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Genetic, physiological, and cellular heterogeneities of bacterial pathogens in food matrices: Consequences for food safety

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Abstract

In complex food systems, bacteria live in heterogeneous microstructures, and the population displays phenotypic heterogeneities at the single-cell level. This review provides an overview of spatiotemporal drivers of phenotypic heterogeneity of bacterial pathogens in food matrices at three levels. The first level is the genotypic heterogeneity due to the possibility for various strains of a given species to contaminate food, each of them having specific genetic features. Then, physiological heterogeneities are induced within the same strain, due to specific microenvironments and heterogeneous adaptative responses to the food microstructure. The third level of phenotypic heterogeneity is related to cellular heterogeneity of the same strain in a specific microenvironment. Finally, we consider how these phenotypic heterogeneities at the single-cell level could be implemented in mathematical models to predict bacterial behavior and help ensure microbiological food safety.

KEYWORDS

food safety, microbiology, microstructure, pathogens

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1 | INTRODUCTION

Food safety is still an important issue all over the world. In Europe, an estimated 23 million people fall ill after eating contaminated food, resulting in 5000 deaths and more than 400,000 disability-adjusted life years every year (WHO, 2017). Ensuring food safety relies on preventing contamination with pathogenic microorganisms, inhibiting their growth, and ensuring their inactivation when necessary. Food products display huge diversity in terms of chemical composition and structure. They provide nutrients essential for bacterial growth which can be heterogeneously distributed. They comprise various structures from liquids to solids, including multiphasic systems (Wilson et al., 2002), they can display textural heterogeneities at the macroscale and microscale level (Verheyen & Impe, 2021), and support dynamic microenvironments due to food microbiota activity (De Filippis et al., 2018). The complexity of these heterogeneous food microstructures leads to difficulties in assessing pathogen behavior (growth, survival, and inactivation) by modeling and thus in controlling microbiological food safety. The behavior of bacteria depends on their living environment, but cell physiology is generally described in terms of the average behavior of the cell population, as summarized by mean and variance values. Consideration of the phenotypic characteristics at the single-cell level can reveal a hidden world of significant biological importance beneath the population average (Koutsoumanis & Aspridou, 2017). Considering a given bacterial species, individual cells within a bacterial cell population can display slightly different to very divergent behaviors, thus giving rise to a wide range of diverse phenotypes; Figure 1). This phenotypic heterogeneity refers to the variability of a particular feature, character or trait observed for a given microorganism. It can result from intrinsic or extrinsic factors involving very diverse molecular mechanisms. The first level of phenotypic heterogeneity that have attracted attention among microbiologists is conceptualized by the notion of bacterial strains (or clones or isolates) (Dijkshoorn et al., 2000). While different techniques and approaches have been developed and used to characterize and differentiate isolates belonging to a given bacterial species (Li et al., 2009), the definition of a bacterial strain is basically and primarily based on the particular type and stable arrangement of genes. These genotype variations between different strains of a given species can be defined as genotypic heterogeneity (Figure 1, section 2). Besides, an extrinsic level of phenotypic heterogeneity can arise among cells of a given bacterial strain in response to different environmental cues. In a heterogeneous ecological niche, where diverse microenvironmental conditions can co-exist, bacterial cells can have different adaptative responses with respect to their direct surrounding environment. This physiological heterogeneity is closely related to the environmental conditions faced in an ecosystem defined by its biotope (area of defined environmental conditions) and biocenosis (living organisms interacting in a biotope, including the microbiota), which can further change over time and location (Ismaili et al., 1996). Here, the bacterial cell response is reviewed according to matrix structure (section 3.1), physicochemical microenvironments (section 3.2), and microbiota (section 3.3.). Cellular heterogeneity is an additional level of intrinsic phenotypic heterogeneity and refers to the stochastic molecular mechanisms and dynamics at play (Avery, 2006). Cellular heterogeneity is an intrinsic property of biological systems where individual cells within one species and one specific environment can exhibit a wide range of divergent molecular characteristics resulting in differential gene/protein expression in the population (Komin & Skupin, 2017). It involves some genomic rearrangements that can generally revert to initial patterns, but when the frequency is low or when the change is irreversible, it can define a new bacterial strain. Thus, cellular heterogeneity can arise from genotypic or nongenotypic heterogeneity, via various molecular mechanisms at different levels leading to different types of heterogeneous phenotypes. Here, the disparity among isogenic bacterial strains under congruent environmental conditions is reviewed through phase variation mechanisms (section 4.1) and transcriptional (section 4.2) or post-transcriptional (section 4.3) regulations. These three levels of heterogeneity can actually account for some phenotypic heterogeneities, which can then either be intrinsic, that is, in the cases of genotypic and cellular heterogeneities, or extrinsic, that is, in the case of physiological diversity. It must be stressed that (i) some molecular determinants can be regulated both in response to environmental conditions and by mechanisms participating to cellular heterogeneity, and (ii) some mechanisms of the cellular heterogeneity, for example, phase variation, can generate more or less stable genetic diversity. These different levels of phenotypic heterogeneity could impact the prediction of the behavior of pathogens in complex food systems with mathematical models (Koutsoumanis & Aspridou, 2017; Verheyen & Impe, 2021). Hence, the consideration of single-cell levels in modelling of bacterial behavior (section 5.1) and risk assessment (section 5.2) is finally reviewed here.

2 | GENETIC HETEROGENEITY: GENOMIC DIVERSITY OF BACTERIAL STRAINS WITHIN A SPECIES

Considering a given bacterial species, different genetic variants can be isolated (Dijkshoorn et al., 2000). While different techniques and approaches are used to

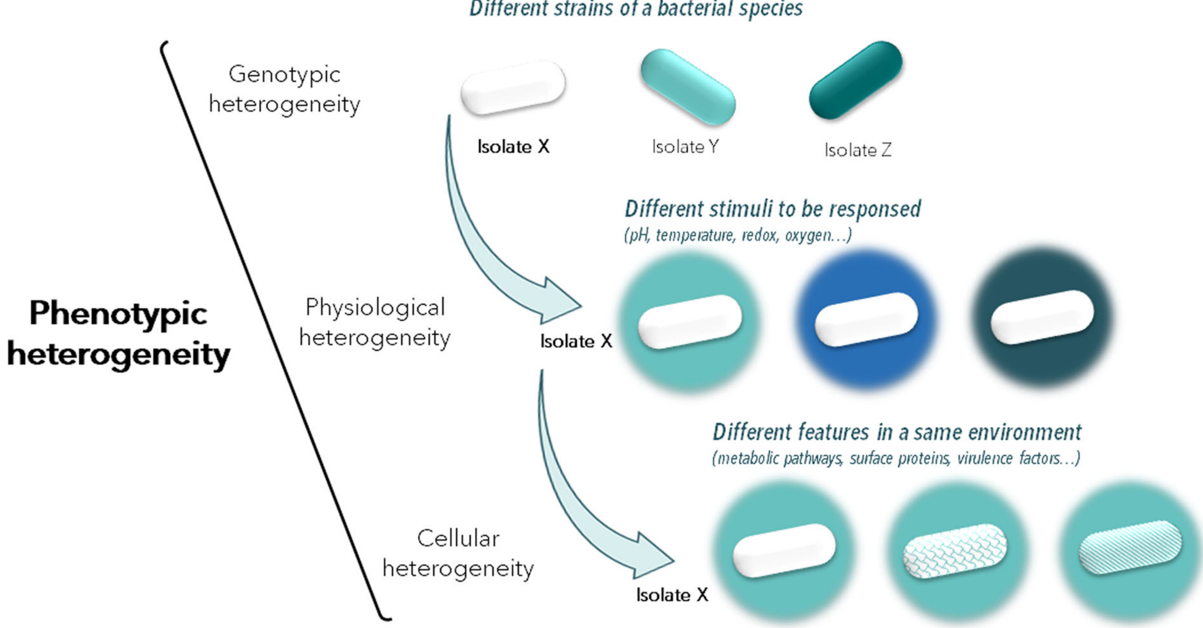

FIGURE 1 The different levels of phenotypic heterogeneity for a bacterial species. Genotypic heterogeneity considers different isolates of the same bacterial species. Physiological heterogeneity considers the responses of one isolate to several environmental conditions. Cellular heterogeneity considers different features of one isolate in a specific environment

characterize bacterial strains (Li et al., 2009), intraspecies diversity mainly results from two main genetic events, namely horizontal gene transfer, also called lateral gene transfer, and/or recombination. Horizontal gene transfer can occur following the exchange of genetic material by (i) conjugation, that is, conjugative plasmids (Virolle et al., 2020) or integrative and conjugative elements (Delavat et al., 2017), (ii) transduction, that is, by bacteriophages (Schneider, 2021), gene-transfer agents (Redfield & Soucy, 2018) or membrane vesicles (Dell'Annunziata et al., 2021), and (iii) competence (also called natural transformation) (Lorenz & Wackernagel, 1994). When a replicative DNA sequence, namely a plasmid, is transferred to a host cell, it can persist and be transmitted to the daughter cells, whereas a nonreplicative DNA sequence needs to be integrated by recombination to be inherited by the descendants. Besides the classical and legitimate homologous recombination involving long homologous sequences and inverted repeat sequences, DNA recombination can be illegitimate and occurs between sequences with little or no homology, by heterologous rearrangement or some site-specific elements (Ehrlich et al., 1993). While the roles of genetic mobile elements, such as transposons, integrative and conjugative elements or insertion sequences, are well known in the acquisition of novel genes and functions, such as antibiotic resistance (Sun et al., 2019), gene conversion by recombination is sometimes overlooked even if it plays an important role in phenotypic heterogeneity (Wisniewski-Dyé & Vial, 2008). A nontoxigenic *Vibrio cholerae* O1 strain was shown to be converted into a toxigenic strain by RecA-mediated acquisition of a cholera toxin-producing prophage (Sinha-Ray et al., 2019). This chitin-induced transformation could explain the emergence of new toxigenic *V. cholerae* strains in chitin-rich aquatic reservoirs. Genetic heterogeneity of antimicrobial resistance among foodborne pathogens, such as *Salmonella* and *Campylobacter* pathogenic strains, is well known and would result from the overuse of antibiotics in agricultural practices (Grant et al., 2016).

In the end, the presence or absence of some genetic determinants by a variety of molecular mechanisms, as well as genomic rearrangements, can reshape the genomes and result in different heritable genetic variations, leading to various strains from the same species (Fraser-Liggett, 2005). While the core genome is shared by all strains, the pan-genome comprises (i) a set of dispensable genes shared by some isolates and (ii) a set of strain-specific genes, contributing to the genetic heterogeneity of the species (Tettelin et al., 2005). Depending on the bacterial species and the molecular mechanisms at play, the diversity of the pan-genome can be quite different (Fraser-Liggett, 2005). *E. coli* is most certainly the bacterial species where the genome plasticity and flexibility has been explored the most (Dobrindt et al., 2010). Intragenic SNP analysis based on microarrays first allowed the assessment of genomic diversity and evolutionary relationships of *E. coli* O157 (Zhang et al., 2006). More recently, multilocus sequence typing on 12 SNPs identified the

geographical origin of *E. coli* O157 food isolates (Liu et al., 2020). Regarding the species *Listeria monocytogenes*, which is divided into four evolutionary lineages, although the pan-genome is highly stable, the accessory genome shows nine hypervariable hotspots, suggesting an ability to adapt at the gene scale (Kuenne et al., 2013). Analysis of the prevalence and distribution of *L. monocytogenes* multilocus sequence typing clones in food and clinical sources grouped hypovirulent or hypervirulent strains (Maury et al., 2016, 2019).

The analysis of genomic diversity by whole genome sequencing (WGS) and single-nucleotide polymorphism (SNP) typing from food or clinical isolates compared to other isolates are now frequently used for source attribution and risk assessment of foodborne pathogens (Franz et al., 2016). SNP-based typing techniques have been developed to classify *L. monocytogenes* strains as slow or fast growers in cold conditions (Fritsch et al., 2019; Hingston et al., 2017) or to decipher the persistence of *L. monocytogenes* in food production environments (Unrath et al., 2021). Genomic and physiological analysis of *Clostridium botulinum* species indicates that type B and F strains can be grouped in one subset and type E strains have distinct characteristics including temperature limits (Stringer et al., 2013). Similarly, D_{120}-values for *Bacillus cereus* spores of various strains vary in a range of 2.2 $\log_{10}$ (Wells-Bennik et al., 2016). This approach could be applied to different foodborne pathogens respective to other areas including bacterial virulence, adaptation, and/or survival to stressful conditions (e.g., low pH, high temperature, high salt concentrations, and tolerance to sanitizers).

