal model	SD: 0–0.15% SL: 0–1.5% T: 8 and 15 °C Vacuum or 40% CO_2 – : 60% N_2	3 replicates	Viable counts	Mejlholm and Dalgaard (2007b)
Listeria monocytogenes 3 strains	TSBYE	Ordinary	pH: 4.8–6.6 a_w: 0.955–0.996 Inoculum :10^0, 10^6 cfu/ml Inoculum type: 1. Detached from SSG 2. Planktonic cells	3 replicates per combination 30 combinations	OD for 30 days	Belessi *et al.* (2011a)
Escherichia coli (STEC) 2 strains	BHI	Ordinary model per time to growth (TTG)	a_w : 0.945–0.995 Undissociale LA : 2–10 mM pH: 4.46–5.24 T: 27 °C	3 replicates per combination 30 combinations	OD for 14 days	Lindbland and Lindgvist (2010)
Listeria monocytogenes 2 strains	TSB, milk, cheese curd	Non-linear cardinal model	pH: 5.5–6.5 (LA) NaCl: 0–8% Inoculation level: 10 and 100 cfu/ml T: 30 °C	6 replicates per combination 20 combinations	Viable counts for 8 h	Schvartzman *et al.* (2010)
Listeria monocytogenes 1 strains	BHI	Ordinary	pH: 5.5–6.5 Citric acid: 0–0.4% Ascorbic acid: 0–0.4% T: 4–30 °C	8 replicates per combination 232 combinations	OD for 21 days	Valero *et al.* (2006)
Escherichia coli 0158:H23 (selected as the serotype growing faster than 059:H21; 055:H6; 0157:H7	TSB	Ordinary with variables scaled in the range 0.1–0.9 $x = (x - x_{min}/x_{max} - x_{min})$	pH: 5.0–7.0 a_w: 0.960–0.999 T: 8–16 °C Inoculum level: 2, 3, 4 log cfu/ml	8 replicates per combination 405 combinations	OD for 30 days	Valero *et al.* (2010)
Staphylococcus aureus 5 strains	TSB	ordinary	pH: 4.5–7.0 a_w: 0.856–0.999 T: 8–19 °C	30 replicates per combination 146 combinations	OD for 36 days	Valero *et al.* (2009)

Aspergillus ochraceus *Aspergillus parasiticus*	Malt extract agar	Ordinary	pH: 6.0–6.2 a_w: 0.80–0.93 (glycerol) T: 10–42 °C	10 replicates per combination 42 combinations	Visual inspection for 90 days	Garcia *et al.* (2011)
Byssochlamys fulva *Byssochlamys nivea*	Malt extract agar	Ordinary	pH: 5.5–6.0 a_w: 0.88–0.99 T: 10–45 °C	8 replicates per combination 48 combinations	Visual inspection for 30 days	Panagou *et al.* (2010)
A. flavus	3% chilli powder extract agar	Ordinary	a_w: 0.85–0.97 Time : 0–90 days	10 replicates × 3 times per combination 6 combinations	Visual inspection for 90 days Ergosterol analysis	Marín *et al.* (2009)
A. carbonarius	Hulled pistachios	Ordinary	Moisture: 12.5–34.8% T: 10–42 °C Time: 17–90 days	10 replicates per combination 35 combinations	Visual inspection for 90 days	Marín *et al.* (2008)
A. carbonarius	Synthetic grape medium	Ordinary	a_w: 0.85–0.96 T: 10–40 °C Time: 0–25 days	4 replicates x 2 times per combination 42 combinations	Visual inspection for 25 days Ochratoxin A determination	Tassou *et al.* (2009)
P. brevicompactum	MY 50	Non-linear	pH: 5.0 Sugar: 500 g/l Sorbic acid: 0–1000 mg/l Benzoic acid: 0–300 mg/l	5 replicates per combination 24 combinations	Visual inspection for 75 days	Membré *et al.* (2001)

products predicted not only the probability of growth but also the time to growth (TTG) of spoilage organisms, such as *Zygosaccharomyces bailii* (Jenkins *et al.*, 2000). The latter response resembles that of the time to toxigenesis (also incorrectly termed "lag time") by *Cl. botulinum*, whereas TTG has also been modeled for *E. coli* (Lindblad and Lindqvist, 2010) and *Geobacillus stearothermophilus* (Llaudes *et al.*, 2001).

Given the variety of existing G/NG models (Table 2.2), recent reports attempted to validate the most commonly used cardinal G/NG models against independent data from literature and international databases, such as ComBase. Representative initiatives include the validation of G/NG and growth models for *L. monocytogenes* in mayonnaise-based salads (Vermeulen *et al.*, 2007c) and ready-to-eat (RTE) meat products and seafood (Mejlholm *et al.*, 2010). More specifically, the collective validation dataset of Mejlholm *et al.* (2010) consisted of 1014 G/NG responses and six different models were evaluated. Notably, it was proven that the most complex models, which accounted for the effect of nine environmental factors, performed better than simpler models, suggesting the usefulness of complexity in model development, when necessary.

Judging by the overview of the existing G/NG models in Table 2.2, it is evident that ordinary models are the most commonly used. This was expected because ordinary logistic regression is often a built-in routine in most commercial statistical software, thereby requiring no code or advanced mathematical skills for their application. Although the high number of models published emphasizes their importance for food spoilage and safety, the marked diversity in strains, experimental conditions and protocols, culture preparations, inoculation levels, and duration of incubation underlines the difficulty in comparison of different models. Furthermore, overlapping between experimental conditions for the same microorganisms by different researchers is also evident. Therefore, systematic grouping of models and available G/NG responses would greatly enhance the application of probability models in daily practice. In this regard, the Microbial Response Viewer (Koseki, 2009) constitutes an updated Combase database of G/NG data for 29 microorganisms in response to temperature, pH, and a_w, fitted to ordinary and square-root cardinal models. An important advantage of this web-based application is the simultaneous illustration of the G/NG interface and the growth rate at growth-supporting conditions, through colored contour plots.

2.2.2 Growth kinetic models

2.2.2.1 Effect of temperature

As nicely detailed by Corkrey *et al.* (2012, 2014) in their seminal meta-analysis article, in their effort to thermodynamically describe the temperature dependence of the growth rate of multiple species from all three domain of life, namely Bacteria, Archaea, and Eukarya: "…temperature governs the rate of chemical reactions including those of enzymatic processes controlling the development and decline of life on earth from individual cells to complex populations and spanning temperatures from well below freezing (e.g., −2 °C) to above the boiling point of water, such as at 122 °C, which is the highest temperature so far known for biological growth". Commonly, the change of growth rate of poikilothermic organisms in the biokinetic ranges has a U-shape characterized by a minimum (suboptimal), maximum (superoptimal) around the optimal for growth. In their work, Corkrey *et al.* (2012, 2014) concluded that the effect of temperature on poikilotherm growth rate is exerted through a single rate-limiting enzyme-catalyzed reaction, also associated with protein denaturation. In this way, the temperature dependence of the growth rate of various organisms can be described by an Arrhenius-type equation that uses the following thermodynamic components in the form of non-linear regression model parameters: (i) the enthalpy (ΔH) of activation of the rate-limiting reaction, (ii) the heat capacity change (ΔC), (iii) denaturation kinetics (D), and (iv) the critical temperature (T_{mes})at which the protein denaturation

is minimized (i.e., corresponding to the theoretical optimal growth temperature).

Temperature is also known to control the shelf life of foods, either by determining the rate of microbial spoilage or the rate of microorganism-independent quality decay reactions, e.g., due to enzymes causing texture breakdown (e.g., pectinolytic, enzymes acting on myofibrillar proteins) and lipid or colour oxidation (e.g., polypheloxodases, esterases, etc.) A shelf life loss kinetic model is characteristic not only for the studied food but equally important to the set of environmental conditions of the experiment. These conditions can determine the reaction rates and have to be defined and monitored during kinetic experiments. Since most environmental factors do not remain constant the next logical step would be to expand the models to include them as variables, especially the ones that more strongly affect the reaction rates and are more prone to variations during the life of the food. The practical approach is to model the effect into the apparent reaction rate constant, i.e., expressing k as a function of E_j: $k = k(E_j)$ (Taoukis *et al.*, 1997):

$$r_A = \frac{-d[A]}{dt} = k[A]^m$$

Secondary models describe the mathematical dependence of parameters (e.g., lag time, growth rate, time-to-certain log increase or reduction) estimated by a primary model on the environmental or cultural conditions, such as pH, a_w, T, E_h, CO_2 concentration in the packaging atmosphere, preservatives, etc. Food product spoilage is dynamic and in some cases relatively small changes in environmental conditions cause a complete shift in the microflora responsible for product spoilage (i.e., the ephemeral or specific spoilage organisms) or in the non-microbial quality decay indices. The modeling procedure includes the mathematical description of growth/inactivation kinetics or the probability of growth for a microorganism of interest, as a function of a specific interpolation range of the environmental variables affecting these kinetics. In 1982, Hauschild assessed the probability of spore germination and the production of toxin in vacuum-packaged bacon and liver sausage. Hauschild and other researchers (Roberts and Gibson, 1986; Tompkin, 1986) also examined the effects of some variables such as salt, nitrite, phosphate and sorbate concentration, formulation, processing techniques, and pH. The most common secondary models are the Arrhenius model, modified Arrhenius, polynomial and square root models. The Arrhenius is an empirical model, based on thermodynamic considerations (Labuza and Riboh, 1982):

$$k = k_0 e^{-E_A/RT}$$

The Arrhenius equation expresses the dependence of the rate (k) of chemical reactions on the temperature T (in absolute temperature kelvin) through the activation energy E_A. Parameter k_0 is the pre-exponential factor or simply the prefactor and R is the gas constant (8314 J/K mol). In the above equation, if values of k are recorded at different temperatures and if ln k is plotted against $1/T$, a straight line is formed with slope $-E_A/R$ (Labuza and Riboh, 1982; Labuza *et al.*, 1992). If the objective is to get a rapid estimation of the expected shelf life of the product, then an accelerated shelf life test (ASLT) can be used in the range of interest. Taking into account the limitations or possible deviations from the Arrhenius law, the ASLT involves the use of the Arrhenius equation at higher testing temperatures in a thorough shelf life study and extrapolation of the kinetic results to normal, non-abusive storage conditions. This procedure is used to substantially reduce the experimental time, through the acceleration of the quality deterioration reactions. The successive steps that outline shelf life determination and ASLT methodology are presented in Taoukis *et al.* (1997). The Arrhenius relation developed theoretically for reversible molecular chemical reactions has been used to describe the effect of temperature on the rate of several reactions of quality loss. It should be noted that

the Arrhenius equation implies that k_A is the value of the reaction rate at 0 K, which is of no practical interest. Alternatively, the use of a reference temperature, T_{ref}, is recommended, corresponding to a representative value in the temperature range of the process/storage of study. The Arrhenius equation is then mathematically transformed as follows:

$$k = k_{ref} \exp\left[\frac{-E_A}{R}\left(\frac{1}{T} - \frac{1}{T_{ref}}\right)\right]$$

where k_{ref} is the rate constant at the reference temperature T_{ref}. The value of E_A is, in that case, calculated from the linear regression of ln k versus $(1/T - 1/T_{ref})$. Alternatively to isothermal kinetic analysis, the study at a single non-isothermal temperature profile is proposed, where temperature varies with time, following a predetermined function of $T(t)$ (Taoukis and Giannakourou, 2004). In that case, the above equation is modified as follows, assuming a first-order reaction:

$$A = A_0 \exp\left[-k_A \int_0^t \exp\left(-\frac{E_A}{R}\frac{1}{T(t)}\right)dt\right]$$

where the integral is calculated by numerical techniques. This approach requires a very strict temperature control and is very sensitive to experimental error in concentration measurements. Although the Arrhenius equation may be applied in most non-microbial quality decay indices, for the bacterial growth extrapolations plots may show non-linearity. Therefore, it cannot fit data well below optimum or above minimum temperatures for growth. The plots are normally only accurate over a limited temperature range for microbial growth (Labuza and Fu, 1993). Fu *et al.* (1991) illustrated this accuracy with Arrhenius plots for *Pseudomonas fragi*.

