Etude de l'organisation spatiale et de l'hétérogénéité phénotypique d'Escherichia coli O157 : H7 et Listeria monocytogenes dans des matrices gélifiées modèles

Cédric Saint Martin

université PARIS-SACLAY

Etude de l'organisation spatiale et de l'hétérogénéité phénotypique d'*Escherichia coli* O157:H7 et *Listeria monocytogenes* dans des matrices gélifiées modèles

Study of the spatial organisation and phenotypic heterogeneity of Escherichia coli O157:H7 and Listeria monocytogenes in model gelified matrices

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Cédric SAINT MARTIN

Composition du Jury

Filipa Lopes Professeure, CentraleSupélec (Université Paris-Saclay)	Président
Frédéric BORGES Maître de conférences, HDR, Université de Lorraine	Rapporteur & Examinateur
Sandrine GUILLOU Ingénieure de recherche, HDR, ONIRIS Nantes	Rapporteur & Examinatrice
Bianca SCLAVI Directrice de recherche, CNRS (Sorbonne Université)	Examinatrice
Romain BRIANDET Directeur de recherche, INRAE (Université Paris-Saclay)	Directeur de thèse
Mickaël DESVAUX Directeur de recherche, INRAE (Université Clermont-Auvergne)	Co-Directeur de thèse

THESE DE DOCTORAT

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Titre : Etude de l'organisation spatiale et de l'hétérogénéité phénotypique *d'Escherichia coli* O157:H7 et *Listeria monocytogenes* dans des matrices gélifiées modèles

Mots clés : matrices alimentaire, hydrogels, pathogènes alimentaire, organisation spatiale, hétérogénéité phénotypique, miscroscopie confocale à balayage laser

Résumé : Les aliments sont les principaux vecteurs de l'ingestion de micro-organismes pathogènes chez l'humain et l'animal. Ces milieux structurés sont hétérogènes et la diversité des micro-environnements favorise l'émergence de sous-populations bactériennes. Dans les matrices alimentaires, les micro-organismes peuvent être isolés sous forme de cellules uniques ou regroupées sous forme de biofilms de surface ou de microcolonies incluses. L'activité locale des micro-organismes génère des gradients de nutriments et de métabolites qui renforcent l'hétérogénéité des biotopes et la diversité phénotypique des microbiotes associés. L'objectif de cette thèse est d'étudier le comportement de deux bactéries pathogènes (*Escherichia coli* O157:H7 et *Listeria monocytogenes*) dans un milieu structuré modèle, une matrice gélifiée transparente de composition modulable. Le couplage de cette matrice hydrogel synthétique à la microscopie confocale laser à balayage (MCLB) nous a permis de mettre en évidence l'influence de l'inoculum initial, la texture, la concentration en NaCl et le pH sur la morphodynamique de ces systèmes bactériens, à l'échelle de la cellule unique. Par ailleurs, l'utilisation d'une fusion transcriptionnelle fluorescente a permis de mettre en évidence une expression localisée en périphérie des microcolonies d'un gène de réponse au stress acide de *E. coli* O157:H7, *gadB*, et ce spécifiquement dans les matrices acides. L'expression forte et spatialisée de ce gène est corrélée avec une plus grande capacité de survie de la population à un stress acide mimant la digestion gastrique. Ces travaux soulignent l'importance des conditions de croissance sur le comportement des bactéries pathogènes dans les matrices alimentaires et suggèrent de nouveaux leviers pour limiter les infections liées à la consommation d'aliments contaminés.

Title : Study of the spatial organisation and phenotypic heterogeneity of *Escherichia coli* O157:H7 and *Listeria monocytogenes* in model gelified matrices

Keywords : Food matrices, hydrogels, foodborne pathogens, spatial organisation, phenotypic heterogeneity, confocal laser scanning microscopy

Abstract : Food is the main vector for the ingestion of pathogenic microorganisms in humans and animals. These structured environments are heterogeneous and the diversity of microenvironments favors the emergence of bacterial subpopulations. In food matrices, microorganisms can be isolated as single cells or aggregated as surface biofilms or embedded microcolonies. The local activity of microorganisms generates nutrients and metabolites gradients that enhance this biotope heterogeneity and the phenotypic diversity of associated microbiota. The objective of this thesis was to study the behaviour of two pathogenic bacteria (*Escherichia coli* O157:H7 and *Listeria monocytogenes*) in a model medium, a transparent gel matrix of adjustable composition. The coupling of a synthetic hydrogel model to confocal laser scanning microscopy (CLSM) allowed us highlighting the influence of the initial inoculum, the texture, the NaCl concentration and the pH on the morphodynamics of these bacterial systems, at the single cell scale. Furthermore, the use of a fluorescent transcriptional fusion has allowed us to demonstrate a localised expression of an acid stress response gene of *E. coli* O157:H7, *gadB*, at the periphery of microcolonies, and specifically in acidic matrices. The strong and spatialised expression of this gene correlates with a greater ability of the population to survive an acid stress mimicking gastric digestion. This work demonstrates for the first time the possibility of inducing and observing pathogenic bacterial cell type diversification and emergent properties in food matrix models. This work highlights the importance of the growth conditions on the behaviour pathogenic bacteria in food matrices and reveals new levers to mitigate infections resulting from the consumption of contaminated foodstuff.

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Acronymes et abréviations

A

-AIEC : *Escherichia coli* Adhérente Invasive

-ANSES : Agence Nationale de Sécurité Sanitaire, de l'alimentation, de l'environnement, et du travail

-ANR : Agence nationale de la recherche

-AOBS : Séparateur de faisceau acoustique "Acousto-Optical Beam Splitter"

-Aw : activité de l'eau, part libre de H_2O pour les réactions "Water activity"

C

-CC : Complexe Clonal

-CDC : Centre de contrôle et prévention des maladies "Center for disease control and prevention" (USA)

-CLSM/MCLB : Confocal Laser Scanning Microscopy (Microscopie Confocale à Balayage Laser)

-CNRS : Centre national de la recherche scientifique

-SNC : Système nerveux central

D

-DAEC : *Escherichia coli* Diffusive Adhérente

-DAM : méthylase de l'adénine sur l'ADN "DNA Adenine methylase"

E

-EAEC : *Escherichia coli* Entéroaggrégative

-*E. coli* : *Escherichia coli*

-EFSA : Autorité européenne de sécurité des aliments "European Food Safety Agency"

-EHEC : *Escherichia coli* Entérohémorragique

-EIEC : *Escherichia coli* Entéroinvasive

-EPEC : *Escherichia coli* Entéropathogénique

-EPS : Exopolymère bactérien "Extracellular polymeric substance"

-ETEC : *Escherichia coli* Entérotoxigénique

F

-FACS : Trie cellulaire activée par fluorescence "Fluorescence activated cell sorting"

-FAST : Activation de fluorescence par décalage d'absorption de sonde "Fluorescence activating and absorption-shifting tag"

-FISH : Fluorescence par hybridation in situ "Fluorescence in situ hybridisation"

-FRET : Transfert d'énergie par résonance de fluorescence "Fluorescence resonance energy transfer"

G

-GABA : acide γ-aminobutyrique "γ aminobutyric acid"