Furthermore, advances in identifying genetic features by statistically correlating genomic data with a particular trait with genome-wide association studies (GWAS) was used to perform a source attribution study for *Campylobacter coli* clinical isolates (Jehanne et al., 2020) and to identify and trace outbreaks linked to *Salmonella* Montevideo (den Bakker et al., 2011).

Nevertheless, genomic analyses alone can never fully correlate the genotypes with the phenotypes in any living cells. This goal is an illusion from the start, as genetics is only the tip of the iceberg of cell physiology (Collado-Vides et al., 2009; Dorman, 2013). While a genome is indeed a blueprint of possibilities (which may or may not occur), the phenotype is the complex resultant of the expression of genes and proteins of diverse functions, which are regulated at very various levels, that is, at pre-transcriptional, transcriptional, post-transcriptional, translational and/or post-translational levels, and in adaptative responses to the environmental conditions (Alvarez-Ordóñez et al., 2015). A particular phenotype can be observed in one condition but absent in another, while the genotype remains the same, and reversibly, a particular genotype does not systematically result in an expected phenotype. The true key in expecting such a correlation with the phenotypes resides beyond the genotypes in understanding the mechanisms of gene/protein expression regulation under environmental dynamic changes.

3 | PHYSIOLOGICAL HETEROGENEITY: BACTERIAL CELL RESPONSE TO FOOD MATRIX MICROENVIRONMENTS

Food systems provide a large diversity of microarchitectures, including aqueous liquid systems with or without thickeners, that is, soups or fruit juices, aqueous gels, that is, pâté, oil-in-water emulsions, that is, salad cream or mayonnaise, water-in-oil emulsions, that is butter, gelled emulsions, that is sausage or cheeses and all food surfaces (Wilson et al., 2002). These systems can contain vegetable or animal cells, particles, granules, strands, crystals, gas, micelles, or droplets and display heterogeneities at the macroscale level (solid particles, macrofibres, and air pockets) or microscale level (fat or air microdroplets) (Verheyen & Impe, 2021). Food products provide nutrients such as proteins, carbohydrates, and vitamins, which are also heterogeneously distributed, such as collagen fibers in ground beef or fats in fish or meat. Thus, a heterogeneous complex matrix can be considered as an assemblage of several systems side by side, each of them with its own physical and chemical characteristics (Figure 2), where microorganisms encounter gradients of available resources and physical conditions due to diffusion kinetics of nutrients, oxygen, metabolites, preservatives, temperature, and so forth (Brocklehurst et al., 1995; Malakar et al., 2000). Bacteria have to adapt and change their metabolic activity according to the local environment (Meldrum et al., 2003; Wilson et al., 2002). Hence, close-by cells could be subjected to different environmental pressures and show heterogeneous physiology and ability to survive and grow. Communities with a vast range of physiological heterogeneities could emerge in a limited zone as in biofilms (Xu et al., 2000). Thus, to understand population heterogeneity in food matrices, we must explore how structural heterogeneity and physicochemical gradients impact cell physiology and induce an array of adaptive mechanisms.

3.1 | Food structure and microstructure

Structural heterogeneity in food includes the different phases, their proportion, composition, and the physicochemical characteristics of each phase (Wilson et al., 2002), together with constraints on the mechanical distribution

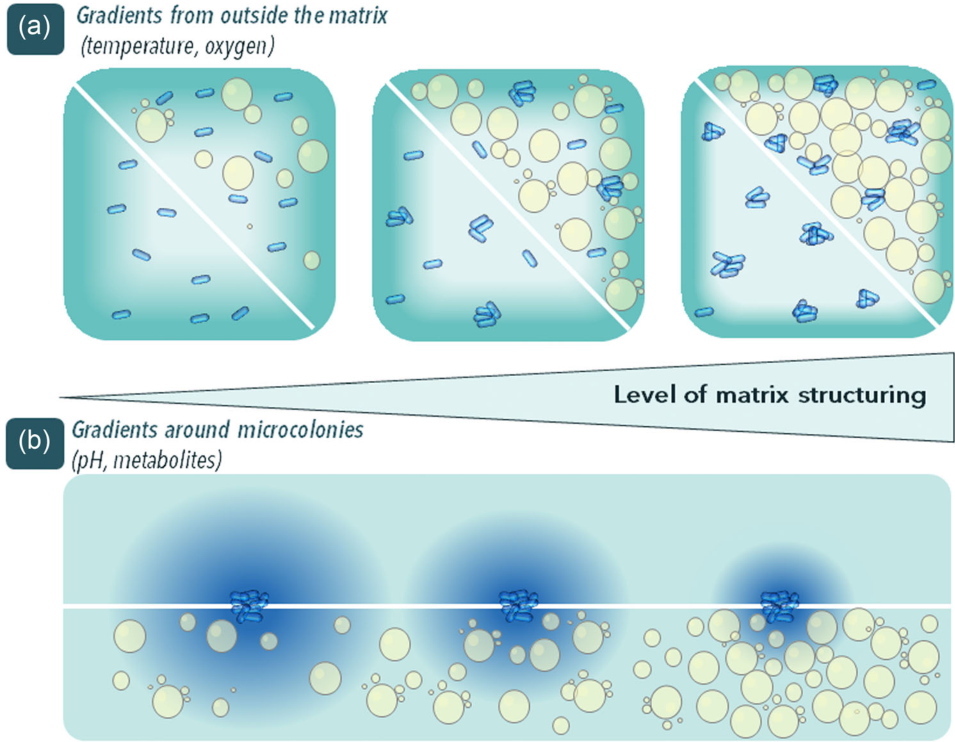

FIGURE 2 Physico-chemical microgradients increase with the level of matrix structuring. (a) when the gradients are reported from outside of the matrix (maximum concentration) to the center, such as for temperature or oxygen, the penetration is lower when the level of structure increases (from the left to the right), with or without oils droplets (emulsions); (b) when the gradients are reported from inside of the matrix (maximum concentration) to the center, such as microbial metabolites, the diffusion is lower when the level of structure increases (from the left to the right), with or without oils droplets (emulsions)

of water (Hills et al., 1997). When a matrix is prepared by mixing culture medium with different ratios of gelatine and dextran, various structures can be obtained. A uniform mixture is obtained with a 1:1 ratio, whereas dextran spheres appear in the gelatine matrix when the gelatine concentration increases (Boons et al., 2013). Doubling the concentrations of dextran and gelatine while keeping the same proportion between them leads to a completely different microstructure as phase inversion happens and gelatine spheres appear in the dextran matrix (Boons et al., 2013). Moreover, in the latter system, the presence or absence of salt has a significant impact on the size of the dextran droplets. Similarly, the addition of acid-modified corn starch above 1% modifies the structure of the gelatine network and allows the appearance of hollow zones containing starch granules (Marfil et al., 2012).

3.1.1 | Impact on bacterial spatial organization

The arrangement of zones determines the overall structure and their relative properties impact the partitioning of solutes, nutrients, and cells. The level of matrix structuring affects the bacterial ability to move within the matrix and to escape from nonoptimal environments (Skandamis & Jeanson, 2015; Wimpenny et al., 1995). Liquid systems allow free motility, and microorganisms can grow in the planktonic state. When the space constraints increase in the matrix, in presence of thickeners for example, bacterial growth turns from planktonic to immobilized, and colonies grow in the bulk of the matrix, or at the air or oil interfaces. Some processing steps in contrast can break solid matrices, like minced meat, and change the matrix properties, thereby enhancing the ability of pathogens to move. In matrices containing both gelatine and dextran phases, *E. coli* preferentially grow as colonies in the dextran phase whatever the dispersed phase (Boons et al., 2013). As a result, when the dextran phase is the dispersed phase in the gelatine matrix, *E. coli* colonies grow as tight spheres inside the dextran drops, but when there is a phase inversion, colonies adopt a diffuse string formation having more space to grow in the continuous dextran phase (Boons et al., 2013). In oil-in-water emulsion, the percentage between the oil and aqueous phases, the type of emulsifier, and the droplet size distribution are fundamental parameters for emulsion stability (Brocklehurst et al., 1995). In this type of matrix, microbial growth occurs

within the aqueous phase, which is usually structured depending on the concentration of fat. The percentage of oil can be 3–5% v/v as in milk or can reach more than 80% v/v as in mayonnaise (Wilson et al., 2002). The aqueous available space in these systems thus differs highly and can impact the microbial mode of life. In emulsified systems with low proportions of fat (e.g., 26.8%), *L. monocytogenes* is localized in the free space between droplets, and the microbial growth remains planktonic (Baka et al., 2017; Wilson et al., 2002). When the proportion of oil increases to 83% in model media and in artificially inoculated fresh and tinned dairy cream, *L. monocytogenes*, *Salmonella* Typhimurium, and *Yersinia enterocolitica* form colonies in the space available for the aqueous phase (Parker et al., 1995). In solid systems, the size and form of the colonies can differ, turning from diffuse to tight according to the level of structural constraints (Saint Martin et al., 2022). When the agar concentration increases from 0 g/L to 75 g/L, the colony areas and the number of *B. cereus* per colony decrease (Stecchini et al., 1998). Bacteria form aggregated clusters based on intercellular signaling in or on a solid structure (Esipov & Shapiro, 1998). Swarming of *E. coli* through semisolid agar revealed that chemotactic activity is not critical, but that tumble frequency was a much better judge of mobility: cells that have no or intermittent tumbling cannot swarm and the higher the tumbling frequency the greater the ability of cells to swarm (Wolfe & Berg, 1989). Heterogeneity in tumbling capacity could lead to heterogeneous abilities of bacteria to reach new environments and thus heterogeneous bacterial distribution in the matrix. Moreover, in solid matrices, when bacteria grow locally as colonies, the relatively high cell density results in local competition for space between neighboring microorganisms. Colonial growth also modifies the microlocal conditions because cell metabolism changes the solute composition around the colony (Wimpenny et al., 1995). In cheese-like model matrices, the spatial distribution of bacterial colonies has been defined according to the size of colonies and the distances between neighboring colonies (Jeanson et al., 2015). Two types of colonies were separated: microcolonies, where microbial growth is similar to planktonic growth and macrocolonies, which have slower growth due to diffusion limitations from outside and into the colony (Jeanson et al., 2015). New approaches and creative experimental designs based on 3D bioprinting are emerging in the study of microbial behavior in controlled spatially structured environments (Kyle, 2018).

3.2 | Impact on bacterial behavior

The structure of the medium in which bacteria are entrapped can affect the efficiency of inactivation processes and/or bacterial growth rates. When thermal treatments are applied to *L. monocytogenes* entrapped in several matrices, the maximum specific inactivation rate K_{max} was higher in the viscous system than in the respective gelled system, demonstrating a protective effect of the gelled matrix (Verheyen et al., 2019). In the presence of fat, the effect of food matrix is more complex depending on the temperature. Below 60°C, the inactivation rate decreased in both emulsions and gelled emulsions with increasing fat content (Verheyen et al., 2020). Similarly, colonies of *L. monocytogenes* and *S.* Typhimurium grown on agar surfaces were more resistant to cold atmospheric plasma than their planktonic counterparts (Smet et al., 2017). The efficiency of inactivation treatments mainly depends on the complex relationship between the structure and composition of the food matrix, thermal conductivity, rheological properties, and inactivation process and level.