Schoolfield *et al.* (1981) reparameterized an earlier equation (Sharpe and De Michele, 1977) into a six-parameter non-linear model shown as follows:

$$\frac{1}{K} = \frac{\rho(25^\circ\mathrm{C})\frac{T}{298}\exp\left\{\frac{\Delta H_A^{\neq}}{R}\left(\frac{1}{298} - \frac{1}{T}\right)\right\}}{1 + \exp\left[\frac{\Delta H_L}{R}\left(\frac{1}{T_{1/2_L}} - \frac{1}{T}\right)\right] + \exp\left[\frac{\Delta H_H}{R}\left(\frac{1}{T_{1/2_H}} - \frac{1}{T}\right)\right]}$$

where T is the absolute temperature, R is the universal gas constant, K is the response (e.g., generation time), $\rho_{(25)}$ a scaling factor equal to the response rate $(1/K)$ at 25 °C, H_A is the activation energy of the rate-controlling reaction, H_L is the activation energy of denaturation of the growth-rate-controlling enzyme at low temperatures, H_H is the activation energy of denaturation of the growth-rate-controlling enzyme at high temperatures, $T_{1/2L}$ is the lower temperature at which half of the growth-rate-controlling enzyme is denaturated, and $T_{1/2H}$ is the higher temperature at which half of the growth-rate-controlling enzyme is denaturated.

The Q_{10} approach in essence introduces a temperature dependence equation of the form:

$$k(T) = k_0 e^{bT} \quad \text{or} \quad \ln k = \ln k_0 + bT$$

which implies that if ln k is plotted versus temperature (instead of $1/T$ of the Arrhenius equation) a straight line is obtained. Such plots are often called shelf life plots, where b is the slope of the shelf life plot and k_0 is the intercept. The shelf life plots are true straight lines only for narrow temperature ranges of 10 to 20 °C (Labuza, 1982). For such a narrow interval, data from an Arrhenius plot will give a relatively straight line in a shelf life plot, i.e., Q_{10} and b are functions of temperature:

$$\ln Q_{10} = 10b = \frac{E_A}{R}\frac{10}{T(T+10)}$$

The variation of Q_{10} with temperature for reactions of different activation energies is shown in

Table 2.3 Q_{10} dependence on E_A and temperature.

E_A (kJ/mol)	Q_{10}			Reactions in E_A range
	at 4 °C	at 21 °C	at 35 °C	
50	2.13	1.96	1.85	Enzymic, hydrolytic
100	4.54	3.84	3.41	Nutrient loss, lipid oxidation
150	9.66	7.52	6.30	Non enzymatic browning

Table 2.3. Similarly to Q_{10} the term Q_A is sometimes used. The definition of Q_A is the same as Q_{10} with 10 °C replaced by A °C:

$$Q_A = Q_{10}^{A/10}$$

Zwietering *et al.* (1991) compared the suitability and usefulness of the Schoolfield model to five other models using *L. plantarum* grown at various temperatures between 6 and 43 °C. It was found that the Schoolfield model described data satisfactorily. Davey (1989) used a modified Arrhenius-type model to describe the effects of water activity and temperature on microbial growth rates:

$$\ln k = C_0 + \frac{C_1}{T} + \frac{C_2}{T^2} + C_3 a_w + C_4 a_w^{\ 2}$$

where T is temperature (K), a_w is the water activity, and C_0, C_1, C_2, C_3, C_4 are coefficients to be determined. Products, such as dried or fermented meats, that have low water activity (e.g., a_w <0.90) may find this equation applicable (Van Gerwen and Zwietering, 1998). However, when water activity is non-limiting the last two terms of the equation can be removed (McMeekin *et al.*, 1992). In the above equation, all parameters appear linearly and thus estimation can be made using multiple linear regression.

Ratkowsky *et al.* (1982) introduced the Belehradek model for the first time in food microbiology. Until then, this model was unknown in the field of microbiology, although it has been published since 1926 (McMeekin *et al.*, 1993; Ross, 1993). This model, which is also known as the "square root model" is shown below:

$$\sqrt{k} = b\left(T - T_{\min}\right)$$

where k is the growth rate, b is a constant, and T is the temperature. The parameter T_{min}, a theoretical minimum temperature for growth, is the intercept between the model and the temperature axis. Given that T_{min} is a model parameter (i.e., theoretical), its value can be 5 to 10 °C lower than the actual lowest temperature at which growth may occur. Ratkowsky tested 50 sets of growth data and found that this model described the microbial growth rate well (Ratkowsky *et al.*, 1982). Moreover, Pooni and Mead (1984) compared the results of the square root model with other models to data from 14 published studies on poultry spoilage and found that the above equation was the most appropriate for predicting spoilage from –2 up to 15 °C. The square root model was later extended in order to include the entire biokinetic temperature range (Ratkowsky *et al.*, 1982), as described by the following empirical non-linear regression model:

$$\sqrt{k} = b\left(T - T_{\min}\right)\left\{1 - \exp\left[c\left(T - T_{\max}\right)\right]\right\}$$

where b is the regression coefficient of k for temperatures $T < T_{\text{opt}}$, c is a parameter allowing the model to fit the data over the optimum temperature, T is the temperature, $T_{\min}$ is the theoretical minimum temperature below which no growth is likely, and $T_{\max}$ is the theoretical

maximum temperature beyond which growth is not possible.

The equation terms T_{min} and T_{max} can be used to classify the microorganisms into psychrophiles, mesophiles, or thermophiles (Ross, 1993). Other researchers have also shown that this equation is reasonably effective in predicting effects of constant storage temperatures on microbial growth rates (Chandler and McMeekin, 1985a, 1985b; Phillips and Griffiths, 1987). The effects of fluctuating storage temperature has been studied by Blankenship *et al.* (1988) who developed a dynamic model for predicting growth of *Cl. perfringens* in cooked meat with chilli during chilling using a time-explicit approach.

2.2.2.2 Effects of other environmental factors

Moisture content and water activity (a_w) are also important factors besides temperature that affect the rate of food deterioration reactions. Water activity describes the degree of bounding of the water contained in the food and its availability to act as a solvent and participate in chemical reactions or support microbial growth (Labuza, 1980). Critical levels of a_w can be recognized above which undesirable deterioration of food occurs. Controlling the a_w is the basis for preservation of dry and intermediate moisture foods (IMFs). Minimum a_w values for growth can be defined for different microbial species. For example, the most tolerant pathogenic bacterium is *Staphylococcus aureus*, which can grow down to an a_w of 0.85–0.86. This is often used as the critical level of pathogenicity in foods. McMeekin *et al.* (1987) modified the classical square root model linking growth rate to temperature by adding the a_w variable. The new model accurately predicted the effect of temperature and water activity on the growth rate of *Staphylococcus xylosus* and *Halobacterium* spp., respectively, on salted dried fish (McMeekin *et al.*, 1987; Chandler and McMeekin, 1989a). However, it was found that the effect of variables were additive, suggesting that T and a_w act independently (Chandler and McMeekin, 1989a):

$$\sqrt{k} = b\left(T - T_{\min}\right)\sqrt{\left(a_w - a_{w\min}\right)}$$

The pH of the food system is another determining factor. The effect of pH on different microbial, enzymatic, and protein reactions has been studied in model biochemical or food systems. Enzymatic and microbial activity exhibits an optimum pH range and limits above and below which activity ceases, much like the response to temperature. The functionality and solubility of proteins depend strongly on pH, with the solubility usually being at a minimum near the isoelectric point, having a direct effect on their behavior in reactions. Examples of important acid–base catalyzed reactions are non-enzymatic browning and aspartame decomposition. Non-enzymatic browning of proteins shows a minimum near pH 3–4 and high rates in the near neutral–alkaline range. Significant progress in elucidating and modeling the combined effect to microbial growth of factors such as T, pH, a_w, or salt concentration has been achieved in the field of predictive microbiology (Ross and McMeekin, 1994; Rosso *et al.*, 1995).

Adams *et al.* (1991) modified a square root model for the combined effects of pH and temperature using a variety of acidulants and showed that the growth rate under varying conditions of suboptimal temperature and pH can be predicted using this modified equation, which gave good fits for three serotypes of *Yersinia enterocolitica*:

$$\sqrt{k} = b\left(T - T_{\min}\right)\sqrt{\left(pH - pH_{\min}\right)}$$

McMeekin *et al.* (1992) suggested that the effects of temperature, a_w, and pH on microbial growth could be described together with the following equation that was subsequently used successfully on growth data for *Listeria monocytogenes* by Wijtzes *et al.* (1993):

$$\sqrt{k} = b\left(T - T_{\min}\right)\sqrt{\left(a_w - a_{w\min}\right)}\sqrt{\left(pH - pH_{\min}\right)}$$

Gas composition also affects certain quality loss reactions. Oxygen affects both the rate and apparent order of oxidative reactions, based on

its presence in limiting or excess amounts. Exclusion or limitation of O_2 by nitrogen flushing or vacuum packaging reduces the redox potential and slows down undesirable reactions. Further, the presence and relative amount of other gases, especially carbon dioxide and secondly ethylene and CO, strongly affects biological and microbial reactions in fresh meat, fruit, and vegetables. The mode of action of CO_2 is partly connected to surface acidification but additional mechanisms, not clearly established, are in action. Different systems require different O_2:CO_2:N_2 ratios to achieve maximum shelf life extension and, often, excess CO_2 can be detrimental. Alternatively, hypobaric storage, whereby total pressure is reduced, has been studied. Modified atmosphere packaging is commonly used with many fresh meats. Recently, Devlieghere *et al.* (1998) replaced the pH terms in the square root model with terms to describe for dissolved CO_2 in modified atmosphere cooked meats. The following equation was proposed for modeling the effect of carbon dioxide-enriched (%CO_2) atmospheres on growth of the specific spoilage organism *Photobacterium phosphoreum* on fish (Dalgaard, 1995; Dalgaard *et al.*, 1997):

$$\sqrt{k} = b\left(T - T_{\min}\right)\frac{\left(\%CO_{2\max} - \%CO_2\right)}{\%CO_{2\max}}$$

Later, similar but square-root-transformed terms were used to model the effect of CO_2 and sodium lactate (NaL) on the growth of *Lactobacillus sake* and *Listeria monocytogenes* at a given pH (Devlieghere *et al.*, 1998, 2000a, 2000b):

$$\sqrt{k} = b\left(T - T_{\min}\right)\sqrt{\left(a_w - a_{w\min}\right)}\sqrt{\left(CO_{2\max} - CO_2\right)}\sqrt{\left(NaL_{\max} - NaL\right)}$$

A more comprehensive square-root-type model that includes the effects of temperature, pH, water activity, and lactic acid has been suggested in a series of publications (Presser *et al.*, 1997; Ross, 1993; Salter *et al.*, 1998):

$$\sqrt{k} = c\left(T - T_{\min}\right)\left(1 - \exp\left(d\left(T - T_{\max}\right)\right)\right)\sqrt{\left(a_w - a_{w\min}\right)} \times \left(1 - \exp\left(g\left(a_w - a_{w\max}\right)\right)\right)\sqrt{1 - 10^{pH\min - pH}}\sqrt{1 - 10^{pH - pH\max}} \times \sqrt{1 - \frac{LAC}{U_{\min}\left(1 + 10^{pH - pKa}\right)}}\sqrt{1 - \frac{LAC}{D_{\min}\left(1 + 10^{pKa - pH}\right)}}$$