I

-INRAE : Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement

L

-*L. monocytogenes* : *Listeria monocytogenes*

-LMPA : Agarose à faible point de fusion "Low Melting Point Agarose"

M

-MLOC : microfluidic lab-on-chip

N

N&D : Nettoyage et Désinfection

P

-pH : Potentiel hydrogène, mesure de l'acidité/alcalinité du milieu "potential of hydrogen"

Q

-QMRA : Évaluation quantitative du risque microbiologique "Quantitative microbiological risk assessment"

R

-RBS : Site de fixation des ribosomes "Ribosome binding site"

S

-STEC : *Escherichia coli* productrice de Shiga Toxine "Shiga toxin *Escherichia coli*"

U

-UCA : Université Clermont Auvergne

Introduction

Ce doctorat est effectué dans le cadre d'une collaboration entre deux unités, d'une part l'unité MICALIS (équipe B3D, Biofilms et Communautés Spatialement Organisées) associant INRAE, AgroParisTech et l'Université Paris-Saclay, et d'autre part l'UMR MEDIS, une unité sous la tutelle d'INRAE et l'Université Clermont Auvergne (UCA). La thèse est co-dirigée par Romain Briandet (MICALIS) et Mickaël Desvaux (MEDIS) et co-encadrée par Maud Darsonval (MICALIS). Cette thèse s'inscrit dans l'école doctorale ABIES avec la mise en place d'un comité de pilotage interne composé de l'équipe d'encadrement ainsi que de Florence Dubois-Brissonnet (MICALIS) et Sabine Leroy (MEDIS), et d'un comité de thèse impliquant également deux rapporteurs extérieurs, Ivan Matic (CNRS) et Pascal Piveteau (INRAE).

Alors que les aliments sont les principaux vecteurs de l'ingestion de micro-organismes pathogènes chez l'humain et l'animal, très peu d'études décrivent la spatialisation, l'hétérogénéité et le comportement des communautés microbiennes dans ces biotopes. Les matrices alimentaires sont hétérogènes et constituées d'un ensemble de micro-environnements créés par de multiples gradients, e.g. acide, oxygène, nutriments (McClements, 2007). Les microorganismes cultivés dans des matrices alimentaires structurées solides ou semi-solides forment le plus souvent des microcolonies de quelques dizaines à centaines de micromètres dont les cinétiques de croissance et la morphologie dépendent du milieu (Antwi et al., 2007; Verheyen et al., 2019). Au-dessus d'une certaine taille, des gradients de nutriments et de métabolites apparaissent au sein et dans l'environnement immédiat des microcolonies (Jeanson et al., 2015). L'exposition de cellules à différents micro-environnements locaux favorise l'apparition de sous-populations par des phénomènes d'adaptation physiologique ou de sélection de l'expression stochastique (Schröter and Dersch, 2019; Stewart and Franklin, 2008). La diversité phénotypique qui en découle peut constituer une "stratégie de minimisation des risques" (*bet-hedging*) au niveau des propriétés de la population

en terme de croissance, de survie ou de virulence dans le cas de microorganismes pathogènes (Rantsiou et al., 2012).

La modélisation du comportement des pathogènes dans les aliments est généralement basée sur l'étude de leur croissance en laboratoire dans des milieux liquides de référence. Bien que ces modèles basés sur des cultures liquides permettent de prédire le comportement des populations, de comprendre l'impact de différents facteurs environnementaux (e.g. pH, température, Aw) sur les paramètres de croissance (e.g. phase de latence, vitesse maximale de croissance), des phénomènes divergents entre milieux liquides et solides peuvent être expérimentalement observés, notamment lorsque l'on se situe dans des conditions proches de la limite entre croissance et survie (Skandamis and Jeanson, 2015; Smith and Schaffner, 2004). En particulier la vitesse de croissance devient de plus en plus variable entre réplicas quand la croissance est faible (Ratkowsky et al., 1991). Il n'existe à notre connaissance aucune étude décrivant des motifs d'expression génétique dans ces microcolonies alors que plusieurs études réalisées dans différents modèles spatialisés tels que le biofilm démontrent un rôle majeur de cette diversification phénotypique dans le comportement de ces communautés (Floyd et al., 2015; Klauck et al., 2018; Lenz et al., 2008).

L'objectif de cette thèse est d'étudier la croissance de deux bactéries pathogènes dans un modèle de matrice gélifiée transparent afin de caractériser les paramètres morphodynamiques et l'expression génétique en microcolonies. L'origine de l'hétérogénéité du comportement des cellules peut être difficile à déterminer quand trop de variables sont en jeu dans le milieu (Bury-Moné and Sclavi, 2017). Pour mieux cerner les effets de la composition et des propriétés de la matrice, un grand degré de contrôle doit être exercé sur ces paramètres, ce qui est difficile en matrices alimentaires réelles compte tenu de leurs complexités et variations entre réplicas. Dans cet objectif de recherche, il a semblé préférable de travailler sur une matrice gélifiée dont la structure est proche d'une matrice alimentaire mais avec une formulation moins

complexe, ce qui permet une démarche ascendante avec une caractérisation du comportement bactérien en matrices et pouvant être étendu en milieu réel lors d'études ultérieures (De Roy et al., 2014).

Les études présentées dans ce manuscrit se concentrent sur les bactéries pathogènes *Escherichia coli* O157:H7 et *Listeria monocytogenes*. Ces bactéries pathogènes alimentaires zoonotiques peuvent être retrouvées dans le même type d'aliments (fromage, viande hachée) et sont parmi les agents bactériens les plus souvent incriminés dans les zoonoses humaine d'origine alimentaires en Europe, respectivement à la 3ème et 5ème position. Ces pathogènes représentent deux véritables enjeux pour la santé publique en France et en Europe. En effet, *L. monocytogenes* est associé à un taux de létalité élevé (entre 18 et 30 %), et *E. coli* O157:H7 est responsable du syndrome hémolytique rénal (SHU) et de séquelles neuromotrices graves notamment chez les enfants (EFSA, 2018a, 2020). Pour comprendre leur comportement en matrice alimentaire et simuler la texture du fromage à pâte molle et de la viande hachée, une matrice hydrogel composée d'agarose à faible point de fusion a été utilisée. Les deux pathogènes ont été inoculés à différentes concentrations dans ces hydrogels, et leur comportement a été étudié en fonction de différents paramètres physico-chimiques de la matrice : pH, concentration en NaCl et texture. La transparence de ces hydrogels, compatible avec l'imagerie de fluorescence, nous a permis d'étudier l'organisation spatial des pathogènes dans ces milieux structurés, mais également l'hétérogénéité d'expression génétique à l'aide de fusions transcriptionnelles fluorescentes.