Several studies have also compared growth rates measured in more or less complex systems to those measured in the liquid culture media. In jellified systems, *B. cereus* cells have a reduced growth rate when immobilized in 10% gelatine compared to the planktonic state, the difference being greater when water activity is decreased by adding sucrose or NaCl (Wilson et al., 2002). Similarly, the growth rate of *L. monocytogenes* at 4°C decreased as gelatine concentration increased (from 0.054 h^{-1} at 0% to 0.015 h^{-1} at 30%) (Aspridou et al., 2014). A secondary model was proposed to describe the effect of gelatine concentration on the bacterial growth rate in a cheese-like medium at 20°C compared to milk (Theys et al., 2009). The observed maximum growth rate (μ_{max}) decreases when gelatine concentration increases from 0 g/L to 300 g/L for both *Listeria innocua* and *Lactococcus lactis*. The addition of 1 or 5% gelatine at 20°C to the growth medium also led to a significant reduction of growth rate and maximal cell density of *S. typhimurium* in all tested conditions of pH (4.5–5.5) and a_w (0.97–0.992) (Theys et al., 2008). Nevertheless, the decrease in growth rate induced by pH or water activity is less pronounced in media containing gelatine, indicating that the microstructure could modulate the effect of gradients encountered (Theys et al., 2008). In contrast, in some conditions, growth in an immobilized state can be similar to planktonic or even favored. Smet et al. (2015) compared the growth rates of planktonic cells, immerged colonies, and surface grown colonies of *S. typhimurium* and *E. coli* at different temperatures. For *E. coli* at low temperatures (8°C), planktonic cells grow a little bit faster than colonies, but the difference is no longer apparent at higher temperature (22°C), and for *Salmonella*, the three states have similar growth rates (Smet et al., 2015). The quantification of bacterial growth parameters in these complex media is controversial, probably because in addition to the level of

structure, many factors differ between studies. It is very difficult to appreciate the impact of each factor independently from physiochemical and compositional aspects of food products. The addition of gelling agents or fats can modify other physicochemical factors. For example, the addition of gelling agents or the presence of fat that replaces an equal amount of water can modify the a_w, and the addition of emulsifiers, which are essential to stabilize the emulsion, can decrease the pH. Even when a gelled emulsion is specifically designed to mimic canned meat, *L. monocytogenes* growth rate is significantly higher in real canned meat, probably due to a different source of proteins (Baka et al., 2016).

The effect of microstructure is more significant under additional stress conditions, which further influence growth kinetics. The effect of structure on growth greatly increases when the temperature is lower (4°C instead of 12°C) and is still measurable at 12°C only for higher concentrations of gelatine (30%) (Aspridou et al., 2014). Moreover, according to the hurdle theory, when stress factors are combined (e.g., low temperatures and a solid environment), a synergistic inhibitory effect on microbial growth is often observed. The combined effect of each factor can be higher than the addition of each effect alone (Leistner, 2000). Consequently, the growth boundaries are narrowed when growth occurs in an immobilized state (Meldrum et al., 2003).

3.3 | Physicochemical microenvironments in foods

Bacterial growth mainly relies on the surrounding physicochemical parameters (pH, water activity, oxygen, temperature, etc.), and the spatial heterogeneity of food means that individual cells encounter various microenvironments with specific growth conditions (constrained vs. free space and specific physicochemical parameters) (Skandamis & Jeanson, 2015). The different types of structure in food systems can affect the distribution of solutes (substrates, metabolites, and inhibitors) within the bulk of the matrix (Figure 2). In a liquid system with shaking, the nutrients and oxygen are homogeneously distributed and their availability will be similar in every point of the system (Dens & Van Impe, 2001). On the contrary, structured systems as semisolid and solid matrices can limit the diffusion of molecules due to physical barriers and ionic forces that prevent homogeneous spread. Concentration gradients can appear and persist and make the food matrices become an assemblage of several environments with different physicochemical characteristics (Dens & Van Impe, 2001). Fick's second law gives predictive results for diffusion at a macroscale but cannot consider the microscale variability due to local change in structure and composition of the matrix. Diffusion coefficients of solutes in complex matrices are poorly studied. Only mass transfer of salt has been studied in depth in cheese (Floury et al., 2010). Nondestructive methods such as magnetic resonance imaging, nuclear magnetic resonance, or fluorescence recovery after photobleaching are being developed to improve the measurement of diffusion coefficients of solutes in complex matrices (Floury et al., 2010).

A large variability of physicochemical factors such as pH, packaging atmosphere (e.g., CO_2 levels), redox potential (Eh), a_w and relative humidity, concentration of antimicrobial compounds, or temperature are applied to specific foods (Jeanson et al., 2015; Møller et al., 2012; Theys et al., 2009). Combinations of factors together with the range of gradients for each factor create microenvironments in food matrices with variability of physicochemical environments at a microscopic scale. Furthermore, conditions are not stable over time and may evolve as chemical reactions and diffusion of different solutes take place. When bacteria reach a new environment, they sense its characteristics with chemoreceptors (Sibona, 2007). Each bacterium has a defined zone, called a comfort zone, where all environmental factors allow growth at a specific rate (Booth, 2002). Out of this comfort zone, there is a survival zone where there is no growth, but the death rate is low. In more extreme conditions, death rates become higher (Booth, 2002). When possible, motility is a valuable escape mechanism, but the structural constraints of the food systems can prevent the motility of bacterial cells that are trapped in the solid phase. Hence, bacterial cells have no other option than to adapt or die. The variety of stresses encountered and the responses available lead to diversification and heterogeneity of phenotypes in the matrix. As phenotypic changes are driven by reshuffling of gene expression in cells, as it is in biofilms (Flemming et al., 2016), recent methodological progress allowing spatiotemporal monitoring of bacterial gene expression at the microscopic scale in three-dimensional systems will be of great interest. Indeed, techniques that give access to local gene expression at the single-cell level by combining fluorescent reporters and imaging by confocal laser scanning microscopy would help to unravel cellular heterogeneity patterns in foods. Nevertheless, transcriptional fusion with fluorescent genes and strain modification by genetic engineering are still under-exploited.

3.3.1 | Microgradients of nutrients or antimicrobials

The heterogeneity of nutrient sources and their distribution in foods can affect bacterial growth and induce growth or survival heterogeneity. Immobilized cells

consume nutrients from their local microenvironment and can show different behaviors due to local nutrient competition. For example, the addition of glucose to gelatine gel increases bacterial growth more than it does in culture broth (Aspridou et al., 2014).

Preservatives are antimicrobial agents added to the food product to slow down alteration caused by spoilage microorganisms until the end of the shelf-life. They are mainly organic acids (e.g., lactic, citric, or acetic acids), plant extracts or essential oils (e.g., thyme, clove or basil), bacteriocins (e.g., nisin), or salts (e.g., sulfites, nitrites). They have different modes of action such as cell acidification, membrane damage, or interference with key bacterial functions and can be bacteriostatic or bactericidal (Lucera et al., 2012). Weak organic acids can be added to foods as preservatives to decrease the pH. Moreover, in foods such as meat and cheese, fermentation of, respectively, glycogen or lactose leads to a progressive and heterogeneous acidification of the matrix (Hamoen et al., 2013). In solid food matrices where bacteria are more or less immobilized, their growth and physiology is impacted by the pH directly around the cell or colony and by the pH microgradients created by metabolic activity within the colony (Floury et al., 2010). The variability of pH in colonies at a microscopic scale was demonstrated to be far higher than at a macroscopic scale (Ferrier et al., 2013). The impact of pH microgradients on bacterial behavior is increasingly studied thanks to a technical shift from the use of microelectrodes to imaging techniques using pH-sensitive fluorescent dyes associated with confocal laser scanning microscopy (Burdikova et al., 2015; Malakar et al., 2000). For example, pH microgradients occur in and around submerged colonies of *S.* Typhimurium bigger than 400 μm in gelatine gel systems, with a range from pH 7 at the edge to pH 4.3 in the center of the colony (Walker et al., 1997; Wimpenny et al., 1995), but no pH microgradients occur around bacterial colonies in a model cheese regardless of the size of the colony (Jeanson et al., 2013). The existence of pH microgradients in real food systems or in small colonies is thus still uncertain. To cope with changes in environmental conditions, bacteria are able to develop a wide range of adaptative strategies to survive and to persist. Acid tolerance response (ATR) is defined as a transient response that induces bacterial adaptation and resistance to sublethal pH, which aims to rebalance and maintain pH homeostasis (Alvarez-Ordóñez et al., 2015). Localized ATR due to the presence of pH microgradients could induce heterogeneity within the food matrix in terms of resistance, growth rate, cell morphology, or metabolic activity of foodborne pathogens (Walker et al., 1997). For example, adaptation to a low pH increased the heat resistance of *Enterococcus faecium* to a different extent according to the type of acid (Fernández et al., 2009). Moreover, heat-treated spores of *B. cereus* show a higher heterogeneity in outgrowth at pH 5.5 than at 7.4 (Warda et al., 2015).

During cheese making and according to the specific technologies, acidic environments can be locally deacidified by acid-tolerant yeasts and molds. In Camembert cheeses, during the ripening period, the pH increases at the surface from 5 to 6 due to the oxidation of lactic acid in CO_2 by *Penicillium camembertii*, in contrast to the cheese bulk where the pH stays at 5 (Back et al., 1993). In these conditions, *L. monocytogenes*, which contaminates the raw milk and survives during the acidification period or contaminates the product during the process, can grow at the surface but not in the bulk of the cheese (Back et al., 1993). Moreover, tomato juice which is contaminated by *Penicillium* showed pH gradients with increased pH nearly reaching neutrality at the surface, while the lower parts stay at acidic pH (Huhtanen et al., 1976). *Clostridium* spores were then able to germinate and grow in the less acidic portions of juice (Huhtanen et al., 1976). Moreover, as a close relationship was shown between complex mechanisms of ATR and virulence in *S.* Typhimurium and *L. monocytogenes* (Álvarez-Ordóñez et al., 2010; Gahan & Hill, 1999), we could easily hypothesize that virulence of these pathogens may be regulated at the microscale level by the heterogeneity of pH values, in particular in natural cheese (Burdikova et al., 2015).

More generally and accordingly to their physicochemical properties, preservatives in complex foods are expected to be more or less trapped by micelles or lipid phases. Hence, the concentration at which they are available in the aqueous phase, where microorganisms are, can be reduced and consequently they lose their efficacy. Moreover, due to the localization and proportion of lipid phases, microorganisms could be exposed to inhibitory or subinhibitory concentrations according to their localization. For example, the antimicrobial activity of clove and thyme oils against *L. monocytogenes* was demonstrated in peptone water but was lost in full-fat hotdogs (Singh et al., 2003). Similarly, in a 30% oil-in-water emulsion, eugenol lost the high inhibitory activity observed in the aqueous phase (Pernin et al., 2019). The $\log P_{oct/wat}$ of eugenol is 2.61 which probably explains why this compound preferentially migrates into the lipid droplets as soon as it is dispersed in the emulsion and why it consequently loses its antimicrobial activity in this system. On the contrary, ferulic acid, which is more hydrophilic, retains its antimicrobial activity, probably because a higher proportion of the compound remains in the aqueous phase of the emulsion (Pernin et al., 2019). In food systems containing fats, the partition coefficient ($\log P_{oct/wat}$) of antimicrobial compounds appears to be a key factor in maintaining their efficacy. Besides interactions with fats, preservatives may also interact with emulsifiers, proteins, or charged polysaccharides

from food (Pernin et al., 2019; Weiss et al., 2015). As a result, antimicrobial agents are less available for inhibition or inactivation of microorganisms.

3.3.2 | Microgradients of temperature and water activity

Temperature is a major extrinsic factor used to control food safety. Thermal processes with high temperature (cooking, pasteurization, and sterilization) or low temperature (refrigeration and freezing) are commonly used to preserve foods. The food characteristics, namely its size, structure, and composition (pH, fat, additives, etc.), interfere with temperature diffusion and thermal gradients can be created. When a muffin batter is baked at 190°C, it takes 15 min for the internal muffin temperature to increase from 20°C to 100°C (Channaiah et al., 2017). In the same way, when submitted to cycles of freezing/thawing or taking in/out of the fridge, the temperature differs between the surface and the core of the products. Hence, the protection of pathogens from the effects of sublethal temperatures varies according to the matrix composition, in particular the water activity or the presence of cryoprotectants as fats. D_{61}-values for *Salmonella* in muffin batter (0.92, pH 6.6) or bread dough (a_w 0.97, pH 5.5) are, respectively, 16.5 and 3.1 min (Channaiah et al., 2016, 2017). The higher fat content in muffin batter probably protects bacterial cells better than bread dough. Sugar and salt enhance the survival of *L. monocytogenes* and *S. aureus* in refrigerated model brines (Bevilacqua et al., 2018). Spatiotemporal heterogeneities of temperature can bring an exposure of microorganisms to temperatures out of their comfort zone and can result in adaptation and acquired thermotolerance. The cold adaptation response counteracts the effects of low temperature on cellular components. At a molecular level, cold exposure has several consequences: (1) reduction of membrane fluidity, thus limiting exchanges with the extracellular environment; (2) stabilization of mRNA secondary structure, which reduces translation efficiency and gene expression, and (3) misfolding of proteins that slows metabolism. Over the past two decades, many reviews and studies have described molecular mechanisms of the cold adaptation response at the molecular level (Alvarez-Ordóñez et al., 2015). The variability of individual cell behavior during the cold chain has received increasing attention in recent years. However, few data address cell heterogeneity in the cold adaptation response or describe growth heterogeneity at the single-cell level.