It has been applied to *Listeria monocytogenes* and *Escherichia coli* growth rates. It was presented in its most complete form in Ross *et al.* (2003); *c*,*d*, and *g* are fitted parameters, [*LAC*] is the lactic acid concentration (mM), $U_{\min}$ is the minimum concentration (mM) of undissociated lactic acid that prevents growth when all other factors are optimal, $D_{\min}$ is the minimum concentration (mM) of dissociated lactic acid that prevents growth when all other factors are optimal, and *pKa* is the pH for which concentrations of undissociated and dissociated lactic acid are equal. The gamma (γ) concept, or concept of dimensionless growth factors, was introduced by Zwietering *et al.* (1992). Later, minor changes and new developments were added (Wijtzes *et al.*, 1998, 2001; Zwietering, 1999; Zwietering *et al.*, 1996). The gamma (γ) concept relies on the following factors:

i. The observation is made that many factors that affect microbial growth rate act independently and that the effect of each measurable factor on the growth rate can be represented by a discrete term that is multiplied by terms for the effect of all other growth rates affecting factors, i.e.:

$$\mu = f\left(T\right) \times f\left(a_w\right) \times f\left(pH\right) \times f\left(\text{organic acid}\right) \times f\left(\text{other factor}_1\right) \times f\left(\text{other factor}_2\right) \times \ldots \left\langle \text{raise} \ldots \right\rangle f\left(\text{other factor}_n\right)$$

ii. The effect on growth rate of any factor can be expressed as a fraction of the maximum growth rate (i.e., the rate when that environmental factor is at the optimum level). Under the gamma concept approach, the cumulative effect of many factors poised at suboptimal levels can be estimated from the product of the relative inhibition of the growth rate due to each factor:

$$\gamma = \frac{\mu_{\max}\left(T, a_w, pH, \ldots\right)}{\mu_{\max\, opt}}$$

In the gamma model approach, the reference growth rate is μ_{max}, so that reference levels of temperature, water activity, etc., are those that are the optimum for growth rate, usually represented as T_{opt}, a_{wopt}, pH_{opt}, etc. The relative inhibitory effect of a specific environmental variable is described by a growth factor "gamma" (γ), a dimensionless measure that has a value between 0 and 1:

$$\gamma(T) = \left(\frac{T - T_{\min}}{T_{opt} - T_{\min}}\right)^2$$

$$\gamma(pH) = \frac{(pH - pH_{\min})(pH_{\max} - pH)}{(pH_{opt} - pH_{\min})(pH_{\max} - pH_{opt})}$$

$$\gamma(a_w) = \frac{(a_w - a_{w,\min})}{(1 - a_{w,\min})}$$

The relative inhibitory effect can be determined from the "distance" between the optimal levels of the factor and the minimum (or maximum) level that completely inhibits growth by recourse to a predictive model. The combined effect of several environmental factors is then determined by multiplication of their respective γ factors:

$$\mu_{\max} = \mu_{\max\, opt}\gamma(\mathrm{T})\gamma(\mathrm{pH})\gamma(\mathrm{a_w})$$

The effect of environmental parameters like carbon dioxide, sodium lactate, and nitrite has also been included in square-root-type models. The absence of these inhibitory substances is optimal for growth. Therefore the calculation of γ factors requires information only about the lowest concentration of each substance that prevents growth. Cardinal parameter models (CPMs) were introduced to predictive microbiology in 1993 and have become an important group of empirical secondary models (Augustin and Carlier, 2000; Le Marc *et al.*, 2002; Messins *et al.*, 2002; Pouillot *et al.*, 2003; Rosso, 1999; Rosso *et al.*, 1993, 1995; Rosso and Robinson, 2001). The basic idea behind CPMs is to use model parameters that have a biological or graphical interpretation. When models are fitted to experimental data by non-linear regression, this has the obvious advantage that appropriate starting values are easy to determine. In addition, the models may be easily adjusted to account for different pathogen–food combinations by introducing the cardinal values and the maximum specific growth rate at optimum conditions (μ_{opt}) of the organisms in the target (e.g., new) food. General CPMs rely on the assumption that the inhibitory effect of environmental factors is multiplicative. CPMs consist of a discrete term for each environmental factor, with each term expressed as the growth rate relative to that when that factor is optimal. At optimal growth conditions all terms have a value of 1 and thus $\mu_{\max}$ is equal to μ_{opt}.

Augustin *et al.* (2005) proposed a new, mechanistically based, CP model. This model includes the effect of six fundamental variables (temperature, a_w, pH, phenol concentration, nitrites, and CO_2) and the interactions between these environmental parameters (Mejlholm *et al.*, 2010):

$$\mu_{\max} = \mu_{opt}\, CM_2(\mathrm{T})\, CM_1(pH)\, SR(a_w)\, SR(nit)\, SR(phe)\, SR(CO_2)\, \xi$$

where a_w is the water activity, *nit* is the concentration (mM) of undissociated sodium nitrite, *phe* is the concentration (ppm) of smoke components (phenol), CO_2 is the CO_2 proportion, and ξ is the effect of interactions between the environmental parameters, especially at combinations near the growth boundaries.

Abbreviations CM_n and SR were included as used by Augustin *et al.* (2005). With this model, both the growth rates and probability of growth of *Listeria monocytogenes* could be predicted accurately in dairy, meat, and seafood products in a single model output. Augustin *et al.* (2005) estimated optimal specific growth rates for each of the product categories by fitting their model to growth data obtained from dairy ($n = 340$), meat ($n = 324$) and seafood ($n = 80$) products:

$$CM_n = \left\{ \begin{array}{l} 0, X \le X_{\min} \\ \dfrac{(X - X_{\max})(X - X_{\min})^n}{(X_{opt} - X_{\min})^{n-1}\left[\begin{array}{l}(X_{opt} - X_{\min})(X - X_{opt}) - (X_{opt} - X_{\max}) \\ \times((n-1)X_{opt} + X_{\min} - nX)\end{array}\right]}, X_{\min} < X < X_{\max} \\ 0, X \ge X_{\max} \end{array} \right\}$$

where X is the temperature or pH; $X_{\min}$, X_{opt}, and $X_{\max}$ are the theoretical minimal, optimal, and maximal values of X for growth of *Listeria monocytogenes*;

$$SR(a_w) = \left\{ \begin{array}{l} 0, a_w \le a_{w_{\min}} \\ \left(\dfrac{a_w - a_{w_{\min}}}{a_{w_{opt}} - a_{w_{\min}}}\right), a_{w_{\min}} < a_w < a_{w_{opt}} \\ 0, a_{w_{opt}} \le a_w \le a_{w_{\max}} \end{array} \right\}$$

where $a_{w\,min}$, $a_{w\,opt}$ and $a_{w\,max}$ are the theoretical minimal, optimal, and maximum a_w values allowing growth of *L. monocytogenes*;

$$SR(c) = \left\{ \begin{array}{l} 1 - \dfrac{c}{MIC}, c < MIC \\ 0, c \ge MIC \end{array} \right.$$

where MIC is the minimum inhibitory concentration of undissociated sodium nitrite (mM), phenol (ppm), or CO_2 (proportion) against *L. monocytogenes* and c is the concentration of undissociated sodium nitrite (mM), the concentration of phenol (ppm), or the proportion of CO_2. The modeling of the interactions between the environmental parameters (ξ) is based on the approach of Le Marc *et al.* (2002):

$$\xi = \left\{ \begin{array}{l} 1, \psi < 0.5 \\ 2(1 - \psi), 0.5 < \psi < 1 \\ 0, \psi \ge 1 \end{array} \right.$$

The ψ-value is determined from sets of environmental parameters and describes how far specific combinations of product characteristics and storage conditions are from the predicted growth boundary (corresponding to a value of ψ equal to 1.0) (LeMarc *et al.*, 2002):

$$\psi = \sum_i \frac{\varphi_{e_i}}{2\prod_{j \ne i}(1 - \varphi_{e_i})}$$

and

$$\varphi(\mathrm{T}) = \left(\frac{T_{opt} - T}{T_{opt} - T_{\min}}\right)^3$$

$$\varphi(\mathrm{pH}) = \left(\frac{pH_{opt} - pH}{pH_{opt} - pH_{\min}}\right)^3$$

$$\varphi(a_{\mathrm{w}}) = \left(\frac{a_{\mathrm{w}_{opt}} - a_{\mathrm{w}}}{a_{\mathrm{w}_{opt}} - a_{\mathrm{w}\min}}\right)^3$$

$$\varphi(nit, phe, CO_2) = 1 - SR(nit)SR(phe)SR(CO_2)$$

The Augustin model has been expanded by introduction of the effect of different organic acids and their interactions, leading to the CPM of Zuliani *et al.* (2007). The new parameters added are acetic acid and lactic acid. The maximum growth rate is expressed by

$$\mu_{\max} = \mu_{opt}\, CM_2(\mathrm{T})\, CM_1(pH)\, SR_1(a_w)\, SR(OA)\, \xi$$

where *OA* represents the concentration (mM) of undissociated acetic or lactic acid. Zuliani *et al.* (2007) suggested that the antimicrobial effect of acetic and lactic acid should be modeled as the effect of the dominating undissociated acid alone:

$$SR\left(AAC_{\mathrm{U}}\right)=\begin{cases}1-\dfrac{\left[AAC_{\mathrm{U}}\right]}{\left[MIC_{\mathrm{U\,acetic\,acid}}\right]}, AAC_{\mathrm{U}}<MIC_{\mathrm{U\,acetic\,acid}}\\ 0 \qquad\qquad\qquad\quad , AAC_{\mathrm{U}}>MIC_{\mathrm{U\,acetic\,acid}}\end{cases}$$

$$SR\left(LAC_{\mathrm{U}}\right)=\begin{cases}1-\dfrac{\left[LAC_{\mathrm{U}}\right]}{\left[MIC_{\mathrm{U\,lactic\,acid}}\right]}, LAC_{\mathrm{U}}<MIC_{\mathrm{U\,lactic\,acid}}\\ 0 \qquad\qquad\qquad\quad , LAC_{\mathrm{U}}>MIC_{\mathrm{U\,lactic\,acid}}\end{cases}$$

where $[AAC_{\mathrm{U}}]$ and$[LAC_{\mathrm{U}}]$ are the concentrations (mM) of undissociated acetic acid and lactic acid, respectively, and [MIC_{U} acetic acid] and[MIC_{U} lactic acid] are the MICs (mM) of acetic acid and lactic acid, respectively, that prevent growth of *Listeria monocytogenes*.