Cette thèse est réalisée dans le cadre de l'ANR PathoFood « ANR-17-CE21-0002 ». Ce projet se déroule sur la période 2018-23 et a été initié en réponse à l'appel « Alimentation et systèmes alimentaires, Défi 5 : Sécurité alimentaire et défi démographique, Axe 1 : Socle de connaissance pour répondre aux enjeux du défi ». PathoFood réunit deux unités de recherche INRAE (MEDIS à Clermont-Ferrand et

MICALIS à Jouy-en-Josas), l'ANSES de Maisons-Alfort, et trois centres techniques d'expertise du réseau ACTIA (ADIV, AERIAL et ACTALIA). Cette ANR est labellisée par les pôles de compétitivité VALORIAL et VITAGORA. PathoFood a pour but d'étudier l'hétérogénéité cellulaire et spatiale de bactéries pathogènes alimentaires, d'observer l'impact des communautés microbiennes sur l'hétérogénéité cellulaire et la distribution spatiale des bactéries pathogènes alimentaires, ainsi que le développement de modèles microbiologiques prédictifs décrivant le comportement de bactéries pathogènes alimentaires. Ce projet de thèse s'inscrit dans la première tâche de l'ANR (Figure 1).

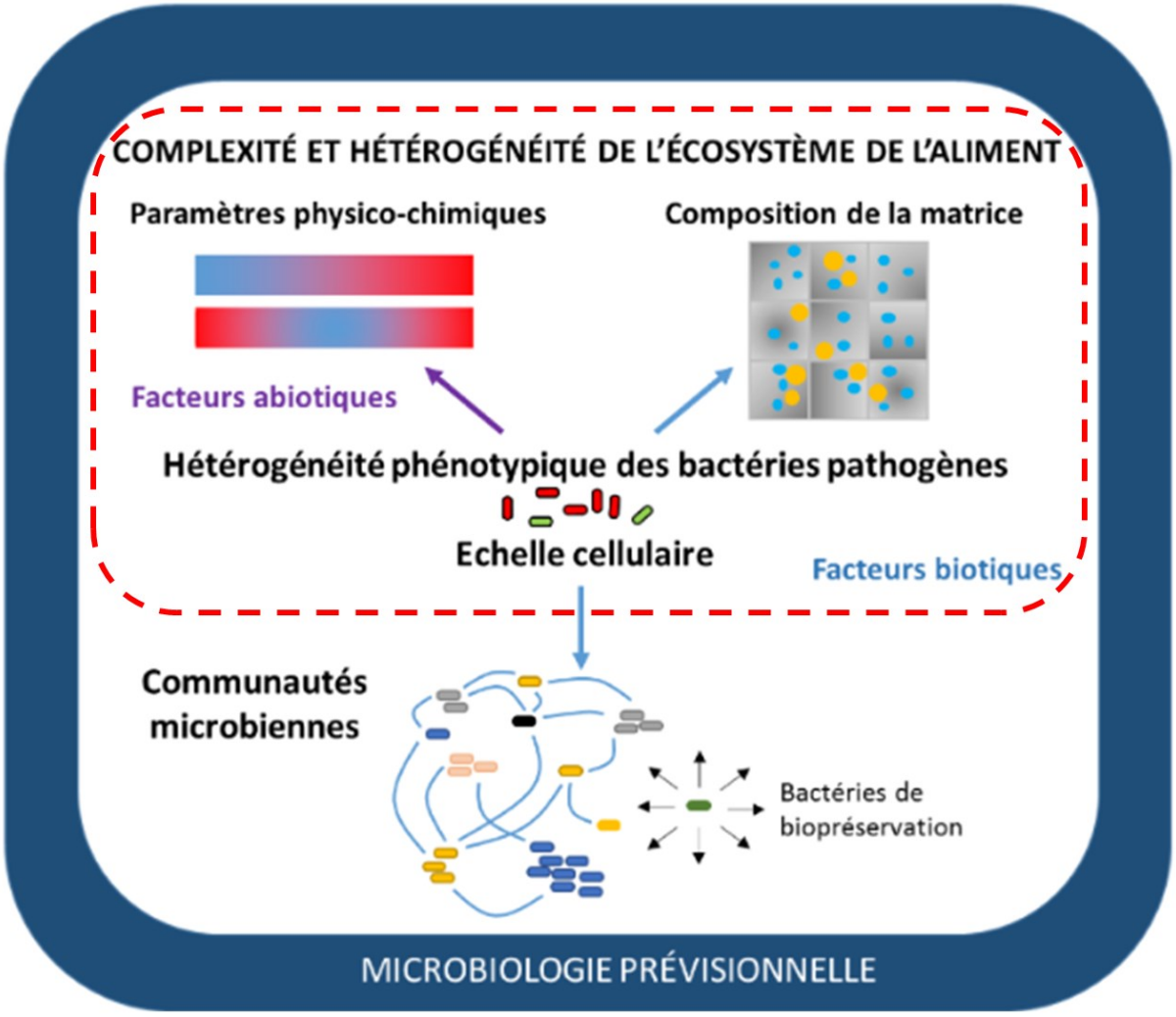

Figure 1 : Schéma récapitulant les grands axes d'exploration de l'ANR PathoFood au sein d'une matrice alimentaire. Le cadre rouge regroupe les sous-tâches de l'ANR abordées lors de cette thèse telles que l'impact des facteurs abiotiques (l'hétérogénéité de composition et les paramètres physico-

chimiques de la matrice) et des facteurs biotiques dus au métabolisme et l'hétérogénéité phénotypique des bactéries pathogènes sur la complexité de l'écosystème alimentaire.

Ce manuscrit de thèse est structuré en trois parties :

La partie I traite du contexte bibliographique du projet. Elle est organisée en trois sous-parties : A) une revue récapitulant les connaissances actuelles sur l'hétérogénéité des matrices alimentaires, l'hétérogénéité cellulaire des pathogènes et les conséquences sur la sécurité sanitaire des aliments; B) une description des techniques expérimentales permettant l'observation et l'analyse en cellule unique des microorganismes et leurs limites respectives; et C) une présentation des bactéries pathogènes modèles de l'étude ainsi que certains mécanismes de résistance au stress acide fréquemment rencontrés dans les aliments.

La partie II présente les résultats expérimentaux développés en deux chapitres : A) l'étude de la spatialisation de microcolonies des pathogènes alimentaires en fonction de la composition des matrices gélosées, et B) l'exploration des motifs d'expression génique dans les microcolonies sur l'exemple de l'expression de *gadB*.

La dernière partie est consacrée à la discussion des résultats et leurs mises en perspective dans le contexte de la sécurité microbiologique des aliments.

Partie I : Etude bibliographique

1) Diversité physiologique et hétérogénéité cellulaire des pathogènes dans des matrices alimentaires et conséquences sur la sécurité sanitaire des aliments

Dans les aliments, les bactéries vivent dans un écosystème avec des biotopes hétérogènes où une même espèce bactérienne peut présenter des hétérogénéités phénotypiques au niveau des cellules uniques constituant la population. Cette revue donne un aperçu des facteurs spatio-temporels de l'hétérogénéité phénotypique des bactéries pathogènes dans les matrices alimentaires à trois niveaux : Le premier est l'hétérogénéité génotypique due à la possibilité pour différentes souches d'une même espèce de contaminer les aliments, chacune d'entre elles ayant des caractéristiques génétiques spécifiques. Ensuite, des hétérogénéités physiologiques sont induites au sein d'une même souche, en raison de micro-environnements spécifiques et de réponses adaptatives hétérogènes en lien avec la microstructure de l'aliment. Le troisième niveau est lié à l'hétérogénéité cellulaire d'une même souche dans un microenvironnement spécifique. Cette revue discute l'importance de ces hétérogénéités phénotypiques au niveau de la cellule unique dans le développement de modèles mathématiques de prédiction du comportement bactérien et ainsi contribuer à assurer la sécurité microbiologique des aliments.