The measurement of a_w in foods determines the amount of free water that is available for microorganisms to use for growth. Most foods exhibit an a_w above 0.95 and so allow bacterial growth. However, a_w measurement only supplies information on the overall water activity in food, whereas the distribution of water in food, which is limited by its physical structure, can create a heterogeneous environment for bacterial growth (Hills et al., 1997; Wilson et al., 2002). The distribution of water in these microenvironments is conditioned by the composition of protein and fat and the pH of the food matrix (Hills et al., 1997; Møller et al., 2012). The probability of *L. monocytogenes* growth was influenced by the a_w of cheese matrix at both low and high contamination levels (Schvartzman et al., 2011). Decreasing the a_w by adding NaCl or sugar induces osmotic stress in bacteria and has a significant impact on the growth of colonies (Theys et al., 2010). If the salt or sugar distribution is heterogeneous, we can easily imagine the heterogeneity in bacterial response to osmotic stress. The resistance of *L. monocytogenes* to osmotic stress encountered in food matrices is associated with several transport systems, such as the OpuCABCD system for carnitine accumulation and the GbuABC and BetL systems for glycine betaine (Bucur et al., 2018).

3.3.3 | Gas microgradients and light

Oxygen availability is a critical factor affecting bacterial growth. The availability of oxygen, that is, aerobic, microaerobic, facultative anaerobic, and anaerobic conditions, impacts the behavior and growth of bacteria according to their respiratory type. Most foodborne bacterial pathogens are facultative anaerobes. Oxygen diffuses in food matrices, where it dissolves. During food processing and storage, bacteria can encounter various oxygen concentrations and exposure to oxidative compounds (Pénicaud et al., 2010). Moreover, vacuum packaging and modified atmosphere packaging of foods are current ways of improving shelf-life and safety by bacterial inhibition (Farber, 1991). Packaging is used to keep oxygen levels low to very low (except for fresh meat products). Protective gas mixes in modified atmosphere packaging are most often combinations of oxygen (O_2), nitrogen (N_2), and carbon dioxide (CO_2). The increase of dissolved CO_2 in the water phase of food products has a direct inhibitory effect on bacterial growth, resulting in an increase of lag phase and a decrease of the maximum growth rate. Various direct or indirect techniques can be used to quantify the O_2 and CO_2 solubility and diffusivity, but these measures in food and especially in solid foods are always a challenge and submitted to many biases and interferences (Chaix et al., 2014). Moreover, the O_2 and CO_2 content changes according to environmental factors, such as temperature, and the composition and structure of food matrices, but also according to microbial metabolism (Chaix et al., 2014).

The colonies formed in solid matrices can be subjected to various oxygen conditions. Oxygen is more available at the surface of the colony. Microgradients of oxygen can form around and in the colony due to the diffusion of oxygen within the matrix and its consumption by the bacterial cells (Wilson et al., 2002; Wimpenny et al., 1995). Microelectrodes were first used to measure oxygen penetration in and around a colony of *B. cereus* on an agar medium showing an aerobic zone only through to a depth of 25–30 μm (Wimpenny & Coombs, 1983). They were also used to investigate the oxygen level around colonies of *B. cereus* and *E. coli* and revealed low or depleted oxygen conditions near metabolically active young colonies. The oxygen level was higher in older colonies as a consequence of a lower metabolism and therefore a low oxygen requirement. So, the depth to which O_2 diffuses depends on the age and shape of the colony and on the microorganism's physiology (Pipe & Grimson, 2008). Measurements of oxygen content by the invasive technique of microelectrodes can be unreliable (Tammam et al., 2001). O_2 and CO_2 concentrations were evaluated by mass spectrometry measurements in a soft-agar medium and in a Cheddar cheese model inoculated with *Lactobacillus paracasei*. This study showed that O_2 remained undetectable at depths of a few millimeters after 24 h of incubation and 15 days of ripening, respectively, in each condition, and that CO_2 increased with both depth and incubation time (Tammam et al., 2001). The distribution of *S. aureus* monitored during the ripening of semihard cheeses shows that *S. aureus* cells are preferentially on the cheese surface rather than in the core, in relation to better aerobic conditions and pH. Moreover, the growth of *S. aureus* on the surface was correlated with the expression and production of the enterotoxin SED (Fleurot et al., 2014).

In the presence of oxygen, bacteria have defense mechanisms that detoxify reactive oxygen species such as oxygen radicals, singlet oxygen, and peroxides. Enzymes such as superoxide dismutases, catalases, oxidases, and peroxidases can counteract oxidative stress. But, interestingly, oxidative stress can induce cell adaptations, such as genetic variability, in bacteria within a biofilm (Čáp et al., 2012). To our knowledge, such oxidative stress-induced adaptation has not been shown for bacterial colony-forming cells in food matrices.

Light is an important energy source for phototrophic microorganisms. It was recently discovered that many chemotrophic bacteria are able to sense light and trigger a photosensitive response (Gomelsky & Hoff, 2011). The main bacterial photosensitive responses described so far concern stress adaptation, lifestyle decision (planktonic vs. biofilm), and virulence. In a pioneering study, it was found that exposure to light strongly enhanced the virulence of *Brucella abortus* (Swartz et al., 2007). More recently, it was fortuitously observed that colonies of *L. monocytogenes* on agar undergo synchronized multicellular behavior in response to light and dark cycles, giving rise to alternating opaque and translucent rings (Tiensuu et al., 2013). Cells in the opaque rings were associated with light cycles and were shown to produce more extracellular polymeric substances required for stress and long-term survival. Light sensing in this pathogen has been shown to be predominantly SigmaB dependent and to be absent at the temperature of the mammalian host (Dorey et al., 2019). It was hypothesized that the blue light LOV sensors of *L. monocytogenes* could also contribute to cold sensing and to the acidic tolerance response, two factors of importance for pathogen behavior in the food matrix (Chan et al., 2013). *E. coli* uses another family of blue light-sensing proteins (BLUF domain photoreceptors) to photoregulate its mode of life (e.g., regulation of curli and colanic acid expression) (Tschowri et al., 2009), and there is a growing suspicion that many chemotrophic prokaryotes commonly use light-sensitive proteins to regulate their activity. Foodborne pathogens are heterogeneously exposed to light in a food matrix. Surface communities are directly exposed to natural or artificial light, which diffuses poorly in most food materials, preventing the exposure of bulk communities and contributing to their physiological heterogeneity. Moreover, foods such as ready-to-eat products (pâtés, ham, and cheeses), which are stored in refrigerated display cases, can be alternatively be exposed to light during the day and to darkness at night.

3.4 | Microbiota effect on food pathogens

3.4.1 | Food matrix as a microbial biotope

A microbial pathogen can colonize a food matrix axenically, for example, when an accidental contamination occurs after thermal sterilization. However, in most situations, a pathogen in a food matrix is likely to interact with an abundant and complex microbiota resulting from the contamination of the raw materials and from the technological process (addition of microbial starters or bioprotective agents, contact with air, water, surfaces, and operators). Within these communities, bacterial pathogens compete with their neighbors for space and resources (Hibbing et al., 2010). Recent reports also demonstrated that life in such structured habitats alters the composition of microbial communities compared to liquid cultures (Kleyer et al., 2021). The recent development of high-throughput DNA sequencing technologies allows a new approach that is free from the limitations of culture-dependent experiments and which describes microbial communities in food matrices with unprecedented

resolution (Ercolini, 2013). These tools are now used to control food fermentation, spoilage, biopreservation, and to track pathogen contamination routes (De Filippis et al., 2018). Food microbiota can be composed of bacteria, yeasts, fungi but also viruses including bacteriophages (Dugat-Bony et al., 2020). The microbial diversity of these communities associated with the heterogeneous nature of their microenvironments drives local interactions, which result in altered pathogen growth and behaviors. Such interactions were recently observed in a model gelled matrix where the presence of *E. coli* stimulates the growth of *L. monocytogenes* in stressful acidic conditions (Saint Martin et al., 2022). On the other hand, the presence of *L. monocytogenes* triggers the motility of individual cells of *E. coli* in a specific condition (e.g., 0.25% agarose hydrogel with 25 g/L NaCl) where they could not swim alone, illustrating the interplay of community members in such structured environments. Recent advances in microbial community bioprinting pave the way to the modeling of interspecies interactions in spatially organized systems (Hynes et al., 2018).

3.4.2 | Spatial and nutritive competition

Several studies have demonstrated that in food matrices where the physicochemical (pH, a_w) and nutritional conditions are favorable to the growth of pathogens, the pathogens could be inhibited by the presence of background microbiota (Lardeux et al., 2015). The formal demonstration of a total or partial the so-called "Jameson effect" suggests nutritional competition for shared limited nutrient resources (Delignette-Muller et al., 2006). The race for nutrients is increased when competing communities are spatially organized: as nutrients can be consumed faster than they diffuse in the colonies, embedded cells at a distance from the nutrient interface can suffer from nutrient deprivation. This was shown to drive the inhibition of *L. monocytogenes* competing for space with the fast-growing *L. lactis* in a millifluidic chamber continuously perfused with fresh nutrients (Habimana et al., 2011).

3.4.3 | Specific interference

Microorganisms from the microbiota growing in the food matrix can produce compounds affecting pathogen physiology, survival, and behavior. This is the case of weak acids produced by colonies of lactic acid bacteria in cheese and many fermented foods (Jeanson et al., 2013). Bacteriocins with specific spectra of activity against foodborne pathogens are also involved in their exclusion from the territory influenced by bacteriocin-producing bacteria (Thomas et al., 1997). For specific bacteriocins, it was recently shown that cell–cell contact is mandatory for the inhibition. This is the case with *Lactococcus piscium* CNCM I-4031 which prevents the growth of *L. monocytogenes* in contaminated peeled and cooked shrimp (Saraoui et al., 2018), but also for a bacteriocin produced by *L. monocytogenes* (Listeriolysin S) that induces membrane permeabilization of *L. lactis* in a contact-dependent manner (Thomas et al., 1997).

Foodborne pathogens can regulate their gene expression through a mechanism of quorum-sensing that is dependent on the local population density (Monnet & Gardan, 2015). The signals are carried by different diffusing molecules, that is, acyl-homoserine lactones derived from S-adenosylmethionine in gram-negative bacteria and small peptides in gram-positive bacteria, with acyl-homoserine lactones being able to pass through the membrane of gram-negative bacteria, whereas the small peptides of gram-positive bacteria trigger a two-component system (Ng & Bassler, 2009; Winzer et al., 2002). The synthesis of many bacteriocins active on foodborne pathogens is activated by such mechanisms (Kareb & Aïder, 2020) and interspecific quorum-sensing systems have been reported in different species (Khan et al., 2018). The concentration and bioavailability of such signaling molecules can be altered in multispecies communities by interactions with specific components of the matrix or the microbiota, for example, interaction with amyloid bacterial decorations (Seviour et al., 2015). Recently, a flavin-based extracellular electron transfer (EET) process was described in *L. monocytogenes* and other gram-positive bacteria (Light et al., 2018). Such EET contributes to syntrophic metabolism between microbiota members and is of significant importance for pathogen respiration and physiology. While the precise role of EET for pathogens in the context of food matrix remains to be explored, it could represent an important mechanism to reduce extracellular ferric iron and to enhance iron bioavailability and iron uptake (Jeuken et al., 2020).