2.3 Thermal inactivation

Thermal processing is today one of the most important preservation methods in a wide variety of foods. Quantification of the microbial destruction during thermal treatment of a food product is one of the main issues tackled by predictive microbiology. Throughout the years, different mathematical models have been developed to describe microbial inactivation based either on empirical or biological insights to ensure food safety and quality. These models could further contribute to the microbiological aspect of HACCP by identifying certain microorganisms that could be potential hazards in the process and define steps at which critical control can be achieved (Valdramidis *et al.*, 2005). The efficacy of thermal processes has been calculated based on the assumption that microbial inactivation is a process following first-order kinetics (Stumbo, 1973). This model is based on the assumption that the inactivation of microbial cells takes place at a constant proportion in each successive time (Li *et al.*, 2007), and it has been proved effective for the last 90 years in the canning industry for high-temperature treatments at sterilization level to control *Clostridium botulinum*. It was stipulated that for a given microorganism a thermal process should be expressed in terms of the number of log reductions that the process is expected to deliver (Heldman and Newsome, 2003). In most cases, 6*D* is the target for pasteurization and 12*D* for commercial sterility (ICMSF, 2005). First-order kinetic models with the derived *D* and *z* values have been extensively employed due to simplicity in the interpretation of kinetic parameters by the industry for a variety of pathogens (Van Asselt and Zwietering, 2006) including *Listeria monocytogenes* in chicken meats (Huang, 2013), traditional sausages (Felício *et al.*, 2011), kiwifruit puree (Benlloch-Tinoco *et al.*, 2014), *Salmonella* spp. in chicken shawirma (gyro) (Osaili *et al.*, 2013), peanut butter (Li *et al.*, 2014), catfish and tilapia (Rajkowski, 2012), *Yersinia enterocolitica* in liquid egg products (Favier *et al.*, 2008), *Escherichia coli* in buffalo Mozzarella curd (Trevisani *et al.*, 2014), and fish (Rajkowski, 2012). However, the concept of *D* value becomes ineffective in the case where deviation from linearity occurs. This deviation from first-order kinetics was tackled in various ways, the most important being to ignore the curvature of the survival curve and take into account only the linear segment, although such as approach would lead to over- or underestimation of the thermal treatment and could jeopardize the

safety of the product (Peleg and Penchina, 2000). From a practical point of view, microbial inactivation is often non-linear and a shoulder phase has been observed before initiation of a decline phase occurs, whereas in many cases a prolonged survival of a low population of the organism is observed (tailing), especially in the case of mild heat treatment. In an extensive survey for over 120 inactivation curves of non-spore-forming bacteria, it was demonstrated that only 5% of them followed a log-linear pattern (van Boekel, 2002). In view of these observations, a number of non-linear models has been developed, as detailed by other authors (Corradini and Peleg, 2007; Smelt and Brul, 2014). Among them, the most popular were the biphasic model (Cerf, 1977), modified Gompertz (Bhaduri *et al.*, 1991), log-logistic (Cole *et al.*, 1993), Whiting and Buchanan (1993), and Baranyi (Baranyi and Roberts, 1994). The modified Gompertz equation was one of the first models employed for the inactivation of *Listeria monocytogenes* during heat treatment and showed better performance than first-order kinetic models (Bhaduri *et al.*, 1991). However, Geeraerd *et al.* (2000) demonstrated that this model was not appropriate to model accurately the linear inactivation phase due to structural limitations and similar conclusions were reported for *Bacillus cereus* spores during heat treatment of *sous vide* foods (Li *et al.*, 2014). The variety of non-linear models in thermal inactivation of microorganisms has been extensively reviewed in the past (Xiong *et al.*, 1999; Li *et al.*, 2007) whereas GInaFiT, an Excel add-in software, has been developed to include nine different model types covering eight different shapes of inactivation curves (Geeraerd *et al.*, 2005) in an attempt to facilitate curve fitting. All the above models are based on the assumption that survival curves can be treated in kinetic terms. Recently, a new category of inactivation models has been developed based on the alternative hypothesis that microbial inactivation is a cumulative form of a temporal distribution of lethal events that represent the spectrum of resistances of the treated microbial population to the lethal agent (Peleg and Penchina, 2000). The Weibull model can be described by the following equation (Mafart *et al.*, 2002):

$$\log\left(\frac{N_t}{N_0}\right) = -\left(\frac{t}{\delta}\right)^p$$

in which N_0 and N_t are the population of the microorganism at time 0 and t, respectively, t is the heating time, and δ, p are the scale and shape parameters; a concave upward survival curve is related to $p < 1$, concave downward for $p > 1$, and linear if $p = 1$. The shape of the curve could provide biologically meaningful information not only as a reflection of the properties of an underlying distribution of lethal events but also as an indication of the cumulative effect of the lethal agent on the surviving microbial population, thus providing a link with physiological effects (van Boekel, 2002). Thus, a concave upward curve ($p < 1$) indicates that as the sensitive members of the population are destroyed, the remaining cells have the ability to adapt to the applied lethal agent and become more resistant. On the contrary, for concave downward curves ($p > 1$) the remaining cells become increasingly damaged by the lethal agent and it takes a shorter time to destroy them (Chen and Hoover, 2004). The majority of thermal inactivation models has been developed in buffer systems under controlled laboratory conditions that may be inappropriate when extrapolated to real food situations. The trend in the last few years is to design experiments on model systems or directly on foods that give reliable information on bacterial behavior. Thus, van Lieverloo *et al.* (2013) investigated the thermal inactivation of *Listeria monocytogenes* in liquid food products by means of multiple regression models, taking into account 51 different strains of the pathogen and 6 cocktails of strains. The food products assayed were dairy (milk, cream, butter), fruit and vegetable juices, liquid eggs, and meat gravy. The purpose of the work was to develop a model that could predict thermal inactivation of the pathogen while

accounting for effects of food composition (pH, sodium chloride, sugar) and processing conditions (storage temperature, heat shock). The authors demonstrated that multiple regression modeling can be used effectively to predict the inactivation of the pathogen with a probability of survival of less than 1 in a billion liters with a limited and realistic uncertainty level while retaining the variability of heat resistance due to the high number of strains assayed. Silva and Gibbs (2004) published a study for the design of pasteurization processes for high-acid shelf-stable fruit products using first-order kinetics modeling and reported the *D* and *z* values for a wide variety of spoilage microorganisms including ascospores of heat-resistant fungi and bacterial spores. Typical *z* values ranged from 7.7 to 12.9 °C depending on the fruit product and spore strain. The authors concluded that the target microorganism in the design of the pasteurization process should be *Alicyclobacillus acidoterrestris,* a high heat-resistant bacterium that causes spoilage to fruit juices, despite the common assumption that microbial spores that can be found in pasteurized fruit products (pH < 4.6) do not grow because of the acidity of the product. The inactivation of *Salmonella* spp. in various food matrices under thermal and emerging treatments has been recently reviewed (Bermúdez-Aguirre and Corradini, 2012). It was reported that *Salmonella* inactivation does not follow first-order kinetics, emphasizing the need for models that adequately describe the survival curves and have predictive ability. Survival of the pathogen was explored with Weibullian and non-Weibullian models reporting the potential and limitations to characterize the survival curves. It was demonstrated that departure from linearity is frequently observed during thermal inactivation and thus model selection should be based on goodness of fit and simplicity rather than preselected kinetics. The thermal pasteurization requirements for *Salmonella* inactivation has also been reported in a variety of meat products (Silva and Gibbs, 2012) and minimum process times at various temperatures for 7*D* inactivation of the pathogen have been indicated. Deviation from linearity was also confirmed and the authors proposed the application of an additional 1 or 2*D* inactivation in *Salmonella* numbers to overcome deviations from linearity and thus avoid underestimation of survivor numbers. Inactivation model fitting has been facilitated in the last few years by the development of specialized software tools such as Combase, GroPin, NIZO Premia, GInaFit, PMM-Lab, Sym'Previus, and FILTREX (Tenenhaus-Aziza and Ellouse, 2014) that estimate inactivation parameters on the user specific experimental data. Normally, the user can upload the dataset, choose the linear or non-linear model, select the temperature of treatment together with other parameters (e.g., pH, NaCl, etc.), and get the fitting estimates. It is expected that the wide use and acceptability of these tools by the industry will help in the design and optimization of thermal processes.

2.4 Non-thermal inactivation and modeling stress-adaptation strategies

Non-thermal inactivation is usually the result of the single or combined effect of low pH (<4.5) or a_w (<0.90) and moisture (<60%) at refrigeration or ambient temperatures in the presence or not of preservative agents close to their minimum inhibitory concentration (MIC). Although the lethality is attributed to heat-independent factors, temperature values within the biokinetic range of growth from the minimum (suboptimal, 0–5 °C) to the maximum (superoptimal: 45–47 °C) value for growth remain the factor governing the non-thermal inactivation rate of bacteria (Shadbolt *et al.*, 1999; Ross *et al.*, 2008; McQuestin *et al.*, 2009; Zhang *et al.*, 2010). The latter studies sufficiently demonstrated this concept for non-thermal inactivation of *E. coli* and *L. monocytogenes* at pH (3.5 to 5.1) and a_w (0.76 to 0.94) combinations commonly applying to various dry and fermented meats. This concept has been known for a long time as the basis

for inactivation of *Salmonella, Clostridium perfringens* and *Staph. aureus* in homemade mayonnaise and related products (Radford and Board, 1993).

It needs to be noted that non-thermal inactivation may also be achieved by non-thermal pasteurization treatments, such as ultra-high pressure, cold atmospheric plasma, intense light pulses and pulsed-electric fields (Rajkovic *et al.*, 2010). However, the challenge in modeling these processes is rather associated with the potential shape of inactivation curves and less with the modeling principle behind that. Such technologies may alter the classical log-linear inactivation curves to concave downward curves in which the linear death is delayed (preceded) by an initial survival (shoulder) period, or concave upward curves with "tailing" due to the emergence of resistant subpopulations that either pre-existed or were induced by the treatment itself. In cases like that, the following non-linear Weibull- or logistic-based models accounting for the distinct inactivation of two subpopulations, one sensitive and one resistant, may be used (Whiting, 1993; Mafart *et al.*, 2002):

$$N(t) = N_0\left(f \times 10^{-(t/\delta_1)p_1} + (1-f)\times 10^{-(t/\delta_2)p_2}\right)$$

where δ and p_1, p_2 are as in the classical Weibull model, and

$$N(t) = N_0\left(\frac{f\left(1+e^{-k_1 t_{lag}}\right)}{1+e^{k_1(t-t_{lag})}} + \frac{(1-f)\left(1+e^{-k_2 t_{lag}}\right)}{1+e^{k_2(t-t_{lag})}}\right)$$

where k_1, k_2 are the inactivation rates for the sensitive (f) and resistant ($1-f$) fraction of the population, after an initial shoulder period represented by t_{lag}.

The non-thermal inactivation is a response worth modeling due to the demands of the consumers for more natural and nutritious foods, which have forced the food industry to confront the challenge of designing minimally processed foods with less preservatives, but without compromising their safety. Practically, this challenge involves formulating foods at conditions approaching the microbial growth/no-growth boundaries, where either microbial inactivation or (slow) growth could occur. Therefore, it is of the utmost importance to understand and quantify the interaction effects of different hurdle technologies (Leistner, 2000) on microbial dynamics instead of empirically adopting the multiple hurdle technology. Few combined models describing changes in a microbial population subjected to conditions that vary from growth to inactivation have been reported (Corradini and Peleg, 2006; Ross *et al.*, 2005; Pin *et al.*, 2011). The work of Coroller *et al.* (2012) presents a modeling approach for non-thermal inactivation based on the Gamma hypothesis that predicts the global behaviour of *L. monocytogenes* in various media. The proposed model postulates that only two microbial responses can be observed: growth or inactivation. When the maximum growth rate (as estimated from the Gamma concept) is greater than zero, microbial growth is predicted. When the maximum growth rate is equal to zero, then the bacterial population is inactivated. The underlying principle is that growth, survival, or inactivation of microorganisms are time-dependent and it can be reasonably postulated that if the microbial behavior was observed in static conditions for an infinite time period, only growth or inactivation would be observed. Microbial survival would therefore be characterized by either slow growth or slow inactivation and the concept of infinite lag would have no meaning in this context. The environmental factors of interest are commonly temperature, pH, sodium chloride salt, a_w, and commonly encountered organic acids such as sorbic acid, lactic acid, and acetic acid. For further application in an industrial setup, the modeling approach of Coroller *et al.* (2012) had to meet the following requirements: (i) the biological parameters that are used in the model should be easily found in publications, or elicited by an expert, and (ii) the model should allow the prediction of change in the number of microbial populations (i.e., log increase or decrease) as a result of food

formulation and storage conditions and vice versa. More specifically, it should suggest specific food properties based on the targeted bacterial behavior or it should provide the storage condition for a given food formulation that leads to the targeted bacterial behavior (Coroller *et al.*, 2012).