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

Cédric Saint Martin[a,b], Grégory Jubelin[b], Maud Darsonval[a], Sabine Leroy[b], Charlène Leneveu-Jenvrin[a,e], Ghaya Hmidene[d], Lysiane Omhover[c], Valérie Stahl[c], Laurent Guillier[d], Romain Briandet[a], Mickaël Desvaux[b]*, Florence Dubois-Brissonnet[a]*

[a] Université Paris-Saclay, INRAE, AgroParisTech, MICALIS Institute, 78350 Jouy-en-Josas France,

[b] Université Clermont Auvergne, INRAE, UMR454 MEDIS, 63000 Clermont-Ferrand, France,

[c] Aerial, Technical Institute of Agro-Industry 250 rue Laurent Fries, F-67412 Illkirch, France

[d] ANSES, Risk Assessment Department, 94701 Maisons-Alfort, France

[e] current address: Association pour le Développement de l'Industrie de la Viande (ADIV). 10, rue Jacqueline Auriol, 63039 Clermont-Ferrand, France

*Co-last author

Corresponding author: Florence.dubois-brissonnet@agroparistech.fr

Université Paris-Saclay, INRAE, AgroParisTech, MICALIS Institute, Domaine de Vilvert, 78350 Jouy-en-Josas France

Short title: Bacterial phenotypic heterogeneities and 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 behaviour and help ensure microbiological food safety.

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 5,000 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), and they can display textural heterogeneities at the macroscale and microscale level (Verheyen & Impe, 2021). Bacterial growth mainly relies on the surrounding physicochemical parameters, but individual cells encounter various microenvironments with specific growth conditions because of the food's spatial microheterogeneity. In addition to such spatial gradients, foodborne pathogens encounter dynamic

microenvironments due to food microbiota activity which provides temporal heterogeneities.

The complexity of these heterogeneous food microstructures leads to difficulties in assessing pathogen behaviour (growth, survival, inactivation) by modelling and thus in controlling microbiological food safety. The behaviour of bacteria depends on their living environment, but cell physiology is generally described in terms of the average behaviour of the cell population, as summarised 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 behaviours, thus giving rise to a wide range of diverse phenotypes. 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 (Figure 1). The first level of phenotypic heterogeneity that have attracted attention among microbiologists is conceptualised 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 characterise 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**. 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). Besides, cells in different growth phases can exhibit different physiological responses for a given environmental stimulus and can induce physiological heterogeneity. **Cellular heterogeneity** is an additional level of intrinsic phenotypic heterogeneity and refers to the stochastic molecular mechanisms and dynamics at play (Avery, 2006; Liao et al., 2012). 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 non-genotypic heterogeneity, *via* various molecular mechanisms leading to different types of heterogeneous phenotypes. Hence, these different levels of phenotypic heterogeneity need to be considered in order to predict the behaviour of pathogens in complex food systems with mathematical models (Koutsoumanis & Aspridou, 2017; Verheyen & Impe, 2021).

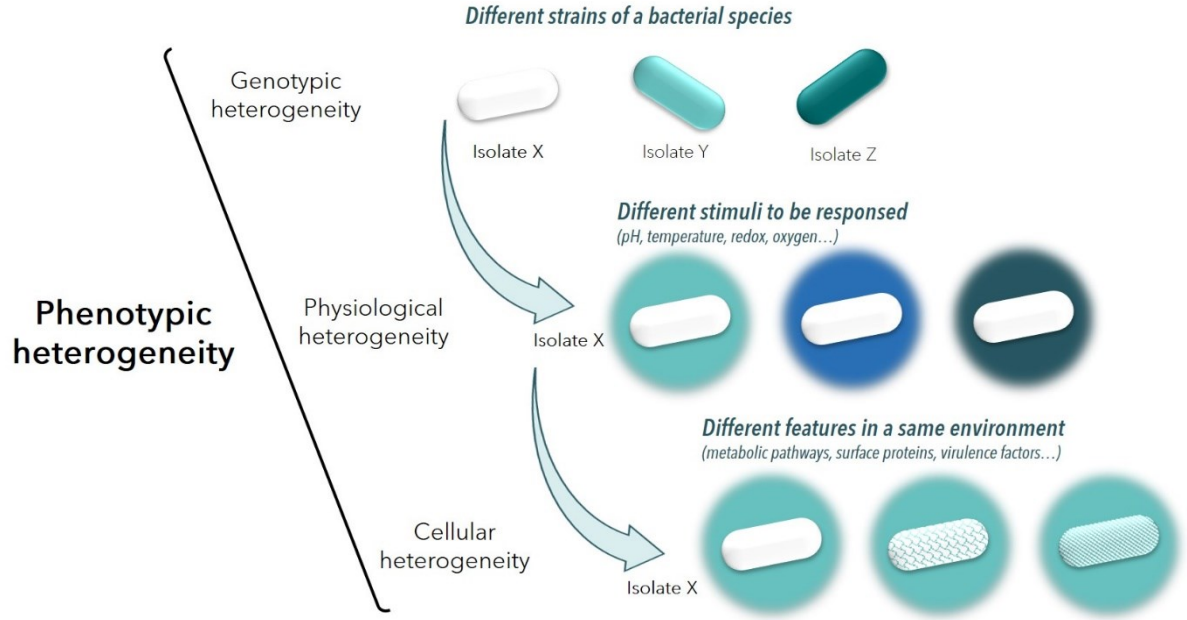

Figure 1: The different levels of phenotypic heterogeneity for a bacterial species. For a same bacterial species, different bacterial strains can be isolated and correspond to different genotypes. Respective to one

bacterial strain isolate, i.e. an isogenic bacterial clone, physiological diversity can arise between different bacterial cells if they are exposed to different stimuli, especially in an heterogenous ecological niche with diverse micro-environments. In the same environmental condition, one isogenic bacterial clone can exhibit different features from one cell to another giving rise to cellular heterogeneity. In other words, while physiological diversity is driven by environmental conditions, cellular heterogeneity exists prior to an environmental change but both levels promote population heterogeneity. These three levels of heterogeneity can actually account for some phenotypic heterogeneities, which can then either be intrinsic, i.e. in the cases of genotypic and cellular heterogeneities, or extrinsic, i.e. 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, e.g. phase variation, can generate more or less stable genetic diversity.

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 characterise 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, i.e. conjugative plasmids (Virolle et al., 2020) or integrative and conjugative elements (Delavat et al., 2017), (ii) transduction, i.e. 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 non-replicative 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.

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). Analysis of genomic diversity by whole genome sequencing 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). *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 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). 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), 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). 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).

It can be stressed, though, that 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, i.e. at pre-transcriptional, transcriptional, post-transcriptional, translational and/or post-translational levels, and in adaptative responses to the environmental conditions (Valentin Ageorges et al., 2020; 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

In complex and structured food environments, microorganisms encounter several types of microstructures (from liquid to solid) and induced gradients of available resources and physical conditions due to diffusion kinetics of nutrients, oxygen, metabolites, preservatives, temperature, etc. (Brocklehurst et al., 1995; Malakar et al., 2000). 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). Bacteria have to adapt and change their metabolic activity and growth 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.