4 | CELLULAR HETEROGENEITY: DISPARITY AMONG ISOGENIC BACTERIAL STRAINS UNDER CONGRUENT ENVIRONMENTAL CONDITIONS

Besides the array of microenvironments encountered by foodborne bacterial pathogens due to the endogenous heterogeneity of most food matrices, a bacterial strain can show some high degree of heterogeneity even in a homogenous medium. Cellular heterogeneity refers to the differences between cells from an isogenic bacterial

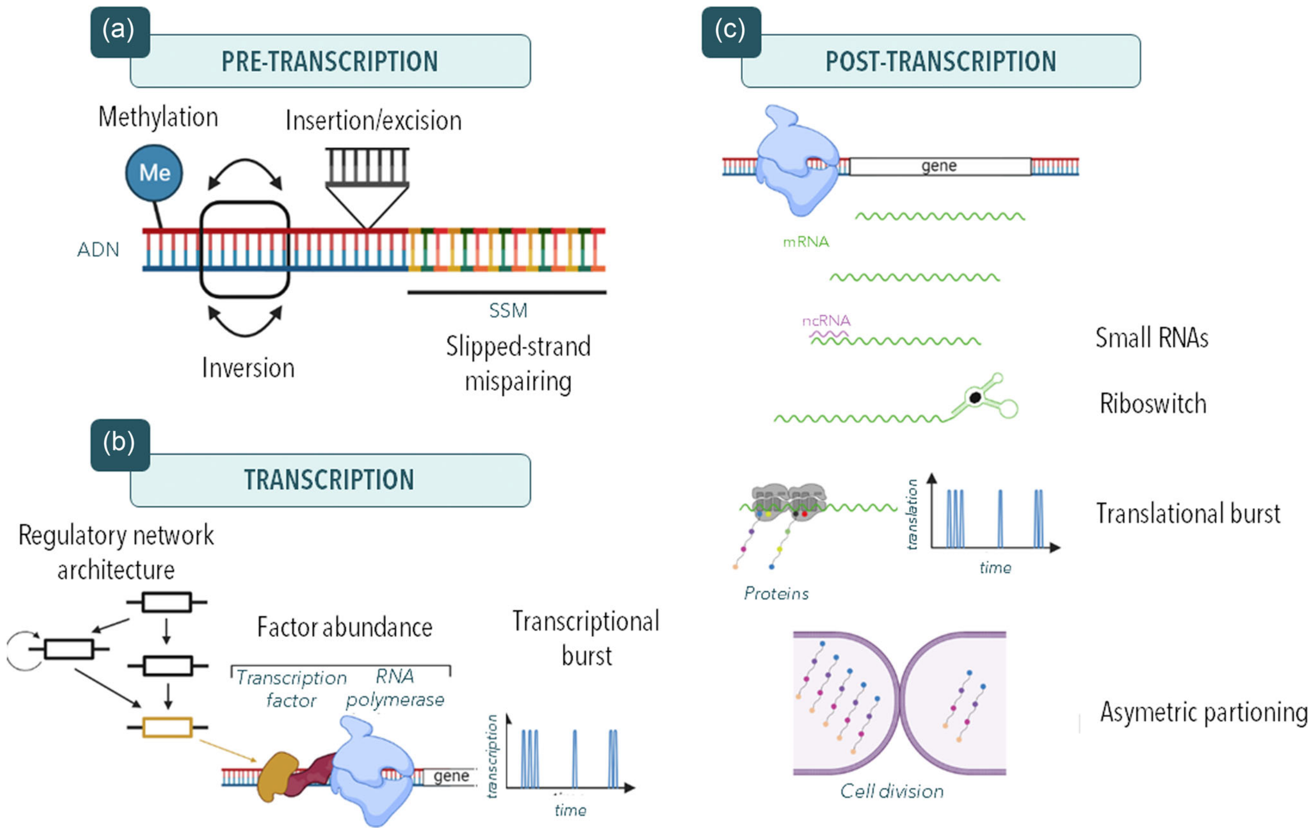

FIGURE 3 Cellular heterogeneity due to different molecular mechanisms of gene expression regulation. (a) Pretranscriptional regulatory mechanisms, (b) Transcription regulatory mechanisms, (c) Post-transcriptional regulatory mechanisms

clone under congruent environmental conditions. Molecular mechanisms at play can occur at pretranscriptional, transcriptional or post-transcriptional levels (Figure 3). Among foodborne pathogens, *S. enterica* is certainly the species where these mechanisms have been the most considered and investigated (García-Pastor et al., 2019). While the information for other foodborne pathogens is scarce, these mechanisms are quite generic as deciphered in model organisms such as laboratory strains of *E. coli*, and it cannot be excluded that they take place in bacterial foodborne pathogens, which would then deserve and trigger further in-depth investigations.

4.1 | Phase variation mechanisms

Some regulatory mechanisms underpinning cellular heterogeneity can occur before the stage of DNA transcription. An important group of pretranscriptional regulatory mechanisms recognized for decades, but sometimes overlooked, belongs to the so-called phase variation mechanisms, which correspond to a reversible ON/OFF switch (Henderson et al., 1999). This molecular regulatory mechanism primarily occurs in the course of DNA replication and has consequences for transcription and/or translation. It is noteworthy that while gene conversion by homologous recombination can sometimes be described as one-way phase variation, this is an oxymoron considering gene conversion is irreversible and is not phase variable by definition. A large majority of genes that are regulated by phase variation are bacterial cell-surface molecular determinants (Owen et al., 1996; Wisniewski-Dyé & Vial, 2008). These mechanisms can thus highly impact the bacterial way of life within heterogeneous food systems and especially the colonization processes.

Since the first mention of phase variation in 1922 for serogroup-agglutination in *Salmonella* (Andrewes, 1922), several mechanisms have been uncovered, namely (i) DNA inversion, (ii) slipped-strand mispairing, (iii) DNA insertion/excision, and (iv) DNA methylation. Contrary to transcriptional repression or induction where there is always a basal gene expression due to promoter leakage, phase variation is absolute with no expression at all when in the OFF phase, which is quite unusual in biology (Henderson et al., 1999; Salaün et al., 2003; Wisniewski-Dyé & Vial, 2008). The collection of phase variation systems found in a single bacterial strain is sometimes referred to as the phasome (Wanford et al., 2018). Moreover, the

different phase variation mechanisms are not equally prevalent in all bacterial species (Van Der Woude & Bäumler, 2004). The frequency of shift in phase variation is quite variable and most certainly dependent on environmental conditions, but is typically asymmetric with generally lower conversion and higher reversion rates (Salaün et al., 2003). Phase variation gives rise to heritable but reversible genotypes. It is often related to bet-hedging strategies where a state of random phenotypic heterogeneity is used to adapt to any unpredictable environmental changes leading subpopulations to express a phenotype deviating from the main population (Woude, 2006). This strategy is generally considered to enhance the chances of survival of one part of the population at a minor energy cost (van der Woude, 2011). For bacterial foodborne pathogens in general, however, the prevalence and contribution of these different levels of phase variation mechanisms linked to different phenotypes have been barely considered and remain an open question, with respect to the ecophysiology of zoonotic etiological agents along the food chain (Marinus, 2010).

4.1.1 | DNA inversion

DNA inversion refers to a DNA segment reversed end-to-end in-site, which generally involves site-specific recombination (Grindley et al., 2006). Inversion of a DNA sequence in the regulatory region of genes is a common way to ensure strict ON/OFF expression. Phase variation of Type 1 pili in *E. coli* is a paradigm for pretranscriptional regulation by DNA inversion (Bryan et al., 2006). As the major pilin, FimA is an important protein involved in adhesion and its production shows a shift towards bacterial colonization of surfaces. The gene encoding FimA is expressed from a promoter region that can undergo inversion of the DNA region upon the activity of recombinases FimB and FimE. FimE only performs an ON-to-OFF switch, whereas FimB can mediate switching in both directions. However, FimB is two orders of magnitude less effective than FimE (Hasman et al., 2000). While FimB is more active between 37°C and 40°C, FimE is progressively inhibited as the temperature increases (Henderson et al., 1999). Close by the promoter region, leucine regulation factor (Lrp) can specifically bind and work in concert with integration host factor (IHF) sites flanking the invertible region to bend the DNA sequence, thus promoting recombination events (Blomfield et al., 1997). Similar regulation also occurs for Type 1 pili expression in some strains of *Shigella flexneri*, *Shigella boydii*, and *Shigella dysenteriae* (Snellings et al., 1997). This regulatory mechanism involving DNA inversion can be even more sophisticated in *S.* Typhimurium as the promoter switch allows the phase variation of two different types of flagella, that is, either constituted of flagellin H1 encoded by *fliC* or flagellin H2 encoded by *fljB* (Bonifield & Hughes, 2003). The single promoter is present in an invertible element containing the *hin* encoding a recombinase and flanked by two homologous sites, *hixL* and *hixR*. Depending on the orientation of the promoter upon DNA inversion, either *fliC* is in the ON phase for transcription and *fljB* in the OFF phase or vice versa (Henderson et al., 1999). Recombination involves the accessory protein Fis which can bind to the invertible region at two locations to stimulate Hin activity, whereas the histone-like HU facilitates bending of the sequence between *hixL* and the Fis sites (Heichman & Johnson, 1990). Fis, HU, and Hin are collectively called the invertasome. Even if the function of this flagellar variation is not completely clear, a role of this serotyping variation in the evasion of the immune system was hypothesized (Ikeda et al., 2001). In *Campylobacter fetus*, the phase variation of the surface layer protein SapA impacts bacterial colonization as well as the immune response and occurs in a RecA-dependent manner (Grogono-Thomas et al., 2000, 2003), although RecA-independent inversion can also arise at a lower frequency (Dworkin & Blaser, 1997; Ray et al., 2000). In *S. aureus*, a small-colony phenotype associated with slow growth (Loss et al., 2019) results from a very large RecA-dependent chromosomal DNA inversion, namely a 1.26 Mb region out of a 2.87 Mb genome, which impacts the expression of over 50 genes linked to metabolism of carbohydrates, vitamins, and ATP together with capsule synthesis (Cui et al., 2012).

4.1.2 | Slipped-strand mispairing

Phase variation by slipped-strand mispairing (SSM) results from variation in the length of short sequence repeats (SSR) that consequently impacts either transcription or translational levels (Bayliss & Palmer, 2012; Henderson et al., 1999). Its occurrence is related to the fidelity of replication and repair of DNA polymerases as well as endonuclease activity which varies between species (Castillo-Lizardo et al., 2014). While DNA regions potentially subject to SSM are theoretically detectable by analyzing genome sequences, in practice, genes prone to such regulation can be easily missed and overlooked (Desvaux et al., 2005; Kapatral et al., 2003).

The occurrence of SSM has been demonstrated in some bacterial foodborne pathogens. In *S. aureus*, SSM phase variation can occur in *icaC* (Brooks & Jefferson, 2014), a gene encoding an integral membrane protein involved in assembly and secretion of the polysaccharide intracellular adhesin (PIA) (Maira-Litrán et al., 2002). This gene possesses 3 to 6 tetranucleoid TTTA repeats in its coding DNA sequence. Only the coding DNA sequence

with three repeats is in the ON phase and gives a full-length protein of 350 amino acids, while other repeats correspond to OFF phases and result in a frameshift with early stop codons leading to a truncated and nonfunctional polypeptide. The change in the number of repeats is reversible and RecA independent. As inactivation of *icaC* results in a PIA-negative phenotype, this phase variation influences adhesion, biofilm formation, and the colonization process, but also fitness and survival (Brooks & Jefferson, 2014) and probably the immune response (Maira-Litrán et al., 2002). In *Campylobacter*, cell motility is subject to phase variation by SSM, but involves different genes depending on the species. In *C. coli*, it results from polythymine in *flhA* encoding flagellin (Park et al., 2000), whereas it results from homopolymeric guanine in *maf1* (motility accessory factor 1) in *C. jejuni* (Karlyshev et al., 2002). In this latter species, phase variation of the lipo-oligosaccharide is rather complex, as it involves SSM in several genes, for example, *wlaN* encoding a β–1,3 galactosyltransferase (Karlyshev et al., 2002) or *cgtA* encoding an N-acetylgalactosaminyltransferase (Guerry et al., 2002).

Genes involved in virulence can also be submitted to SSM phase variation. In *V. cholerae, tcpH* (toxin co-regulated pilus protein H) encoding a transcriptional regulator appears to be subject to SSM with a base shift frequency of 10^{-4} that can regulate the cycle of virulence by repressing surface protein expression (Carroll et al., 1997). An SSM identified in *L. monocytogenes* was shown to regulate phase-variable expression of hemolytic activity and virulence in mice by reversibly inactivating *prfA* (Lindbäck et al., 2011). Moreover, SSM occurs for the expression of *inlA* (internalin A) due to the polyadenine homopolymeric tract (Manuel et al., 2015; Orsi et al., 2010), as well as *flaR* involved in flagellar motility (Orsi et al., 2008, 2010).

4.1.3 | DNA insertion/excision: Involvement of insertion sequences

Insertion or excision of DNA sequences can also result in phase variation. In *S. aureus*, the insertion sequence element IS256 in *icaC* and *sarA* (staphylococcal accessory regulator) impacts biofilm formation (Kiem et al., 2004). First deciphered in *S. epidermidis*, this phase variation associated with colony morphology and PIA production results from the insertion of IS256 at different sites within the *ica* operon, preferentially in *icaC* (Ziebuhr et al., 1997, 1999), but also in genes involved in transcriptional regulation, that is, *rsbU* or *sarA* (Conlon et al., 2004). While occurring at a low frequency (less than 10^{-8}), reversion mostly results from IS256 excision and the formation of an episome (Conlon et al., 2004). In *S. aureus*, the σ^{B} transcription factor appears to negatively regulate the IS256 activity, which influences the switch to the biofilm-negative phenotype (Valle et al., 2007). A potential link with RsbU, a positive σ^{B} regulator, was suggested but requires further investigation to be demonstrated. In *V. cholerae*, the induction and excision of a prophage in the proximity of genes involved in purine (*pur*) and capsule (*cap*) biosynthesis lead to phase variation of the capsule biosynthesis and a switch from opaque to translucent colonies (Smirnova et al., 1996).