A similar approach has been proposed by Pin *et al.* (2011) for various *Salmonella* serovars, based on literature reports that have been uploaded in the ComBase database. Three discrete models, namely a growth, a probabilistic, and an inactivation model, the first two having cardinal parameters, were fitted with non-linear regression to three corresponding datasets of 707 growth or inactivation rates of *Salmonella*, extracted from ComBase in response to temperature, pH, and a_w. Growth and inactivation responses were also translated into a consolidated binary dataset assigning values of 0 and 1 to no growth and growth responses, respectively, in order to fit the probabilistic (growth/no growth) logistic regression model for *Salmonella*. The aforementioned models were combined into a three-component unified model, which works as follows: the T, pH, and a_w for consecutive short time intervals (*dt*) are introduced into the probabilistic model and if the probability of growth corresponding to the momentary combination of the three independent variables is predicted as ≥ 0.5 then the change in numbers of *Salmonella* is predicted by the growth model, whereas if the expected growth probability is <0.5 then the population of *Salmonella* is predicted by the inactivation model.

Apart from the potential risk by insufficient control of microbial growth, mild processing, including the contemporary non-thermal preservation technologies, may also increase the risk of emerging stress-hardened pathogens, as a result of the repeated exposure of microbial cells to sublethal conditions, which impart tolerance to lethal stresses (Rajkovic *et al.*, 2009). Therefore, there is an imperative need to encompass the microbial adaptive responses in the existing models predicting microbial dynamics below, above, and across the growth boundaries. The next paragraphs provide a detailed overview of current advances in this area.

2.4.1 Modeling the adaptive responses of pathogens to inimical factors

Bacterial "stress-hardening" is a response resulting from exposure of cells to sublethal stresses for short (shock) or long (habituation) periods of time. Exposure to one stress induces resistance to the same (homologous) or different (heterologous) stress, the latter also termed cross-protection. The microbial response may be perceived either as increased survival against lethality treatments or as minimization of lag time and faster growth upon shift to growth-permitting conditions. Although numerous studies have investigated the adaptive response of pathogens to various stresses, especially low pH, few have systematically modeled this process by mathematically describing the shape of survival curves and estimating the inactivation kinetics of adapted pathogens (Greenacre *et al.*, 2003). By modeling the dependence of inactivation rate as a function of exposure to sublethal acidity, it was concluded that the acid tolerance response (ATR) is strongly dependent on the duration of previous exposure (e.g., 2–3 h) to sublethal acidity (e.g., pH 5–5.5) by different acids (e.g., HCl, lactic or acetic acid) before challenge against lethal pH (i.e., <3.5; Greenacre *et al.*, 2003; Shadbolt *et al.*, 2001).

Another paradigm of active microbial adaptation strategy associated with self-adjustment (reduction) of the growth rate in order to combat stress is the "stringent response" and the emergence of persister cells within a population (McMeekin *et al.*, 2013; Lewis, 2007). A stringent response is the transition of cellular metabolism from the relaxed (growth oriented) state to the survival state, in response to nutrient (e.g., carbon, amino acid, and iron) starvation. It is usually perceived as entrance of cells into the viable but non-culturable (VBNC) state and is induced by the sudden accumulation of

intracellular signaling compounds, called alarmones, such as guanosine tetra- or pentaphosphate (ppG[p]pp). Persistence is the result of phenotypic switching between fast and slow growing cells, with the latter being less metabolically active and thus less susceptible to stresses, especially antibiotics (Lewis, 2007). Contrary to antibiotic resistance, which is commonly controlled by a genetic factor, persister cells are genetically identical to non-persisters and emerge, as a dormant subpopulation, from the same population forming a distinct stress-resistant phenotype (Ayrapetyan *et al.*, 2014). Fractionation of growth rates within homogeneous populations may resemble the persistence and enable modeling the spread of stress resistance within microcolonies, such as those of a biofilm on inert (e.g., stainless steel) or living (e.g., vegetable) surfaces (Nguyen *et al.*, 2011).

As far as growth modeling is concerned, bacterial adaptation to a new environment is manifested by the lag phase, representing the period during which the effect of the previous environment on microbial growth is gradually diminished and cells are prepared (in an "adjustment" process) to enter a new log phase. The original definition of a population lag time was "geometrical" and equaled the time when the tangent of the population growth curve with the maximum slope crosses the initial level of the population. Nowadays, mechanistic approaches have also been introduced, considering that: (i) lag time is dependent on the physiological state of cells (commonly represented by the parameter q_0 or p_0) and the work needed (h_0) for growth initiation, carried out at a constant rate ($h_0 = lag \times \mu_{max}$), or (ii) at a certain time-point, a bacterial population consists of a subpopulation in the exponential phase and a no-growing fraction of cells in the lag phase either preparing for growth or incapable of initiating growth because the prevailing conditions are below their growth boundaries (Baranyi and Pin, 2004; Koutsoumanis, 2008; McKellar and Knight, 2000; McKellar, 2001; Robinson *et al.*, 1998; Standaert *et al.*, 2007).

In a dynamic environment, for example, under fluctuating temperatures, it is assumed that the growth rate of cells is instantaneously adapted to momentary temperature changes, whereas there is contrasting evidence for induction of short lag times before growth is resumed (Alavi *et al.*, 1999; Mitchell *et al.*, 1994, 1995). Following the initial lag phase, changes of environmental factors may induce a so-called "intermediate" lag phase (Swinnen *et al.*, 2005), the duration of which depends on the magnitude and direction of the environmental shift (Belessi *et al.*, 2011b; Le Marc *et al.*, 2010; Muñoz-Cuevas *et al.*, 2010). Quantification of the effect of temperature shifts on the lag time of bacteria has received more attention (Augustin *et al.*, 2000a, 2000b; Mellefont and Ross, 2003; Swinnen *et al.*, 2005, 2006; Whiting and Bagi, 2002; Zwietering *et al.*, 1994) than the corresponding effect of osmotic shifts (Mellefont *et al.*, 2003, 2004, 2005; Mellefont and Ross, 2003).

The most widely used approach for quantifying the effect of sudden changes in the environment on bacterial lag time is based on the amount of work that cells need to undertake and the rate at which this work is accomplished (work to be done, h_0; Mellefont *et al.*, 2003, Robinson *et al.*, 1998). The adaptation rate follows the environmental fluctuations, whereas the adaptation work may be reduced or increased if microorganisms adapt faster or become injured, respectively (Belessi *et al.*, 2011b; Le Marc *et al.*, 2010; Muñoz-Cuevas *et al.*, 2010). From a mathematical standpoint, if changes in the environment do not pose additional adaptation work, then the inverse of lag time (the so-called "lag rate"; Mellefont and Ross, 2003) and growth rate should follow the same trend as a function of the shift and they should be linearly correlated. This may be true for shifts of low magnitude (e.g., 3–5 °C) that are far from the growth boundaries. However, linearity is lost when the intensity of the stress imposed is so high that the bacteria cannot adapt to the new conditions and require additional effort to overcome the imposed stress.

This is the case with osmotic or acid shifts, which are considered more severe stresses than temperature (Robinson *et al.*, 1998; Shabala *et al.*, 2008) and are known to induce adaptation work (Mellefont and Ross, 2003; Mellefont *et al.*, 2003, 2004, 2005) or with shifts close to the growth boundaries. Mitchell *et al.* (1995) stressed that extended exposure to temperatures that do not support growth of *Salmonella* may affect subsequent growth of the bacterium at growth permitting temperatures. Existing reports also suggest that preincubation of *Listeria monocytogenes* at 4 °C results in a faster growth rate at 7 °C than the preculture at 7 °C (Membré *et al.*, 1998). More recent papers have shown that the adaptation work and the physiological state parameter of cells under fluctuating conditions can be effectively modeled as a function of both the magnitude and the direction of the environmental changes. Such methodology may then be coupled with the differential form of primary models, such as the Baranyi model, and allow for simulation of the growth of pathogens in response to up- or down-shifts in pH, a_w, temperature, etc.

Following the classical viable count-based modeling, the state-of-the-art in adaptation modeling is the development of pure mechanistic models relating the effect of "cell history" on the potential of the pathogen to survive food-processing challenges and eventually cause illness. Detailed insights into the uncertainty of microbial responses from farm to fork can be provided by adopting systems biology principles and functional genomics (Brul and Westerhoff, 2007). These models are based on the hypothesis that the environment significantly influences the dynamic expression and assembly of all components encoded in the genome of an organism into functional biological networks. The ultimate goal is to integrate top-down approaches, which use "-omics" induction to identify underlying mechanisms, and bottom-up approaches, which explain or predict the overall cellular behavior (including "*omics*") beginning with the molecular level (Bruggeman and Westerhoff, 2007). This may be implemented through the development of an environmental and gene regulatory influence model (Mensonides *et al.*, 2002, 2005) for different stages of the food chain. Use of microarray data coupled with bioinformatic approaches may enable modeling of the global gene expression in response to environmental challenges and predict the lag time or even the virulence of pathogens based on their "history" (King *et al.*, 2014).

2.5 Fermentation: a dynamic environment for microbial growth and pathogen inactivation

Fermentation is one of the oldest food-processing technologies based on the biological activity of specific microbial groups that can produce a range of metabolic compounds that suppress the growth and survival of pathogenic and spoilage microbiota, thus ensuring the microbiological stability and extending the shelf life of foodstuffs (Caplice and Fitzgerald, 1999; Ross *et al.*, 2002; Bourdichon *et al.*, 2012). From the biochemical point of view, the main impact of fermentation is focused on food preservation through the development of inhibitory metabolic compounds (e.g., organic acids, ethanol, bacteriocins), often in combination with a decrease in water activity through drying or salting (Lee, 2004; Gaggia *et al.*, 2011; Peres *et al.*, 2012). The formation of these compounds results in the inhibition of food-borne pathogens, thus improving food safety (Adams and Nikolaides, 2008; Grounta *et al.*, 2013), and also in the improvement of the nutritional value and sensory attributes of the final product (van Boekel *et al.*, 2010; Lanza, 2013).

The use of mathematical models to describe the growth, survival, or death kinetics of bacteria has been extensively employed in a wide variety of food products and processes and the principles of predictive microbiology have been well established and elucidated in a series of articles (Buchanan, 1993; Ross and McMeekin, 1994;

Baranyi and Roberts, 1995; Whiting, 1995; McDonald and Sun, 1999). The most common approach to describe microbial growth and death under given ecological conditions is by means of a sigmoidal curve that approximates quite accurately the changes in bacteria cell population over time. However, on a real food matrix, bacteria do not grow independently from their environment but are influenced by the background microbiota (Malakar *et al.*, 2003) and the structure of the matrix (oxygen availability, water distribution, organic acids, food preservatives, etc.) (Noriega *et al.*, 2010). It is worth noting that single species growth can be differentiated in terms of a lag phase, an exponential phase, and stationary phase, a distinction that is not very clear for multiple species growth. Moreover, different kinds of interactions can be encountered on a food system, among which antagonistic interactions are most important from the food safety perspective, and, consequently, special models must be employed to describe the complex nature of interspecies interactions. Thus, dynamic models have been developed to describe the bacterial kinetics of two or more competitive species (Vereecken *et al.*, 2000; Pommier *et al.*, 2005; Mejlholm and Dalgaard, 2007a, 2007b) while other models take also into consideration substrate consumption and product inhibition of each culture (Sodini *et al.*, 2000).