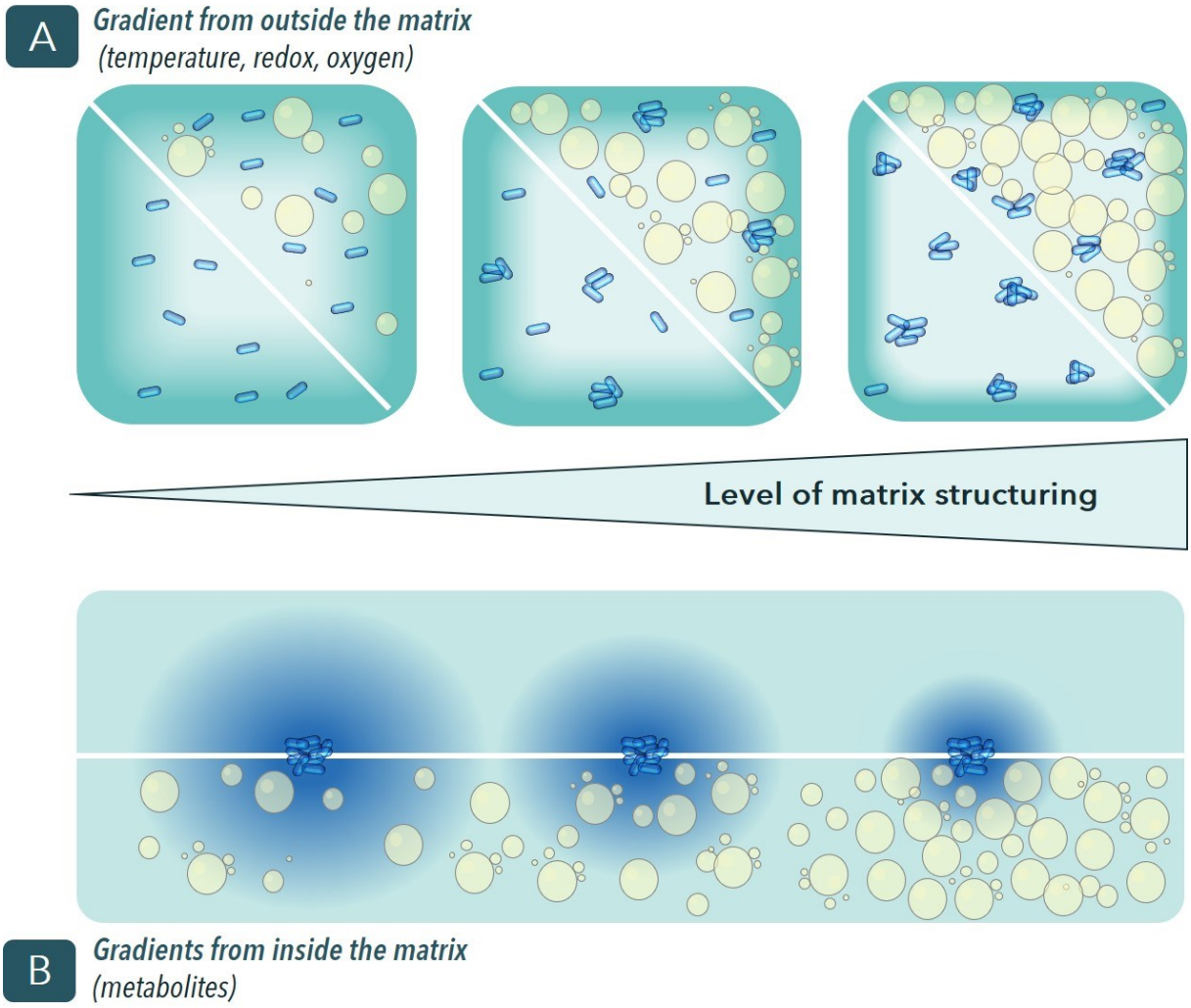

Figure 2: Microgradients increase with the level of matrix structuring: (A) gradients from outside the matrix (B) gradients from inside the matrix. Lipids droplets (yellow bubbles); bacteria (blue rods); gradients of physico-chimical parameters (gradients of green/blue). White lines segregate mono- and di-phasiques exemples.

3.1. Matrix structure

Among food systems, there is a large diversity of micro-architectures, including aqueous liquid systems with or without thickeners, *i.e.* soups or fruit juices, aqueous gels, i.e. pâté, oil-in-water emulsions, i.e. salad cream or mayonnaise, water-in-oil emulsions, i.e. butter, gelled emulsions, i.e. 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 (Heertje, 1993) and display heterogeneities at the macroscale level (solid particles, macrofibres, 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 fibres in ground beef or fats in fish or meat. 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 of water (Hills et al., 1996, 1997). The structure of a matrix is dependent on the ratio of each element, but can also depend on their net abundance. 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. Microstructures in foods and bacterial organisation

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 bacterial ability to move within the matrix and to escape from non-optimal 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, bacterial growth turns from planktonic to immobilised, and colonies grow in the bulk of the matrix, or at the air or oil interfaces. Bacterial behaviour is thus more impacted by the place of contamination. In the presence of thickeners, such as additives in sausages, or due to the denaturation of

proteins in cheese, microorganisms are immobilised and grow as colonies. 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 to 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 localised in the free space between droplets and the microbial growth remains planktonic (Baka et al., 2017b; 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 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 signalling in or on a solid structure (Esipov & Shapiro, 1998). The meshes composing the structured system hinder bacterial movements, but channels in the structure need to be smaller than the bacterial size to impact individual cell motility (Biondi et al., 1998). Swarming of *E. coli* through semi-solid 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). Cells that spend time tumbling travel more slowly than other types, but in complex structures this allows them to bypass obstructions and find channels in the mesh. 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 neighbouring microorganisms. Colonial growth also modifies the microlocal conditions because cell metabolism changes the solute composition around the colony (Wimpenny et al., 1995). For example, bacterial immobilisation affects bacterial metabolism by inducing random fluctuations in the percentage of ATP that result in a slower growth rate (Walker et al., 1998). In cheese-like model matrices, the spatial distribution of bacterial colonies has been defined according to the size of colonies and the distances between neighbouring 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 behaviour in controlled spatially structured environments (Kyle, 2018; Moon et al., 2016).

3.1.2. Bacterial growth parameters in structured foods

Environmental structure can affect bacterial growth rates together with physiological responses and susceptibility to antimicrobials. Several studies have compared growth rates measured in more or less complex systems to those measured in the liquid culture media. In emulsified systems with low proportions of fat (e.g. 26.8%), where growth occurs planktonically, the *L. monocytogenes* growth rate is higher than in continuous phase at low temperature (4°C) (Baka et al., 2017a). This is probably due to the

cryoprotective effect of fats. At higher temperatures, namely 8°C and 12°C, growth rates are similar in both systems (Baka et al., 2017a). In emulsions containing over 80% of oil and with small droplets (2 µm), the growth rate of colonies stays stable if the pH is neutral, but decreases when the pH is 5 or below (Brocklehurst et al., 1995). In jellified systems, *B. cereus* cells have a reduced growth rate when immobilised 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 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 Aw (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 immobilised state can be similar to planktonic or even favoured. Smet et al (2015) compared 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) (Smet et al., 2015). For *Salmonella*, the three states have similar growth rates (Smet et al., 2015). Finally, *L. monocytogenes* growth rate and maximum population are even higher in aqueous gels (0.7% agar and 0.5% guar) and gelled emulsions which mimic fish pâtés than in liquids (Baka et al., 2017a).