4.1.4 | DNA methylation: Bacterial epigenetic regulation

Phase variation by DNA methylation implies methylation of some nucleotides that will in turn impact the fixation of transcriptional factors on the DNA strand (Blow et al., 2016). While all cells employ C^5-cytosine methylation and some bacteria share with some eukaryotes the ability to use N^6-adenine methylation, only bacteria have been shown to use N^4-cytosine methylation (Sánchez-Romero et al., 2015).

The expression of Ag43 (antigen 43) in *E. coli* can be considered as a paradigm of phase variation by DNA methylation in bacteria (Henderson et al., 1999). Ag43 is a well-described cell-surface autotransporter belonging to the Type V, subtype *a*, secretion system (T5aSS) involved in autoaggregation of bacterial cells in a self-recognition-mediated mechanism (Ageorges et al., 2019; Henderson et al., 2004; van der Woude & Henderson, 2008). Phase variation of Ag43 is the result of competitive binding between the Dam methyltransferase and the oxidative stress response (OxyR) transcriptional regulator (Owen et al., 1996). The Dam is not part of the R-M system and specifically targets GATC sites for N^6-adenine methylation (Barras & Marinus, 1989; Collier, 2009; Løbner-Olesen et al., 2005). In the promoter region of *agn43*, three GATC sites prevent binding of the OxyR repressor when methylated by Dam and transcription can occur (ON phase). In the OFF phase, methylation by Dam does not occur and OxyR binds to the GATC sites, thus repressing transcription (OFF phase) (Henderson & Owen, 1999; Owen et al., 1996). Even if OxyR is involved in the oxidative stress response (Chiang & Schellhorn, 2012; Storz et al., 1990), there is no link between the phase variation of Ag43 and the redox-sensing function of OxyR, which regulates *agn43* expression in both reduced and oxidized forms (van der Woude & Henderson, 2008). Hemimethylated GATC sites can still be bound by OxyR, but with a lower affinity (Haagmans & Van Der Woude, 2000). While it is considered not to be required for transcriptional activation of *agn43* (Correnti et al., 2002), SeqA (sequestration protein

A) can also bind specifically to hemimethylated GATC and prevents OxyR from interacting with the unmethylated strand of GATC.

Phase variation involving dynamic Dam/OxyR regulation was also demonstrated in *S. enterica* where it impacts the production of the LPS O-antigen by regulating the *opvAB* locus controlling the length of the LPS (Cota et al., 2012). Besides modulating the virulence level, it allows the cell to elude bacteriophage infection (Cota et al., 2012).

As a general trend and unlike eukaryotes, N^6-adenine methylation is considered as the main signal for epigenetic regulation in bacteria, as it was shown to influence virulence of several foodborne pathogens, namely *E. coli* O157:H7 (Campellone et al., 2007), *S. enterica* (Giacomodonato et al., 2009), *C. jejuni* (Kim et al., 2008), and *V. cholerae* (Julio et al., 2001). C^5-cytosine methylation by DNA cytosine methyltransferase (Dcm), recognizing CC(A/T)GG sites, was shown to regulate the expression of the stress response sigma factor RpoS and many of its targets (Kahramanoglou et al., 2012).

In *C. jejuni*, a single phase-variable gene (*cj0031*) that encodes a methyltransferase and an endonuclease of a Type IIG R-M system influences the gene expression pattern (Anjum et al., 2016). The R-M is a system of endonucleases that target specific short sequences (4–6 bp often palindromic), associated with foreign DNA and is an effective defense against bacteriophages. Actually, and as a general trend, such a regulon involving phase variable DNA methyltransferases of the R-M system is called a phasevarion (Bickle & Kruger, 1993). It generally leads to genome-wide modification of gene expression (Atack et al., 2018). Out of the four known R-M systems, only three can lead to phasevarion, namely Types 1, 2, and 3 R-M. DNA methyltransferases of Type 1 R-M can further be under the control of DNA inversion mechanisms as originally deciphered in *Bacteroides fragilis* with *hsdS*, which is partially reshuffled through recombination of inverted repeat sequences (De Ste Croix et al., 2017). Such DNA inversion appears widespread and is found in multiple pathogens, for example, *L. monocytogenes* (Atack et al., 2018; De Ste Croix et al., 2017). DNA methyltransferases of Type 3 R-M are the most represented cases of phasevarion with an estimated 20% of their genes containing an SSR sequence (Atack et al., 2018).

4.2 | Transcriptional regulations

In all living cells, stochasticity is inherent to gene expression due to the binding and dissociation of various factors in the course of transcription (Bury-Moné & Sclavi, 2017). At a transcriptional level per se, several mechanisms can induce cellular heterogeneity, resulting in phenotypic heterogeneity, namely the transcriptional burst, transcription factor abundance, and regulatory network architecture.

4.2.1 | Transcriptional burst

In *E. coli*, transcriptional bursts were reported over relatively short periods of high activity following long periods of inactive transcription (Golding et al., 2005). Such transcriptional bursts would result from the combination of four parameters, (i) binding and retention of the sigma factor at the promoter, (ii) interactions between sigma factor, RNA polymerase and DNA, (iii) changes in DNA structural conformation modulating the access of RNA polymerase to the promoter, and (iv) the transcription initiation of RNA polymerase inducing pauses in mRNA elongation (Mitarai et al., 2008; Wang et al., 2019). Transcriptional bursts were demonstrated to contribute to cellular heterogeneity (So et al., 2011). This mechanism is considered as a universal feature in bacteria and independent of gene-specific promoter architecture or regulation (Sanchez et al., 2013; So et al., 2011). Nonetheless, some studies suggest that some promoters display a specific level of transcriptional burst since it could be varied by changing repressor-binding affinity or repressor concentration (Sanchez et al., 2013; Sepúlveda et al., 2016). As shown in model *E. coli*, DNA topology can be modulated by gyrase and topoisomerases (Drlica, 1992), which can affect transcription regulation but also induce cellular heterogeneity (Mitarai et al., 2008). An increase in positive DNA supercoiling was shown to significantly decrease the transcription initiation, which could be modulated by gyrase and thus modulates the switch between ON and OFF transcription states from one cell to another (Chong et al., 2014). Besides transcriptional bursts, protein bursts can occur at the level of translation with interplay at the transcriptional level (see below). As a generic mechanism, a transcriptional burst can potentially occur in any bacteria, although its contribution to cellular heterogeneity in bacterial foodborne pathogens has not yet been questioned, but undoubtedly requires further attention.

4.2.2 | Transcription factor abundance

If noise in gene expression results from stochasticity, it is well known that transcriptional processes play an important role in noise level and duration. Among the features contributing to noise, the abundance of proteins involved in transcription plays a substantial role. If molecules such as transcription or sigma factors are at low concentration in a cell, the opportunity for molecules (proteins and DNA) to interact by random collisions is less frequent, and this

contributes to rare events of gene activation and therefore noisy expression (Roberfroid et al., 2016). Recent studies have shown that bacteria capitalize on this feature to regulate the level of transcription variability of some genes in order to improve their fitness in certain situations.

4.2.3 | Regulatory network architecture

The regulatory network controlling transcription has been shown to influence stochasticity in gene expression, and it has been proposed that diverse gene network architectures have evolved in bacteria either to amplify or limit transcriptional variability (Alon, 2007). From the description of synthetic networks and biological networks naturally found in bacteria, several features have been described to modulate transcriptional variability, in particular the length of the regulatory cascade and feedback loops.

The first parameter affecting the degree of expression variability for a gene is the length of the regulatory network controlling its transcription. Measurement of an output signal resulting from engineered cascades with one, two, or three regulatory steps in *E. coli* revealed that longer cascades increase the response sensitivity to input variations and strongly amplify cell–cell variability, particularly in the intermediate region of response (Hooshangi et al., 2005). Such properties are a direct consequence of the propagation of noise along the cascade with each cascade element adding its respective noise (Pedraza & Van Oudenaarden, 2005). A typical example of this trend in nature is the flagellar regulon consisting of more than 30 genes regulated in a highly hierarchical manner and involved in the biogenesis of a flagellum for bacterial swimming and chemotaxis. High levels of cellular heterogeneity have been reported for flagellar genes in several bacterial species including foodborne pathogens such as *S. enterica* (Cummings et al., 2006; Jubelin et al., 2013; Saini et al., 2010).

Negative autoregulation is a common feedback motif in the architecture of bacterial gene circuits and it occurs for example in about half of the repressors in *E. coli* (Shen-Orr et al., 2002). This motif usually reduces expression noise for targeted genes and therefore heterogeneity between isogenic cells (Alon, 2007). In some rare cases, self-repressor circuits have been shown to promote variability under specific conditions (Jiang et al., 2019). In contrast, positive autoregulation of a transcription factor tends to increase the expression noise of target genes (Alon, 2007; Avery, 2006). If autoactivation is weak, the transcription level of target genes presents a higher variability between cells in comparison to the same regulation pattern, but without the feedback loop. In the case of a strong positive autoregulation, expression of target genes often follows a bimodal distribution. When the regulator concentration reaches a certain threshold in a cell, the positive feedback loop amplifies its own transcription and keeps it high for a long time. The expression of downstream genes is then altered and the population bifurcates into two distinct states depending on whether the threshold has been reached in bacterial cells. If the regulator also requires a high cooperativity to bind DNA and induce transcription, this makes the response hypersensitive to changes in regulator concentration at the threshold level and leads to biological systems with bistable outputs and hysteretic properties (Viney & Reece, 2013). These systems are used by bacteria to make decisions with a low probability of reversion in the short term, engaging the cell in long-term processes such as competence, persistence, or sporulation (Balázsi et al., 2011). One such system controls the conversion between lysogenic and lytic cycles of lambdoid prophages and involves the regulatory proteins CI and Cro. CI governs the transcription of the two oppositely oriented *cI* and *cro* genes by binding cooperatively to three adjacent operators present in the intergenic region (Dodd et al., 2004). In the lysogenic state, the steady-state level of CI represses *cro* transcription, while *cI* is expressed to maintain continuous CI production. In conditions where the concentration of CI decreases in a cell, transcription of *cro* takes place and Cro inhibits *cI* expression while activating the expression of genes required to initiate the lytic cycle (Bednarz et al., 2014). This cellular heterogeneity-inducing mechanism is highly implicated in the virulence of the foodborne pathogen enterohemorrhagic *E. coli* (EHEC) since Shiga toxin-encoding genes are located within lambdoid prophages. Expression of *stx* genes is therefore dependent on induction of the phage lytic cycle, and Stx production is typically heterogeneous in bacterial populations (Imamovic et al., 2016).

4.3 | Post-transcriptional regulation

Several regulatory mechanisms can occur after mRNA transcription, including at post-transcriptional, translational, post-translational, translocational, and post-translocational levels (Ageorges et al., 2020). With respect to cellular heterogeneity and at this particular regulation level, several molecular mechanisms are now known that can induce cellular heterogeneity and ultimately phenotypic heterogeneity in bacteria, namely riboswitch, small RNA, translational burst, and asymmetric partitioning. As described here below, most of these regulations have been characterized in a very limited number of bacterial species, such as laboratory *E. coli* strains, and/or have been observed by chance in specific species. Considering their generic nature, their occurrence and contribution to cellular heterogeneity and phenotypic heterogeneity in

bacterial foodborne pathogens are most likely, but require focused investigations.

4.3.1 | Riboswitch

Riboswitch regulation can occur at a post-transcriptional or translational level with ligands either inducing termination hairpin in mRNA and stopping the transcription, or changing the mRNA fold and preventing the binding of the ribosome, respectively (Pavlova et al., 2019; Serganov & Patel, 2007). While at a transcriptional level the riboswitch function is generally described as a two-state secondary structure model with a transcription OFF with a bound ligand and ON when unbound (Barrick & Breaker, 2007; Blouin et al., 2009; Huang et al., 2012), there are only a few examples of involvement in cellular heterogeneity. In *L. lactis*, such riboregulation was shown to give rise to cellular heterogeneity with respect to the import of methionine, an amino acid for which this bacterial species is auxotrophic (Hernandez-Valdes et al., 2020). Under limited methionine conditions, one subpopulation relies on a high-affinity transporter (i.e., high expression of the ABC Met-transporter), whereas the other one imports the methionine via a low-affinity transporter (i.e., the branched-chain amino acid permease BcaP, coupled to low expression of the ABC Met transporter). Remarkably, these two isogenic subpopulations exhibiting clearly distinct phenotypes were apparent at colony level, stable over bacterial culture time and even inherited for several generations. It was demonstrated that this phenotypic heterogeneity resulted from a T-box riboswitch located in the 5′ leader regions of mRNAs in the *met* operon encoding the ABC Met-transporter (Hernandez-Valdes et al., 2020).