Table olive fermentation is one of the most dynamic environments for microbial growth, where diverse microbial groups from the indigenous microbiota of olives compete for nutrients with a variety of contaminating microorganisms from fermentation vessels, pipelines, and pumps in contact with olives and brine. Those microorganisms best adapted to the food substrate and process parameters during fermentation dominate the process. It has been reported that in olive fermentation, despite the relatively low initial population of lactic acid bacteria and yeasts compared to Gram-negative bacteria, they eventually become the dominant groups driving fermentation (Sánchez Gómez *et al.*, 2006). The effect of environmental factors driving the fermentation process on the microbial association and the survival of pathogenic bacteria has been modeled through a variety of kinetic and probabilistic models, whereas other approaches include the application of artificial neural networks (ANNs) and susceptibility models for the determination of MIC and NIC values of antimicrobial compounds (for a review see Arroyo-López *et al.*, 2010). However, these models do not take into consideration the dynamic interactions prevailing in the process environment. In a recent work (Skandamis and Nychas, 2003), a dynamic model was developed based on six differential equations to describe the evolution of the fermenting microbiota and the survival of the pathogenic bacterium *Escherichia coli* O157:H7 during the inoculated fermentation of green olives with *Lactobacillus plantarum* in brines supplemented with different amounts of fermenting substrates. The dynamics of the fermentation process is described by the following equations:

For lactic acid bacteria: $$\frac{dN}{dt} = r_{s_{max}} N\left(1 - \frac{Y}{Y_{in}}\right)$$

where N is the population of *L. plantarum* in the brine (cfu/ml), r_{smax} is the maximum specific growth rate (days^{-1}) for the starter culture, Y is the population of yeasts (cfu/ml), and Y_{in} is the minimum population of yeasts (cfu/ml) that inhibits the growth of *L. plantarum.*

For yeasts: $$\frac{dY}{dt} = r_{y_{max}} N\left(1 - \frac{Y}{Y_{max}}\right)Y$$

where r_{ymax} is the maximum specific growth rate for yeasts (day^{-1}) and Y_{max} the maximum cell population of yeasts (cfu/ml). Apart from the above equations that describe the changes in microbial dynamics, two more equations were taken into consideration for fermentable substrate consumption and lactic acid formation.

For fermentable substrates: $\frac{dS}{dt} = \left(-m_s N - \frac{1}{Y_{Y/S}} \frac{dY}{dt} \right) S$

where S is the residual concentration (%) of fermentable substrate in the brine during the process, $Y_{Y/S}$ is the yeast yield coefficient (cfu/ml per mM of assimilated fermentable substrate), and m_s is the maintenance coefficients for *L. plantarum.*

For lactic acid formation: $\frac{d\mathrm{HA}}{dt} = -Y_{\mathrm{HA/S}} \frac{dS}{dt}$

In the above equation, lactic acid (in protonated form) is directly related to the consumption rate of fermentable substrates (dS/dt), with $Y_{\mathrm{HA/S}}$ being a yield coefficient representing the conversion of fermentable material to lactic acid. Finally, as fermentation is a dynamic process and the value of pH changes over time, the changes in proton concentration were modeled as a function of HA as follows:

For proton concentration: $\frac{dP}{dt} = a\left(1 - \frac{P}{P_{\max}} \right) \mathrm{HA}$

where a is a coefficient, P is the concentration of protons, and $P_{\max}$ is the maximum predicted concentration of protons corresponding to the predicted minimum value of pH in the fermentation process. Apart from the evolution of the indigenous microbiota in the process, it would be of great importance to investigate the fate and behavior of pathogenic bacteria in the case of contamination. It should be noticed that table olives are considered as a ready-to-eat food, thus making contamination with pathogens a potential public health concern, although no serious outbreaks have been reported so far due to the fact that the brine environment does not support the growth and survival of pathogenic bacteria (Medina *et al.*, 2013). To investigate the dynamics of pathogenic bacteria in the fermentation process, the brine was inoculated at the onset of fermentation with *E. coli* O157:H7 and the changes in cell population were modeled by the following equation:

$$\frac{dN_e}{dt} = k \frac{dP}{dt} N_e - f \frac{HA}{S} N_e$$

where N_e is the population of the pathogen (cfu/ml) and k, f are coefficients. This equation is based on the assumption that the survival of the pathogen is directly proportional to the production rate of protons (P) and lactic acid (HA), and inversely proportional to the concentration of fermentable substrates. Yeasts were potential competitors of *L. plantarum* despite the fact that the latter species dominated the process (Figure 2.1). The growth of yeasts retarded the growth of the starter but not its metabolic activity, as evidenced by the increasing levels of lactic acid produced throughout the process. Finally, a progressive reduction in the population of the starter was observed at the end of fermentation that could be attributed to the reduction of available fermentable substrate due to antagonism by yeasts. Concerning the behavior of *E. coli* O157:H7, its inactivation pattern could be discriminated into three distinct stages. Specifically, the pathogen presented a rapid decrease within the first five days of the process, which coincides with a rapid drop in pH values in the same period. A short survival period was observed afterwards between 5 and 10 days, when the pH of the process reached a plateau and was close to the pK_a value of lactic acid, with the undissociated fraction of the acid to be apparently below lethal concentrations. Finally, a second death phase was observed from day 20 onwards due to the increased concentration of undissociated lactic acid, with a concurrent reduction in fermentable substrates. The results of the developed model proved the effectiveness of the modeling approach to describe quite accurately the fermentation process as a whole, taking into account the different biochemical mechanisms such as the production of microbial metabolites, consumption of fermentable substrates, as well as microbial effected changes in pH. Since fermentation remains one of the

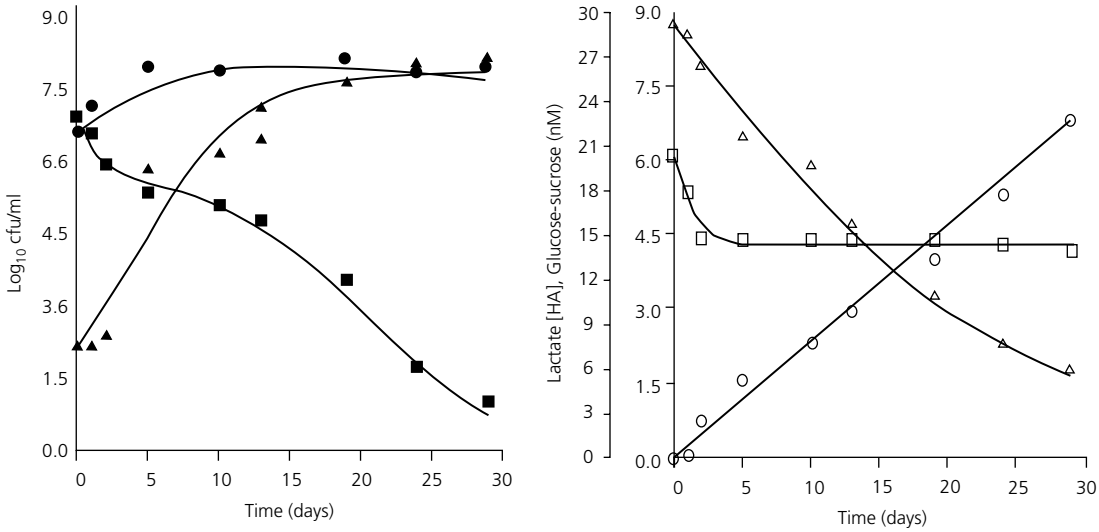

Figure 2.1 Changes in population of lactic acid bacteria (●), yeasts (▲), *E. coli* O157:H7 (■), pH (□), glucose concentration (Δ), and undissociated lactic acid (○) during the inoculated fermentation of green olives with *Lactobacillus plantarum*.

basic processes in food production, similar approaches could be extended to the survival of any food-borne pathogen on other fermented foods such as vegetables, meats, and dairy products.

2.6 Colonial versus planktonic type of growth: modes of microbial existence on surfaces and in liquid, semi-liquid, and solid foods

2.6.1 Biofilm formation on biotic and abiotic surfaces

During processing of contaminated raw materials and food preparation, microorganisms entrapped into the food residues may be transferred to the equipment surfaces (abiotic or inert surfaces) or cross-contaminate other foods (biotic surfaces). In the context of good hygienic and good manufacturing practice, processing plant surfaces are cleaned and disinfected after their use, in order to remove food soil and eliminate the transferred microorganisms. Inadequate disinfection may give rise to biofilm formation. In addition, depending on the bacterial growth rate, the surrounding nutrient status, the attachment ability of the microorganisms, and the adaptive responses triggered by exposure to sublethal inimical factors, there may be an increase in microbial resistance to sanitizers and the establishment of persistent strains. Settlement of bacterial cells on solid surfaces leads to biofilm formation activity. Biofilms are referred to in the literature as biologically active matrices of cells and extracellular substances in association with a solid surface (Kumar and Anand, 1998). Placed in the solid–liquid interface, bacterial cells, irreversibly attached to the food surface, are organized in multistructural communities, embedded into a glykocalyx (Kumar and Anand, 1998). Capillary water channels are part of that porous structure of biofilms, and distribute water and nutrients to the included microorganisms (Poulsen, 1999). Three critical steps are the content of the biofilm formation activity: (i) attachment, (ii) microcolony and EPS production, and (iii) maturation (Davey and O'Toole, 2000; Chmielewski and Frank, 2003). Bacterial cells may actively or passively adhere to the

surfaces, which depends on the bacterial cells surface properties, and the attachment can be reversible or irreversible. The attachment and biofilm-forming capabilities of bacteria depend on the interaction of multiple factors, including the surface characteristics and cell–surface interactions, the presence of other bacteria, the temperature, the availability of nutrients and pH, the production of extracellular polysaccharides, and cell-to-cell communication (Chmielewski and Frank, 2003; Van Houdt and Michiels, 2010).

2.6.2 Growth rate of microorganisms in different forms of growth

Microbial growth in foods occurs in the aqueous phase. The structural characteristics (also called "microarchitecture") of this phase, in combination with the total concentration and dispersion of water compared to the fat phase, determine the form and rate of growth, i.e., the spatiotemporal microbial dynamics. Food may be characterized as liquid (e.g., juices, milk), gelled (e.g., jellies, cottage, marmalades), oil-in-water emulsions (e.g., mayonnaise) or water-in-oil emulsions (e.g., butter and margarine), and the composite form of gelled emulsions (i.e., an immobilized oil-in-water emulsion). Apparently, the type of emulsion determines the distribution of available water. The growth rate of microorganisms in response to food structure, for a given set of intrinsic and extrinsic parameters, is dependent on the motility of cells in the aqueous phase, the extent of resulting (micro-)colony immobilization, and the diffusion kinetics of nutrient, oxygen, and metabolites. Growth of microorganisms in a liquid aqueous phase typically is planktonic and the motility of microorganisms may enable access to certain nutrient-rich sites of the food (Wilson *et al.*, 2002). Access of cells to nutrients and transfer of metabolites away from cells contribute to the formation of a temporarily uniform environment, until the resources are depleted or the microbial metabolites are accumulated at self-toxic levels. If aqueous phase is structured, e.g., due to addition of thickeners or gelling (structure-inducing) agents, such as gelatin, pectins, starch, gums, etc., microorganisms are immobilized in the gelled regions and constrained to grow as colonies, with their growth rates tending to be lower than that of planktonically growing cells (Theys *et al.*, 2008; Aspridou *et al.*, 2014; Wilson *et al.*, 2002; Boons *et al.*, 2013a, 2013b, 2014). This can be further enhanced by increasing the fat concentration on the expense of the water phase, thereby increasing the size of oil droplets with a concomitant trend of reversal of the oil-in-water emulsion. At low fat concentrations, the water phase is enough to allow cell motility that resembles planktonic growth. As the fat concentration increases and compresses water, growth is constrained and becomes colonial (actually spherical). A similar effect can be obtained by adding a structure-inducing agent, such as gelatin. On the surface of foods, such as meat and vegetables, growth is also colonial initially in two dimensions (mono-layer), whereas the center of the colony gradually develops in the third dimension, most likely upward, depending on aeration and nutrient availability. Replenishment of nutrients takes place only from the bottom or the perimeter of the colony and soon cells in the center of the colony experience starvation and self-toxication. This places growth constraints on the colony as a whole and causes suppression of the growth rate as compared to immobilized growth in the food matrix or planktonic growth. Thus, the growth rate of the aforementioned different forms of growth is known to follow the order: planktonic $\geq$ immobilized $>$ surface (Wilson *et al.*, 2002; Theys *et al.*, 2008).