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 Aw, and the addition of emulsifiers, which are essential to stabilise 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). Temperature decrease can lead to higher polymer chain rigidity and lower molecular mobility, which increase the mechanical constraints on the bacterial cells applied by the gel network (Ferry, 1948). 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 immobilised state (Meldrum et al., 2003).

3.2. Physicochemical microenvironments

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 versus free space, specific physicochemical parameters), depending on where they contaminate (Skandamis & Jeanson, 2015). The different types of structure in food systems can affect the distribution of solutes (substrates,

metabolites, 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; McMeekin & Ross, 2002). On the contrary, structured systems as semi-solid 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 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). Non-destructive 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), Aw and relative humidity, concentration of antimicrobial compounds, or temperature are applied to specific foods (Jeanson et al., 2015; Mejlholm et al., 2010; 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. In some ways, cell environments within complex food systems can be compared to those within mature biofilms which are well-known structures offering distinct chemical niches (Ramsing et al., 1993; Santegoeds et al., 1998). As phenotypic changes are driven by reshuffling of gene expression in cells (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.

3.2.1 Nutrient availability

The heterogeneity of nutrient availability is the first factor that can affect bacterial growth and induce growth heterogeneity. Cells grown as colonies consume nutrients from their local microenvironment and can show different behaviours 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).

3.2.2. pH microenvironments and gradients

The pH in food matrices mostly ranges from neutral to acidic, scarcely to alkaline and can evolve during the food process and storage. In foods such as meat and cheese, fermentation of respectively glycogen or lactose leads to a progressive and heterogeneous acidification of the matrix (Giraffa, 2004; Hamoen et al., 2013). In addition, weak organic acids can be added to foods as preservatives to decrease the

pH. Locally, pH can be influenced by other factors such as temperature, the buffering ability of the matrix, the level of substrate available for fermentation and possibly de-acidification by other reactions as maturation progresses (Burdikova, Svindrych, Hickey, et al., 2015). Hence, bacterial cells can be exposed to suboptimal or extreme pH conditions, pushing them out of their comfort zone for growth and survival (Booth, 2002). In solid food matrices, bacteria are more or less immobilised and their growth and physiology will be impacted by the pH directly around the cell or colony. Later, the growth will also be impacted by the pH microgradients created by metabolic activity within the colony, involving substrate consumption and production of end-products. (Floury et al., 2010; Katsaras & Leistner, 1991). 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 behaviour is increasingly studied thanks to a technical shift from the use of microelectrodes to imaging techniques. Microelectrodes were first used to measure pH in and around colonies and it appeared that 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 centre of the colony (Walker et al., 1997; Wimpenny et al., 1995). Moreover, pH gradients are dependent on the presence of fermentable sugars, a sharp pH drop close to the colony being observed in the presence of glucose. Similarly, pH gradients due to the production of lactic acid were observed within colonies of *Lactobacillus curvatus* when they reached 100 µm in the gel cassette system (Malakar et al., 2000). These pH gradients could be the consequence of biochemical heterogeneity of cells in the colony, the cells being at different stages of metabolic activity. Later, it was reported that no pH microgradient occurs around bacterial colonies in a model cheese, regardless of the size of the colony. Acidification kinetics highlighted that lactic acid did not accumulate at the edge of the colony (Jeanson et al., 2013). These observations were consistent with another study showing that diffusion of lactic acid in a buffered gel system is Fickian (Malakar et al., 2002). Nowadays, pH measurement at the cell scale

uses pH-sensitive fluorescent dyes associated with confocal laser scanning microscopy and imaging analysis (Burdikova, Svindrych, Hickey, et al., 2015; Malakar et al., 2000). pH microgradients were observed by fluorescence lifetime imaging in cheese matrix, but not particularly around colonies (Burdikova, Svindrych, Pala, et al., 2015). Regardless of the technique, pH microgradients have only been recorded in and around large colonies (Jeanson et al., 2015) and the existence of pH microgradients in small colonies or in real food systems is 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). Localised ATR due to the presence of pH microgradients could induce heterogeneity within the food matrix in terms of growth rate, cell morphology or metabolic activity of foodborne pathogens, but further studies are needed to explain heterogeneity at the level of gene expression (Walker et al., 1997). The spatiotemporal evolution of a *Staphylococcus aureus* population was monitored during the manufacture and ripening of semi-hard cheese by combining fluorescent reporters, confocal laser scanning microscopy and flow cytometry (Fleurot et al., 2014). The authors showed that *S. aureus* cells preferentially localise at the surface, mostly because of higher pH and favourable aerobic conditions (Fleurot et al., 2014). More recently, the transcriptional fusion of a green fluorescent reporter (*gfp*) with a pH responsive gene *atpB* promoter allowed simultaneous monitoring of changes in spatiotemporal pH and bacterial activity in three-dimensional communities (Hwang et al., 2016). Moreover, the heterogeneity of spore outgrowth varies according to the pH medium. For example, heat-treated spores of *B. cereus* show a higher heterogeneity in outgrowth at pH 5.5 than at 7.4 (Warda et al., 2015).

Over the past decade, numerous studies have reported a close relationship between complex mechanisms of ATR and virulence in *S.* Typhimurium and *L. monocytogenes* (Álvarez-Ordóñez et al., 2010; Gahan & Hill, 1999). Five serovars of *Salmonella* pre-

adapted in acid fruit juices were shown to enhance their acid resistance, suggesting that exposure to an acidic environment during processing could enhance their ability to survive in the human stomach (Yuk & Schneider, 2006). As micro-heterogeneity in pH values from 4.0 to 5.5 exists in natural cheese (Burdikova, Svindrych, Hickey, et al., 2015), we could easily hypothesise that acid tolerance capabilities and virulence of pathogens may be regulated at the microscale level by the heterogeneity of pH values induced in particular by the presence of nearby acid-producing bacteria.

During cheese making and according to the specific technologies, acidic environments can be locally de-acidified by acid-tolerant yeasts and moulds. 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).

3.2.3. Water activity

Moisture in food refers to the measurement of the Aw, which determines the amount of free water that is available for microorganisms to use for growth. Most foods exhibit an Aw above 0.95 and so allow bacterial growth. However, Aw 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., 1996; 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). It can be

characterised in food matrices by proton nuclear magnetic resonance T2 relaxation measurements (Møller et al., 2013).

The Aw is the parameter that most influences the growth rate of *S. typhimurium* in broth and in gelatinised media (Theys et al., 2008). The probability of *L. monocytogenes* growth was influenced by the Aw of cheese matrix at both low and high contamination levels (Schvartzman et al., 2011). However, variability was observed from the two-strain mix of natural isolates tested and one strain had a greater ability to grow and survive in this matrix (Schvartzman et al., 2011).