4.3.2 | Small RNA

As a post-transcriptional regulatory mechanism, attenuation collaterally affects translation, but additional molecular mechanisms can indirectly control protein synthesis in bacteria. The most widely considered are certainly some noncoding RNAs (ncRNAs), which hybridize with mRNA to block the binding of the ribosome in a mechanism analogous to RNA interference (RNAi) in eukaryotic cells (Fischer, 2015), namely the antisense RNA, including the small RNA (sRNA) (Simons, 1988). The role of sRNAs in cellular heterogeneity has been described for more than a decade, especially regarding phenotypic heterogeneity of bacterial cells in biofilm (Coenye, 2010). In *S. epidermidis*, the sRNA RsaE (RNA from *S. aureus*) was shown to regulate the release of extracellular DNA (eDNA) and production of PIA but was heterogeneously expressed, thus inducing cellular heterogeneity in biofilm (Schoenfelder et al., 2019). Recently, it was shown that food preservatives and food residues in the food chain can change the sRNA expression patterns in *Salmonella enterica*, thus impacting its biofilm formation abilities (Lamas, Paz-Mendez, et al., 2018; Lamas, Regal, et al., 2018). Nineteen novel biofilm-associated sRNA candidates have been identified in *S. enteritidis* exposed to meat-juice stress (meat thawing loss broth [MTLB]) thanks to a transcriptomic analysis (Hu et al., 2020). Four specific deletion mutants were shown to have improved abilities in motility (swimming and swarming), autoaggregation in MTLB as well as adhesion and biofilm formation on abiotic surfaces. This study shows the importance of unravelling a complex regulatory network of biofilm formation in the food industry and food safety context (Hu et al., 2020).

4.3.3 | Translational burst

Considering the rates of transcription of mRNA molecules from the template DNA strand and the rate of translation of protein from each mRNA molecule as well as the rates of degradation of mRNA and proteins, a so-called protein burst can occur (Ozbudak et al., 2002; Thattai & Van Oudenaarden, 2001).

In *B. subtilis*, using the green fluorescent protein-encoding gene controlled by an inducible promoter, the variability in protein expression level was demonstrated to depend on the underlying biochemical rates of transcription and translation (Ozbudak et al., 2002). While high transcription rates associated with low translation rates result in slight fluctuations in protein expression, low transcription rates coupled to high translation rates instead induce large fluctuations in the amount of protein expressed (Thattai & Van Oudenaarden, 2001). Besides random intrinsic fluctuations, which are inherent to transcription and translation rates, stochastic gene expression can result from extrinsic fluctuations due to variations in the number of enzymes or ribosomes, for instance (Rosenfeld et al., 2005). Modeling flux balance analysis to a population of *E. coli* cells, the stochasticity of protein expression was shown to induce metabolic heterogeneity and a few genes appeared sufficient to capture most variability of the entire cell population (Labhsetwar et al., 2013).

While tryptophan was the predominant amino acid that was incorporated in place of the UGA codon, stop-codon readthrough was demonstrated to be heterogeneous in the cell population. Stop-codon readthrough resulted from fluctuations in the concentrations of translational components, leading to phenotypic heterogeneity of genetically identical populations (Fan et al., 2017).

4.3.4 | Asymmetric partitioning

While DNA is universally known as the heredity factor enabling information to pass from parent to progeny cells at each cell division following replication of this genetic material, cell division also involves the partitioning of all other biochemical molecules (Huh & Paulsson, 2011). However, the partitioning of cell proteins including enzymes occurs randomly and can be asymmetric, which would then cause some cellular heterogeneity among isogenic daughter cells (Huh & Paulsson, 2011; Lloyd-Price et al., 2014). Such asymmetric partitioning can affect the distribution and localization of bacterial–cell surface proteins (King & Roberts, 2016).

In *E. coli*, a strong uneven partitioning was observed for the multidrug efflux pump AcrAB-TolC (Bergmiller et al., 2017) a tripartite exporter of toxic compounds (Kobylka et al., 2020), which was found to be accumulated at old cell poles of mother cells over cell divisions (Bergmiller et al., 2017). This cellular heterogeneity leads to a significant phenotypic difference in drugs. Importantly, the resulting cellular heterogeneity was not transient but was predicted with long lived and highly heterogeneous phenotypes of significance in the emergence of multidrug resistance bacteria (Bergmiller et al., 2017).

5 | CONSEQUENCES FOR FOOD SAFETY AND RISK ASSESSMENT

The mathematical modeling of bacterial growth in foods provides fundamental information to ensure the safety of food products. Predictive microbiology models are used to calculate shelf-life, establish time-temperature inactivation treatment, or formulate the product to prevent the growth of pathogens (Tenenhaus-Aziza & Ellouze, 2015). Moreover, they are fundamental tools in assessing exposure and quantitative risk (Fritsch et al., 2018). The following sections cover first how the physiological and cellular heterogeneities of bacterial pathogens are considered in modelling and then how they are used in risk assessment.

5.1 | Considering single-cell behavior in modeling

5.1.1 | The challenge of data acquisition about pathogen behavior in foods at the single-cell level

The growth, survival, and inactivation of pathogens in food directly depend on food properties. The simplest way to evaluate the behavior of pathogens in foods would be to follow the kinetics of the pathogens in naturally contaminated foods. However, as the prevalence of most pathogens in foods is low, it is difficult to rely on ageing tests. Pathogen behavior can be more efficiently assessed through the use of challenge tests. Challenge tests are microbiological laboratory-controlled studies that consist in monitoring the evolution of a microbial population intentionally added to food. In a challenge test, the volume of the inoculum should not exceed 1% of the volume of the test unit and the inoculation level should be set at approximately 100 CFU/g (Álvarez-Ordóñez et al., 2015). While this level guarantees being above the limit of quantification for most microbiological methods, it deviates from the real level of pathogen contamination in foods, which usually occurs with very few cells (Teunis et al., 2010). Moreover, the preparation of the inoculated cells does not reproduce the real physiological state of the cells contaminating the food products, which is largely unknown (Guillier & Augustin, 2006). The challenge is thus to consider pathogen behavior at the single-cell level so as to consider the heterogeneity of both the population and the food environment.

To characterize individual pathogen growth in foods, two factors should be evaluated, individual cell growth probability and lag time. The growth rate of cells following the lag phase is considered to be similar to the growth rate measured for populations (Akkermans & Van Impe, 2021). Experiment at single-cell levels showed that between cell variability of generation times can be observed for exponentially growing cells, but the variation is limited, and the mean generation observed for these cells is consistent this the growth rate observed at population level (Kutalik et al., 2005). The inoculation of food with low levels of cells leads to a paradigm shift in growth monitoring. A set of enumerations carried out on a dozen samples initially contaminated with a few cells usually results in "noisy" kinetics that can be explained by the individual cell behaviors (Gnanou Besse et al., 2006). Figure 4 illustrates the impact of using enumerations of samples inoculated with single cells to construct a growth kinetics. First, the growth was simulated in eight samples (Figure 4a). Then, each sample was used to carry out an enumeration along the kinetics (Figure 4b). Using common exponential growth models appears to be not relevant for such situations in which samples are inoculated with few cells presenting large differences in lag times. To evaluate the lag times of individual cells, the number of samples at each sampling time should be increased to estimate the variability of levels along the growth curve (Baranyi et al., 2009; D'Arrigo et al., 2006). If the growth rate is known, the individual lag times of cells can then be inferred from the variability of contamination levels for a given time (Figure 5a) (D'Arrigo et al., 2006) or from the variability of detection times for a given level (Figure 5b). These methods are called the

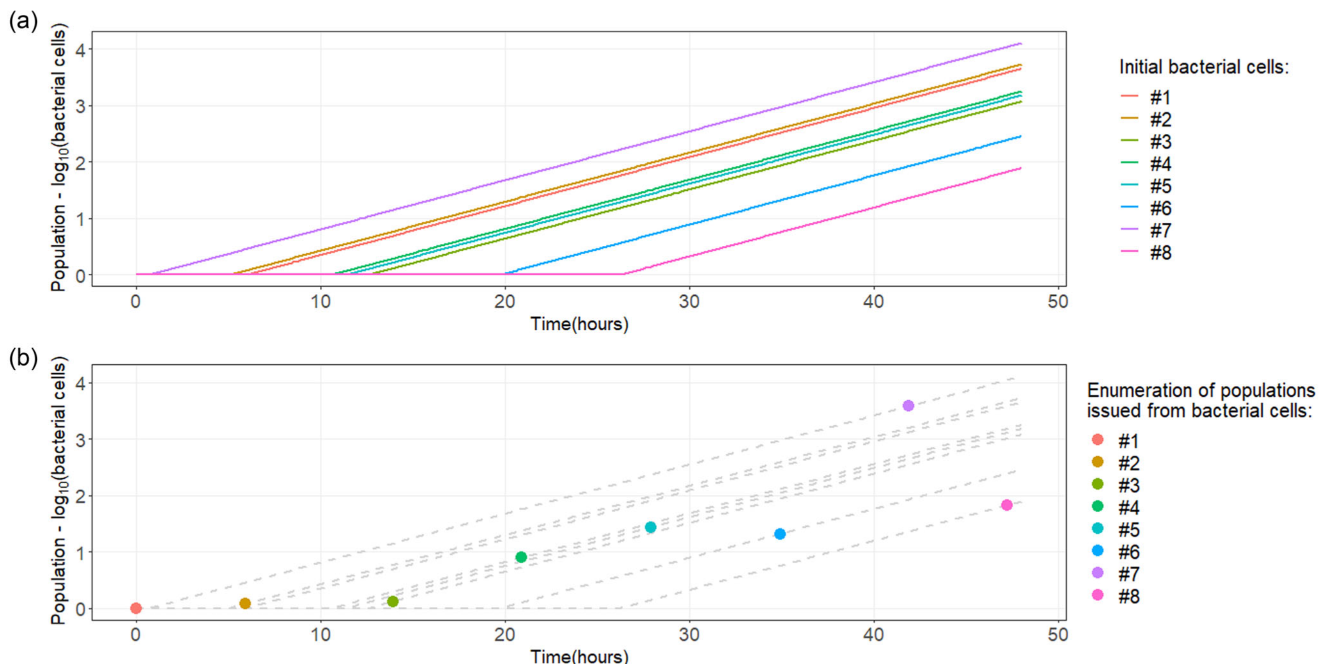

FIGURE 4 (a) Growth kinetics in environments inoculated with a single bacterial cell that present an exponential growth rate of 0.2 h–1 and a variable individual lag time (values were randomly drawn from exponential distribution of mean 10 h). (b) Enumerations carried in each of the environments at different sampling times

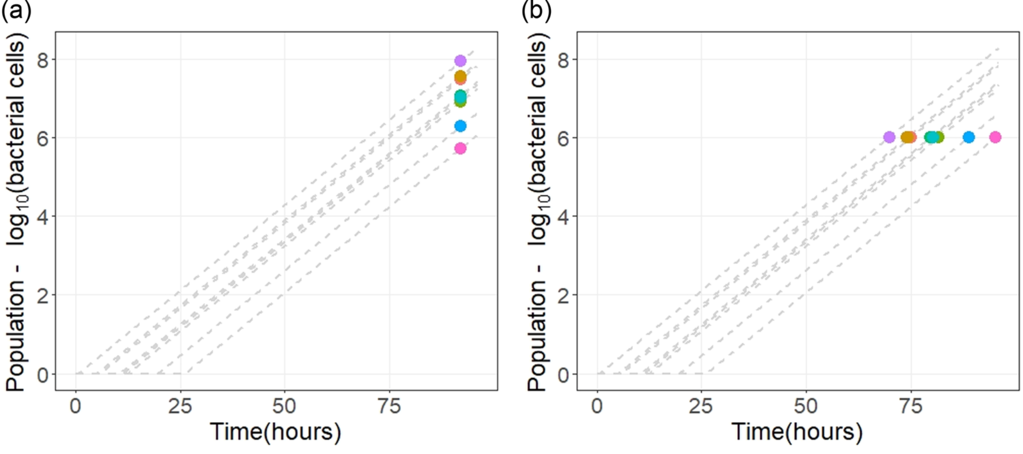

FIGURE 5 Schematic representation of the methods to estimate individual lag times through enumerations. (a) In the "vertical" method, a large number of samples are enumerated at one time. (b) In the "detection time" method, times to reach a given threshold are used. In both cases, the estimation of individual lag times relies on is based on the knowledge of the growth rate and the control of contamination by a single cell of the inoculated environments

"vertical" method and the "detection time" method, respectively. The estimation of individual lag times is only possible because the growth rate variability has been demonstrated to be far less important that individual lag time variability (Guillier et al., 2005).