These observations have also been explored in relation to the stochastic behavior of individual cells growing in liquid media or immobilized inside or on the surface of solid media. Starting from the single cell level and simulating the formation of a colony or the proliferation to high numbers in the planktonic state may assist in drawing useful conclusions on the expected behavior of large populations. These aspects are further discussed in the following paragraphs.

2.6.2.1 Individual-based modeling of floating or immobilized cells

The growth of a microbial population depends on the cumulative behavior of individual cells. It is well known that a great variability exists in the growth responses, i.e., lag time, generation time, and probability of growth among individual cells. This biological variability (also termed "*biovariability*"; Billon *et al.*, 1997) markedly impacts the dynamics, e.g., geometrical lag, germination time, and time to reach detectable levels of low populations, such as 1–50 cells and increases with the intensity of environmental stresses (Billon *et al.*, 1997; Dupont and Augustin, 2009; Francois *et al.*, 2005, 2006a; Guillier *et al.*, 2005; Guillier and Augustin, 2006; Smelt *et al.*, 2002, 2008). For instance, the distribution of germination times (lag) of individual *Cl. botulinum* spores became less peaky (i.e., with a lower kyrtosis coefficient) and less positively skewed (i.e., a skewness factor close to 1) as the incubation temperature decreased from 37 to 20 °C, suggesting that the variance in germination times increased with the intensity of temperature stress (Billon *et al.*, 1997). However, the variable behavior of single cells is masked by the massive behavior of large populations, e.g., >500 cells, or it is almost eliminated at optimal conditions (Llaudes *et al.*, 2001; Métris *et al.*, 2006; Smelt *et al.*, 2002). Most of the available predictive models quantify the response of high microbial populations at a given set of conditions, which may be constant or varying with time. In order to model the variability of single cells (or single spores), stochastic modelling may be applied, which is also the common approach in quantitative microbial risk assessment. Therefore, deterministic models apply to the population level, whereas stochastic models may describe the population dynamics taking into account the variability in the responses (e.g., lag time and generation time) of individual cells.

Although deterministic models average the behavior of individual cells, the characteristics of the latter cannot be deduced from population measurements (Kutalik *et al.*, 2005). Indeed, the growth of a population may be simulated by superimposing the evolution of independent subpopulations derived from single cells, each receiving a lag time value, also termed "physiological lag" (Baranyi *et al.*, 2009; different from the geometrical population lag), from a specific probability distribution. The evolution of a microbial population can be modeled as a Poisson birth process with the constant birth intensity parameter μ (Baranyi, 1998; Baranyi and Pin, 2001). A cell is divided after an initial delay, which consists of the physiological lag and the generation time of the cell. Then each cell produces a subpopulation, which consists of cells growing independently in the same habitat with a constant growth rate (Baranyi, 1998; McKellar, 2001). To model this process, it is assumed that (Baranyi, 2002; Métris *et al.*, 2003): (i) after the first division of each cell, the daughter cells enter directly the exponential phase and (ii) daughter cells do not interact by any means, e.g., competition or quorum sensing. Both assumptions were reasonable when the experimental method used to describe the variability in lag times was the time to detect visible changes in the optical density of the liquid medium containing a single cell derived by a series of two- or tenfold dilutions of a standard concentrated microbial suspension, or even by sorting with flow cytometry (Baranyi *et al.*, 2009; Francois *et al.*, 2003; Smelt *et al.*, 2002, 2008; Standaert *et al.*, 2005). Individual lag times commonly follow Weibull, Gamma, exponential or normal distributions (Kutalik *et al.*, 2005; Francois, 2005, 2006a, 2007; Métris *et al.*, 2006; Standaert *et al.*, 2007). The development of sophisticated image analysis systems for real-time monitoring of single cell division (or spore germination) under the microscope, during continuous exposure of attached cells to flowing liquid media, allowed further insight into the variability assessment of single cells (Billon *et al.*, 1997; Elfwing *et al.*, 2004). By targeting specific cells, it was observed that the generation time of daughter cells, which were removed after division, are not the same for all cells but they follow a distribution, the

variance of which decreases with the number of consecutive divisions (Pin and Baranyi, 2006; Métris *et al.*, 2005, 2006; Kutalik *et al.*, 2005).

Exposure of bacterial populations to sublethal stresses, such as chlorine, heat shock, pH, osmotic stresses, as well as suboptimal conditions in a new environment, shift the distribution of the time to the first division to higher levels (i.e., right) and the increase in its variance (Dupont and Augustin, 2009; Francois *et al.*, 2005, 2006a; Guillier *et al.*, 2005; Guillier and Augustin, 2006). Furthermore, stress may decrease the probability of a single cell to initiate growth and increase the number of cells needed for growth initiation (Dupont and Augustin, 2009; Koutsoumanis, 2008). As a result, both the extension of individual lag times and the reduction in single cell growth probability may lead to false negative detection, due to insufficient growth above the threshold level of enrichment or no growth at all during enrichment (Dupont and Augustin, 2009). Given that stress increases the biological variability, interactions between cells within colonies (e.g., due to competition for nutrients or the release of inhibitory metabolic products) may be an additional indigenous stress factor, which possibly increases cell lag variation, while retarding the growth of the total population (Guillier *et al.*, 2006).

Even though the variability of planktonic cells has been extensively characterized with OD or microscopic measurements, the variability in colonial growth associated with intracolony cell-to-cell interactions cannot be quantified in liquid cultures, nor by direct imaging of cells, when the daughter cell is removed after division. Alternatively, direct time-lapse imaging of microbial populations growing on agar surfaces of different intrinsic properties has enabled the characterization of population heterogeneity taking into account the interactions between adjacent cells (Guillier *et al.*, 2006; Koutsoumanis and Lianou, 2013). Experimental protocols for direct imaging of surface-growing cells include the gel-cassette system (Brocklehurst *et al.*, 1997), the systems introduced by Billon *et al.* (1997), Guillier and Augustin (2006), and later on adopted by Koutsoumanis and Lianou (2013), consisting of an agar layer on top of a microscope slide, covered by a cover slip, sealed with paraffin wax, and placed under the microscope, and, more recently, the anopore strips (Ingham *et al.*, 2005). Applications of these methods at single cell or colony level may be found for *E. coli* O157:H7, for which a comparison between growth rates estimated from viable count data and changes in colony area (in pixels) is made (Skandamis *et al.*, 2007a), *Bacillus cereus* in response to salinity (den Besten *et al.*, 2007, 2010), and *Salmonella* showing the distribution of living and dead cells during the evolution of colonies in time and space (Theys *et al.*, 2009). These techniques may also allow for identification of injured or even dead subpopulations within bacterial microcolonies as a result of exposure to stresses or entrance into the stationary phase of growth. This is achievable through the use of well-established fluorophores, such as SYTO 9, which stains living cells, and propidium iodide, which stains dead cells or cells with impaired membranes. Experimental protocols involving microscopic observations have also been used to monitor the kinetics of single fungal spores, in response to temperature, a_w, pH, and ethanol stress (Dantigny *et al.*, 2005; Judet *et al.*, 2008; Gougouli and Koutsoumanis, 2013). Fungal spore kinetics include the germination time, which is the time until the length of the germ tube equals the diameter of the spore, the growth rate of the germination tube, and the percentage of germinated spores. Such data may serve as a basis in stochastic modeling for predicting the time until spoilage occurs, in the form of visible mycellium. Indeed, the variability in the germination time and rate of single fungal spores has been reflected on the lag time (i.e., the time to visible detection), the radial increase of *Aspergillus*, and *Fusarium* mycelia grown on corn solid media (Samapundo *et al.*, 2007). Advances in individual-based modelling (IbM) have suggested that, apart from the population

measurements, the complete characterization of lag time also requires the evolution of total biomass and thus the geometrical definition of lag time is not quite reliable (Prats *et al.*, 2008). Furthermore, since geometrical lag depends on the time required by the total viable population to exceed the detection limit of the enumeration method, a part of geometrical lag does not have practically biological meaning (also termed "pseudo-lag"; Koutsoumanis, 2008), because growth initiation of a fast-growing subpopulation, which will eventually give the detection signal, might have started quite earlier.

2.6.2.2 Individual-based modeling in foods

The well-established variability of single cells in laboratory media is expected to be even more pronounced in natural food ecosystems. This may be attributable to the combination of multiple stress factors in foods, such as limitations in nutrient diffusion, competition with natural flora, accumulation of inhibitory metabolic products, structural constraints, etc. Despite the low number of studies dealing with single cell variability in foods, a common conclusion is that the behavior of low inocula (e.g., <10 cfu/g) cannot be accurately approximated by models based on the responses of higher inocula on the same food nor by broth-based models (Manios *et al.*, 2013). For instance, the time that *L. monocytogenes* required for a hundredfold increase on vacuum packaged frankfurters stored at 4 and 8 °C, starting from 0.007–0.1 cfu/g, was markedly higher than that expected based on the responses of 10–20 cfu/g on the safe food (Pal *et al.*, 2009). Likewise, the simulated variability in log-numbers of *L. monocytogenes* cells in liver pâté at 7 °C or lettuce and cabbage fresh-cut salads, based on broth data, differed from the observed number (Francois *et al.*, 2006b; Manios *et al.*, 2013). Notably, Monte Carlo simulation based on stochastic description of lag times of individual *L. monocytogenes* cells from broth data slightly overpredicted the growth of single cells of *L. monocytogenes* after 12 days on lettuce. For instance, the model predicted that there is a 60% likelihood for a single cell of the pathogen to reach 1.5 log cfu/g, while the observed growth under the same probability was 1 log cfu/g (Manios *et al.*, 2013). In contrast, remarked underestimation of the observed growth in cabbage was recorded, as the predictions showed that 60% of the individual cells could grow at 0.5 log cfu/g, whereas the observed growth was 2.6 log cfu/g. This deviation of the broth-based predictions from the observed growth suggests poor transferability of broth-based data to foods, because such models do not adequately encompass the effect of the epiphytic flora, the microstructure, or the scattered availability of nutrients. Thus, extrapolating broth-based predictions of microbial growth from single cells to foods may lead to either fail-safe or fail-dangerous predictions. As shown in Figure 2.2, the presence of antimicrobials and the competitive growth of indigenous microflora of ham slices increased the variability in a log-increase of *L. monocytogenes* single cells per slice at 10 °C, as compared to higher initial populations, e.g., 100 cfu/slice. Furthermore, even competition among strains at the single cell level may inhibit growth initiation of each strain in the composite, especially when antimicrobials are added in the product formulation (Figure 2.3). In this case, a higher number of cells is needed so that one cell capable of overcoming strain competition and initiate growth is present in the population (Figure 2.3). Therefore, it is imperative that the evaluation of the response of single cells in foods should receive more focus in parallel to the optimization of laboratory media assays, which provide further theoretical aspects under controlled conditions.