Adding NaCl or sugar to improve the sensory properties or to extend the shelf-life by decreasing the Aw induces osmotic stress in bacteria and has a significant impact on the growth of colonies (Theys et al., 2010). To cope with osmotic stress, bacterial cells try to maintain or restore turgescent pressure by accumulating compatible solutes, such as betaine and carnitine, which are widely found in foods of plant and animal origin (Galinski, 1993). *L. monocytogenes* responded to elevated concentrations of salt by the intracellular accumulation of glycine betaine in the cytoplasm using a specific transport mechanism (Ko et al., 1994). 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). To adapt to osmotic stress, an increased expression of transporter genes associated with the accumulation of these compatible solutes was observed, but so too was a down-regulation of *PTS* genes, leading to decreased carbohydrate uptake and reduced cell growth (Bae et al., 2012).

3.2.4. Preservatives

Preservatives refer to agents added to the food product to slow down alteration caused by spoilage microorganisms until the end of the shelf-life. These molecules can have bacteriostatic or bactericidal properties. Preservatives have different modes of action such as cell acidification (organic acids), membrane damage (essential oils) or interference with key bacterial functions (sulphites, nitrites...)(Cammack et al., 1999).

When these molecules are added to a complex multiphasic system, they will interact differently with the phases present according to their physicochemical properties. Compounds that are sparingly soluble in water tend to become associated with surfactant micelles or to partially migrate into the lipid phase. Consequently, food preservatives are expected to be more or less trapped by micelles or lipid phases accordingly to their physicochemical properties. 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 localisation and proportion of lipid phases, microorganisms could be exposed to inhibitory or sub-inhibitory concentrations according to their localisation. 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. When emulsifiers, such as whey proteins or Tween 80, are added to tryptone soya broth, the antimicrobial activity of eugenol is decreased (Pernin et al., 2019). Tween 80 forms micelles in the aqueous phase where hydrophobic compounds can be trapped, making them unavailable for antibacterial activity. To a lesser extent, proteins can also trap antimicrobials. Food proteins, especially those present in meat products, carry a negative charge above their isoelectric point and some preservatives, such as lauric arginate, carry positive charges in the range of food pH. Consequently, proteins can

trap negatively charged antimicrobials to form soluble or insoluble complexes (Weiss et al., 2015). As a result, antimicrobial agents are no longer available to interact with microorganisms and inhibit their growth. The antimicrobial effect of oregano and cranberry in combination with sodium lactate against *L. monocytogenes* was significantly reduced when 1 mM proline was added to the growth medium (Apostolidis et al., 2008). Similarly, the inhibition of this pathogen is lower in cooked ground beef tips compared to broth. The proline or proline precursors in meat (arginine, glutamate) help *L. monocytogenes* to recover from phytochemical inhibitory activity.

3.2.5. Temperature

Temperature is a major extrinsic factor used to control food safety. Thermal processes with high temperature (cooking, pasteurisation, sterilisation) or low temperature (refrigeration, freezing) are commonly used to preserve foods. Routinely, high temperatures are used to inactivate bacterial cells, viruses or parasites, freezing stops bacterial growth and inactivates some parasites, and refrigeration slows down and inhibits bacterial growth. Temperature control during food processes and storage time is thus critical to control foodborne pathogens. Structured and complex food matrices interfere with temperature diffusion and thermal gradients are created depending on the food structure. 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. In both cases, spatiotemporal heterogeneities of temperature are created which are strongly influenced by the food characteristics, namely its size, structure and composition (pH, fat, additives, etc.).

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 (Aw 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. *L. monocytogenes* growth at 4°C is promoted in emulsified fish-protein systems containing fats compared to liquid systems without fats, suggesting a cryoprotective effect of the matrix (Baka et al., 2017b). Sugar and salt enhance the survival of *L. monocytogenes* and *S. aureus* in refrigerated model brines (Bevilacqua et al., 2018). *S. aureus* is more likely to be killed during freeze-thaw cycles in ground beef when the pH is low (Demchick et al., 1982). Similarly, *S.* Typhimurium on chicken wings is less resistant to 5 freeze-thaw cycles when the process is associated with lactic acid (Olson et al., 1981). When *B. cereus* was cultivated in microcolonies at its minimum temperature, 11% of the microcolonies failed to grow (Den Besten et al., 2010). This growth heterogeneity could partly be explained because a portion of cells lose their membrane integrity. This chill-induced heterogeneity is limited when the concentration of *B. cereus* is high.

Exposure of microorganisms to temperatures out of their comfort zone can result in adaptation and acquired thermotolerance. This 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) stabilisation 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. The cold adaptation response is a universal mechanism in bacteria, but has in particular been studied in psychotropic bacteria such as *B. cereus* and *L. monocytogenes* (Alvarez-Ordóñez et al., 2015). Loss of membrane fluidity is the first consequence to be addressed by the cells. *L. monocytogenes* adapts to low temperature in particular by increasing the ratio of anteiso15:0 branched fatty acid in its membrane to counterbalance the loss of fluidity (Julotok et al., 2010). However, the freeze-thaw tolerance of *L. monocytogenes* was lower at 4°C than at 37°C, suggesting

that cold adaptation does not necessarily induce protection against temperature cycles (Azizoglu et al., 2009). The variability of individual cell behaviour 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.

3.2.6. Oxygen and carbon dioxide

Oxygen availability is a critical factor affecting bacterial growth. Most foodborne bacterial pathogens are facultative anaerobes. The availability of oxygen, i.e., aerobic, microaerobic, facultative anaerobic and anaerobic conditions, impacts the behaviour and growth of bacteria according to their respiratory type. 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. The solubility of CO_2 is increased by low temperature, neutral pH and high water activity (Devlieghere et al., 1998; Farber, 1991). 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 (Peters et al., 1987; 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 mm 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 depletion of oxygen in combination with nutrient limitation and accumulation of metabolites can contribute to the reduction of the growth rate of bacteria (Wilson et al., 2002). As already mentioned in 3.2.2, the distribution of *S. aureus* monitored during the ripening of semi-hard 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. The growth of *S. aureus* on the aerated surface was also correlated with the expression and production of the enterotoxin SED (Fleurot et al., 2014).

In the presence of oxygen, bacteria have defence 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.

3.2.7. Light

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 versus 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). In the case of mammalian bacterial pathogens, it is hypothesised that light could serve as an indicator of whether the pathogen is inside or outside the host (Purcell & Crosson, 2008). More recently, it was fortuitously observed that colonies of *L. monocytogenes* on agar undergo synchronised multicellular behaviour 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 hypothesised 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 behaviour 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, cheeses), which are stored in refrigerated display cases, can be alternatively be exposed to light during the day and to darkness at night.

3.3. Microbiota effect on food pathogens

3.3.1. Food matrix as a microbial biotope

A microbial pathogen can colonise a food matrix axenically, for example when an accidental contamination occurs after thermal sterilisation. 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 neighbours 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 (Chaillou et al., 2015; De Filippis et al., 2018; Wiernasz et al., 2020). 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 behaviours. 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 modelling of interspecies interactions in spatially organised systems (Hynes et al., 2018; Krishna Kumar et al., 2021).

3.3.2. Spatial and nutritive competition

Several studies have demonstrated that in food matrices where the physicochemical (pH, Aw) and nutritional conditions are favourable to the growth of pathogens, the pathogens could be inhibited by the presence of background microbiota (L. Guillier et al., 2008; Lardeux et al., 2015). The formal demonstration of a total or partial 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 organised: 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).