The determination of individual cell growth probability is even more challenging in foods. When using a large inoculum, the presence of nongrowing cells results in an additional delay of population growth. The measured lag of the inoculated population is the sum of the lag of the growing cells and the "pseudo-lag" of the nongrowing cells (Aguirre & Koutsoumanis, 2016). Characterizing cellular growth probability in foods involves the inoculation of numerous samples with a few cells per sample. Samples are inoculated with a low concentration of cells and enumerated after an incubation period. The incubation period

should be long enough to distinguish between samples that contain cells that are still in the lag phase and those that contain cells that will never initiate growth (Augustin et al., 2015).

5.1.2 | Data acquisition in culture medium for the construction of growth models at the single-cell level

Considering the above-mentioned difficulties in measuring single-cell behavior in food, most models have been established on broth media. The use of food media is mainly considered for validating the models in real food matrices. In this section, we present the experimental methods used to work with single cells as well as the direct and indirect methods used to acquire data on individual cell lag times and growth probabilities. The first experimental challenge is to obtain single cells. Two methods are commonly applied to isolate single cells in liquid media in the context of acquiring lag times and growth probabilities. In the first method, an inoculum is serially diluted in a microplate in order to maximize the chances of obtaining single cells in the last dilutions (Augustin & Czarnecka-Kwasiborski, 2012). In the second method, a solution is diluted to a single very low concentration so that when it is dispensed onto a microplate, the chance of isolating single cells is maximized (Akkermans & van Impe, 2021). In both cases, some of the samples will inevitably contain more than one cell. Working with low inocula increases the probability of having no cells at all and therefore decreases the yield of experimental data. In this context, a mathematical optimization was proposed to determine the concentration of the stock suspension that best estimates the probability of growth while maximizing the experimental yield (Buss da Silva et al., 2019). Inoculation in solid media is also a challenge. Working with too high cell densities can create interference between colonies (Fritsch et al, 2021; Guillier et al., 2006). Here again, the choice of inoculum is a compromise between the efficiency of the experiments and the disturbances created by too high densities. For the determination of single-cell lag time in culture medium, different direct or indirect methods are available. One commonly relies on the measurement of the optical density in broth media over time (Smelt et al., 2002). This technique requires that one cell develops a high number of generations to reach the detection level for turbidity measurement. Other indirect methods to estimate the individual lag time of bacterial growth rely on the detection time of colony appearance on solid media (Guillier et al., 2006; Levin-Reisman et al., 2014). The determination of lag time based on these methods requires (i) fine determination of the concentration of cells at the detection limit (N_d), (ii) insurance that the probability of having a single cell is high, and (iii) precise knowledge of the growth rate (μ_{max}) (Akkermans & Van Impe, 2021; Baranyi et al., 2009). If these conditions are met, the estimation of individual lag times can be derived from the following equation:

$$lag_i = T_d - \frac{Ln\,(N_d) - Ln\,(N_0)}{\mu_{max}}$$

In addition, some direct methods have been proposed based on time-lapse microscopy (Elfwing et al., 2004; Koutsoumanis & Lianou, 2013). A technique based on the monitoring of the consecutive divisions of a single cell attached to a solid surface in a flow cell chamber was developed (Elfwing et al., 2004). A simpler system using contrast phase microscopy was also described (Koutsoumanis & Lianou, 2013). This method allows direct follow-up from one cell to a microcolony in real time. The single-cell lag time can be thoroughly derived by measuring the first division time. A method was recently developed to derive individual lag time variability from the distribution of microcolony size, similarly to the vertical distribution method described in Figure 4 (Fritsch et al., 2021). The individual cell lag time lag_i was determined using the time of observation T_{obs}, the cell number per micro colony (ln(x)) and the growth rate (μ_{max}).

$$lag_i = T_{obs} - \frac{\ln\,(N_{Tobs})}{\mu_{max}}$$

Besides the measurement of individual lag times, single-cell growth probability can also be measured with direct or indirect methods. The single-cell growth probability of *L. monocytogenes* was studied under different conditions (i.e., temperature, pH and water activity) in broth media (Augustin & Czarnecka-Kwasiborski, 2012). The probability for a single cell to initiate growth, p, in nonoptimal conditions, is equal to

$$p = C_i / C_{ref}$$

where C_i is the cell concentration in tested condition i and C_{ref} is the concentration in the optimal condition as reference (Dupont & Augustin, 2009). Generally, concentrations are estimated using the most probable number approach. The precision in the estimated probability of growth is greatly influenced by the number of wells used to estimate the concentration and by the proportion of positive wells (Buss da Silva et al., 2019).

Direct microscopy methods can also be used to determine the proportion of non-growing cells after a long enough incubation time (Fritsch et al., 2021; Koutsoumanis & Lianou, 2013). The principle relies on observing more

than one hundred cells immobilized on fine agar slides. The growth probability is simply determined by dividing the number of cells that have not generated a colony by the number of cells that are present at the beginning of the experiment. The density of cells on the agar slides should not be too high so as to prevent the possibility that single nongrowing cells are submerged by colonies initiated by other cells.

5.1.3 | Models for individual lag time and individual growth probability

The determination of individual lag times for bacterial cells in a particular physiological state (k) can be used to predict the individual lag times of these cells in other conditions as long as the growth rate is known in the new conditions (Guillier & Augustin, 2006).

$$\mu_{max,A} \cdot lag_{i,A} = \mu_{max,B} \cdot lag_{i,B} = k$$

As there are many models for predicting the growth rate according to environmental conditions (temperature, pH, water activity, etc.), individual lag times are simply predicted from the distribution of k values. Many statistical distributions have been used to describe the distribution of k or individual lag time values. The distributions generally correspond to right skewed distributions such as exponential, lognormal, gamma, Weibull, and extreme-value distributions (Guillier et al., 2005). So far, very few papers have considered modelling cell growth probability according to environmental conditions (Aguirre & Koutsoumanis, 2016; Augustin & Czarnecka-Kwasiborski, 2012). The decision support tool Sym'Previus is a software which has integrated a single-cell growth probability module (Tenenhaus-Aziza & Ellouze, 2015). The development of such models would require the development of methods for high throughput phenotypic data generation at the single-cell level (Fritsch et al., 2021).

5.2 | Risk assessment and behavior of single cells

In food safety, risk assessment is achieved by evaluating the probability of a hazard occurrence together with the severity of adverse health effects resulting from the hazard exposure. It can help to implement appropriate management measures for food safety management. Risk assessment should start with the identification and description of pathogenic microorganisms in food, followed by an exposure assessment, which is the evaluation of the number of pathogens ingested by the consumer, and then a hazard characterization, which is the characterization of the dose–response relationship to estimate the probability and severity of adverse effects based on the ingested dose. Finally, risk is estimated and characterized from exposure and the dose–response relationship (Augustin et al., 2011).

Exposure assessment consists of the evaluation of the probability that the consumer will ingest microbiological hazards. This is started by assessing the probability of occurrence of the microorganism in food and its quantity and then by assessing the quantity of food ingested by the consumer. Predictive microbiology provides tools to estimate the level of the pathogen in food. It can also describe the evolution of the concentration of the pathogen along the food chain by modeling its growth and its inactivation during the implementation of some control measures. Quantitative risk assessment may include different sources of variability. Indeed, it generally presents the geographical, temporal and/or individual heterogeneity of risk in a given population. In addition, in food safety risk assessment, it is important to consider variability in individual cell behavior, which is likely to impact the microbial response within the food.

An individual-based modeling approach was developed that considers the characteristics of the microenvironment of individual bacterial cells to study the variability in their growth in smear soft cheese (Ferrier et al., 2013) or smoked salmon (Augustin et al., 2015). The behavior of each cell present on the food products was considered. The combination of measurement of microscale pH variability by microelectrodes with individual-based modelling was shown to be effective in predicting the behavior of *L. monocytogenes* (Augustin et al., 2015; Ferrier et al., 2013). The single-cell growth probabilities, individual lag times, and growth rates were predicted according to microenvironmental characteristics (pH and water activity) encountered by the cells on the products. The authors compared the usefulness of this approach versus the population-based approach. In the population approach, the cellular growth probability was not considered, the lag time was set at the same values for all cells, and the microlocal variability was ignored (the mean value was considered). Then, they noted that in the context of microbial risk assessment, the type of approach had a large impact on exposure estimates. The population-based approach was found to overestimate exposure compared to individual-based modeling. The main reason for the incapacity of the population-based approach to predict observed experimental behavior was associated with the absence of consideration of the probability growth of single cells.

The European Food Safety Authority (EFSA) has underlined the importance of considering single-cell behavior (EFSA, 2018) and conducted an in-depth study on a model

which was used to identify factors in the food chain as potential drivers for *L. monocytogenes* contamination of ready-to-eat foods and listeriosis. Factors were related to the host, in particular the population size of elderly and/or susceptible people with underlying conditions, to the food, notably *L. monocytogenes* prevalence and concentration in ready-to-eat food at retail, storage conditions after retail and mode of consumption, and/or to bacterial virulence. One of its outcomes is that deterministic models which provide knowledge only of the mean population are not sufficient for management decisions on microbial safety risk. EFSA insists on the fact that contamination generally occurs at very low level and recommends the development of models based on stochastic approaches which describe the variability of single-cell behavior in order to obtain a realistic risk estimation. Several studies have included the individual cell models for lag in the exposure assessment step for *L. monocytogenes* in ready-to-eat foods (Duret et al., 2014; Pouillot & Lubran, 2011). The inclusion of cellular behavior is a real advance in the understanding of adaptive mechanisms in food and necessarily reduces uncertainty in risk assessments. However, the above-mentioned studies that incorporated cellular behavior did not see their estimates change dramatically. Sensitivity analysis has shown in these studies that considering individual cell behavior was found to have a smaller impact on the output than other sources of variability of the model (especially the variability of time and temperature of storage of the products or the variability of concentrations reached in food during the stationary phase).

6 | CONCLUSION AND PERSPECTIVES

The physiology and behavior of pathogens in a food matrix are extremely complex due to various factors as genetic features at the strain level, physiological characteristics due to incredibly changing signals in the near bacterial surrounding environment, and cellular process adjustment at the single-cell level. These three levels of heterogeneity result in huge variability which is very difficult to predict and include in risk assessment modelling. The risk-based approach to managing food safety has prompted food business operators to adapt methodologies to guarantee the quality and safety of their products (FAO & WHO, 2006). As this complexity is very hard to consider at the same time, many studies examine bacterial growth and stress response in simplified matrices with defined varying factors. Further studies are needed to include complexity in risk assessment, notably by taking advantage of technological advances. For example, the development of microelectrodes together with real-time tracking technologies could play an important role in making precise and dynamic maps of food gradients such as pH, osmotic pressure, redox potential, or temperature. Next-generation sequencing methods in food science have essentially been used to identify foodborne contamination events in a traceability context or to support food microbiological sampling programs in a quality assurance context. Yet, early applications of next-generation sequencing emphasized its great potential to complete characterization of well known, emerging, or re-emerging foodborne pathogens in the food supply. Metagenomics, transcriptomics, or metabolomics could also be useful tools and are increasingly accessible to research labs for acquiring information on the food microbial consortiums and the dynamics of their metabolic activities (Lamas et al., 2019). In the end, big data manipulation systems become easier and help in managing acquisition and management of physicochemical and physiological data at a single-cell level.

Finally, it is important to keep in mind that food microbiological safety is a continuous daily challenge in each production system. Despite efforts to consider variability in foodborne pathogen behavior, the emergence of new bacterial adaptations and microbiological risks can occur. In this context, the main objective of health authorities is to ensure that the management systems are sufficiently responsive to quickly contain an emergent epidemic when it occurs.

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AUTHOR CONTRIBUTIONS

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CONFLICT OF INTEREST

None.

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