2.7 Modeling microbial transfer between processing equipment and foods

The recent deeper insight into bacterial transfer phenomena, along with the pronounced necessity of incorporating them in risk

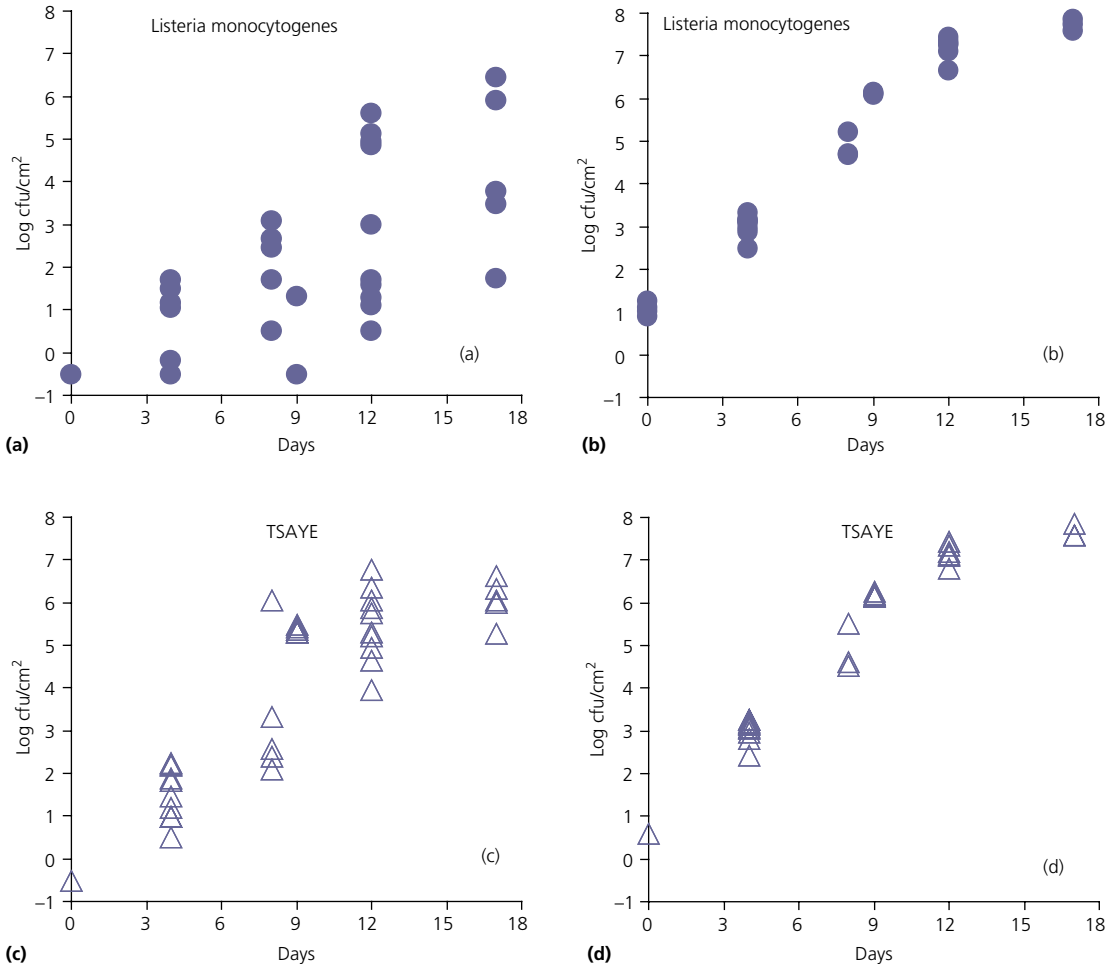

Figure 2.2 Growth of *L. monocytogenes* single cells (a), or 100 cells/slice (b), and the natural mesophilic flora (c, d) on the corresponding slices of ham formulated without antimicrobials, packaged under vacuum and stored at 10 °C (Skandamis and Sofos 2010; unpublished data).

assessment studies, has implemented predictive microbiology with a new type of model: bacterial transfer models. Modeling bacterial transfer can be considered as a challenge for predictive microbiologists. The significance of choosing the right model to describe a microbiological process is well known and the choice of the appropriate parameters as model inputs is decisive for the outcome of the prediction. This fact makes construction of bacterial transfer models a complex task. As Pérez-Rodríguez *et al.* (2008) state, not only do unknown factors govern bacterial transfer but also the known factors (contact time, bacterial strain properties, pressure, etc.) that are involved in cross-contamination events cannot be easily controlled. The challenge in modeling bacterial transfer in food-processing environments, such as the common house-kitchen or a food industry, is the dynamic conditions that dominate those environments. At the very same time, a recipient surface can become the donor surface, in terms of contamination. Furthermore, this event can take place at every possible location. The

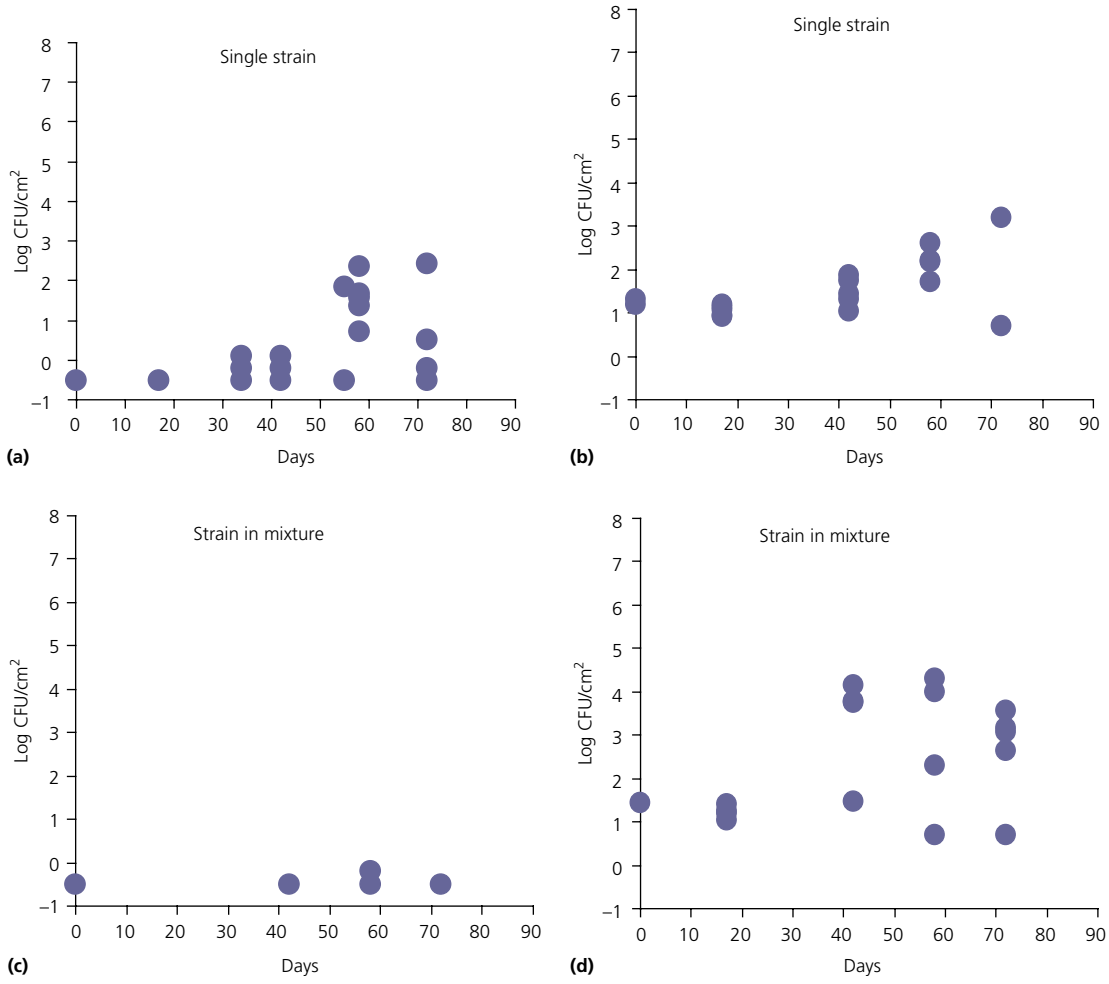

Figure 2.3 Growth of *L. monocytogenes* single cells/slice (a, c) or 100 cells/slice (b, d) as single strain (a, b) or in a mixture of three strains (c, d) on slices of ham formulated with antimicrobials, packaged under vacuum and stored at 10 °C (Skandamis and Sofos 2010; unpublished data).

contamination status changes constantly and each new generated pathway becomes the new starting point for more transfer scenarios.

As aforementioned, the first step to modeling transfer is the calculation of the transfer rates as the percentage of cells transferred from the source to the destination of contamination. It has also been mentioned that the multitude of factors that govern the bacterial transfer lead to a high variability regarding this phenomenon and subsequently regarding the data obtained from relevant studies. Therefore, this variability, which is reflected in estimated transfer rates, can be depicted through probability distributions. For many authors, probability distributions can be constructed based on the log-transformed transfer rates instead of the transfer rates. In fact, normal distribution is considered as the most suitable to best fit the log-transformed transfer rates. Hoelzer *et al.* (2012) used available scientific data to produce probability distributions and models in order to describe the transfer of *L. monocytogenes* between foods and surfaces or during slicing, as well as the impact

of sanitation methods on subsequent cross-contamination events. Normal distributions were also chosen to fit the log-transformed transfer data. In a similar study carried out by Chen *et al.* (2001) to determine transfer rates of *E. aerogenes* between food and surfaces used for food preparation, normal distribution was chosen as the most suitable and statistically convenient way to represent the data. Similarly, Kusumaningrum *et al.* (2004) used the Anderson–Darling criteria and decided that the normal distribution gives the best fit to the log-transformed transfer rates. Alternatively, logistic distributions were ranked first, when normal ones did not give the best results.

Research advances include attempts to construct models that mathematically describe cross-contamination scenarios. Usually, the models are empirical due to the lack of concrete knowledge on bacterial transfer phenomena. The interest has mainly been focused on pathogen transfer that takes place between cutting, shredding, or slicing equipment and foods. The particular interest in those processes is apparently attributed to the employed equipment (e.g., knife or slicer blades) or the type of foods involved (e.g., sliced RTE meat products, fresh-cut salads, and salmon) and consequently the risk implied. The models developed to describe those experimental data represent transfer as a function of the cut/slice number or inoculation level. In respect to this, both Pérez-Rodríguez *et al.* (2007) and Vorst *et al.* (2006) agreed that, when different transfer scenarios were investigated, the logarithmic decrease that was observed in the numbers of the microorganisms under study could be efficiently described by the log-linear model. The goodness of the log-linear model to fit the experimental data was only comparable to that of the Weibull model as reported by Pérez-Rodríguez *et al.* (2007).

The empirical models used so far have been proven sufficiently accurate and promising for risk assessment studies (Aarnisalo *et al.*, 2007; Sheen, 2008; Sheen and Hwang, 2010). A more mechanistic approach was introduced by Møller *et al.* (2012) to treat their experimental data. They developed a model simulating the transfer of *S.* Typhimurium during the grinding of pork. The proposed model was an improved version of the model presented by Nauta *et al.* (2005), taking into account the tailing phenomenon that is observed after sequential slices during a grinding process. The authors claim that this type of model, though similar to empirical models such as that of Sheen and Hwang (2010), can more efficiently describe all the events that take place in a grinder. It also introduces the idea of the two environments existing in the grinder that are responsible for two different transfer rates occurring during the process. This was successfully applied by Zilelidou *et al.* (2014) in modeling the transfer of *Salmonella* Typhimurium and *L. monocytogenes* between lettuce and knives and vice versa in a simulated process of fresh-cut salad preparation in the domestic or catering environment, which involved 50 consecutive cuts with a knife that was first contaminated by cutting a contaminated batch of lettuce leaves. Finally, a mechanistic approach was also chosen by Aziza *et al.* (2006) to explain cross-contamination during cheese-smearing industrial operations. In this study, a binomial distribution was applied and the constructed model reflected the potential risk associated with this type of cheese manufacturing.

The approach of Aziza *et al.* (2006) was based on certain assumptions, e.g., even bacterial distribution throughout the food matrix. Several authors (Hoelzer *et al.*, 2012; Kusumaningrum *et al.*, 2004; van Asselt *et al.*, 2008) have made some of the following assumptions for the setup of the experiment and the subsequent buildup of the model; no bacteria are lost during processes, no cleaning of the equipment takes place in between the use, no new contamination is introduced in the processing equipment for a certain number of operations, and the time after which the transfer event will take place does not have any impact on the event. Such assumptions seem to be necessary for the development of bacterial transfer models since the complexity