3.3.3. Specific interference

Microorganisms from the microbiota growing in the food matrix can produce compounds affecting pathogen physiology, survival and behaviour. 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 permeabilisation 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, i.e. 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; Surette et al., 1999; 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; Thompson et al., 2016). The concentration and bioavailability of such signalling molecules can be altered in multispecies communities by interactions with specific components of the matrix or the microbiota, e.g. 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 (Hederstedt et al., 2020; 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 clone under congruent environmental conditions. Molecular mechanisms at play can occur at pre-transcriptional, 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.

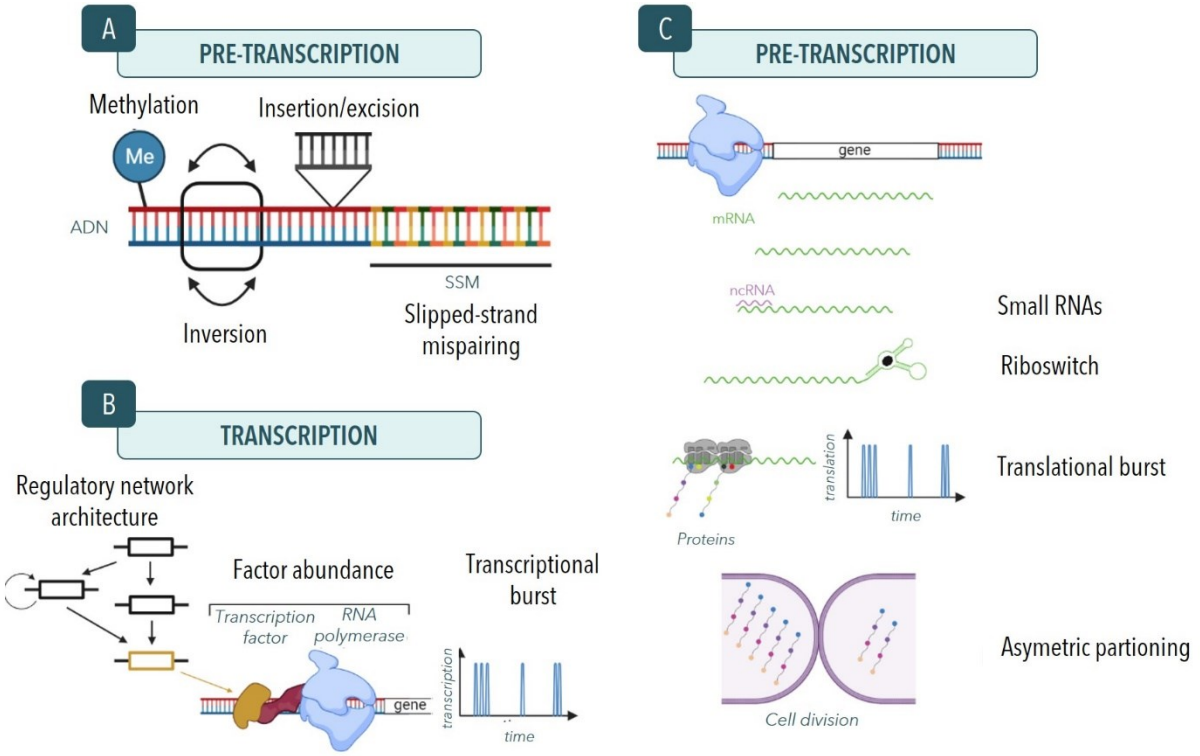

Figure 3: Cellular heterogeneity due to different molecular mechanisms of gene expression regulation occurring at pre-transcriptional level (A), transcriptional level (B) and post-transcriptional level (C). (A) Pre-

transcriptional regulatory mechanisms mainly belong to phase variation mechanisms which correspond to a reversible ON/OFF switch of DNA transcription. Expression switch due to phase variation involve changes in DNA molecule: DNA inversion, slipped-strand mispairing (SSM), DNA insertion/excision, and DNA methylation (See part 4.1.). (B) Transcription regulatory mechanisms are due to the binding and dissociation of various factors, their abundance and the associated regulatory network architecture. Transcriptional burst over the time also contribute to cellular heterogeneity (See part 4.2.). (C) Post-transcriptional regulatory mechanisms involve modifications at mRNA level by the action of small RNAs and riboswitch or at translational level by protein translation level and asymmetric portioning during cell division (See part 4.3.).

4.1. Phase variation mechanisms

Some regulatory mechanisms underpinning cellular heterogeneity can occur before the stage of DNA transcription. An important group of pre-transcriptional regulatory mechanisms recognised 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 colonisation 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. Expression switch due to phase variation is unusual in biology. 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 (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 sub-populations 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 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 pre-transcriptional regulation by DNA inversion (Abraham et al., 1985; Bryan et al., 2006). As the major pilin, FimA is an important protein involved in adhesion and its production shows a shift towards bacterial colonisation 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 37-40°C, FimE is progressively inhibited as the temperature

increases (Henderson et al., 1999). Close by the promoter region, Lrp (leucine regulation factor) can specifically bind and work in concert with IHF (integration host factor) 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* (Ditto et al., 1994; 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, *i.e.* 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 hypothesised (Ikeda et al., 2001). In *Campylobacter fetus*, the phase variation of the surface layer protein SapA impacts bacterial colonisation 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; van Belkum et al., 1998). 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 analysing 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* (Baselga et al., 1993; Brooks & Jefferson, 2014), a gene encoding an integral membrane protein involved in assembly and secretion of the polysaccharide intracellular adhesin (PIA) (Gerke et al., 1998; 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 3 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 non-functional 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 colonisation 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 poly-thymine in *flhA* encoding flagellin (Harris et al., 1987; 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, e.g. *wlaN* encoding a β-1,3 galactosyltransferase (Karlyshev et al., 2002) or *cgtA* encoding an N-acetylgalacto-saminyltransferase (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 haemolytic 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 poly-adenine 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 IS*256* 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 IS*256* at different sites within the *ica* operon, preferentially in *icaC* (Ziebuhr et al., 1997, 1999), but also in genes involved in transcriptional regulation, i.e. *rsbU* or *sarA* (Conlon et al., 2004). While occurring at a low frequency (less than 10^{-8}), reversion mostly results from IS*256* excision and the formation of an episome (Conlon et al., 2004; Loessner et al., 2002). In *S. aureus*, the σ^B transcription factor appears to negatively regulate the IS*256* 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 (Adhikari & Curtis, 2016; Blow et al., 2016; Palmer & Marinus, 1994; Putnam, 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 (Beaulaurier et al., 2019; 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 (Valentin 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 OxyR (oxidative stress response) transcriptional regulator (Owen et al., 1996). 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 oxidised forms (van der Woude & Henderson, 2008; Wallecha et al., 2003). 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; Lindberg, 1973).

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; Murphy et al., 2008), *S. enterica* (Giacomodonato et al., 2009), *C. jejuni* (Kim et al., 2008) and *V. cholerae* (Julio et al., 2001). C^5-cytosine methylation by Dcm (DNA cytosine methyltransferase), recognising 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; Militello 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 defence 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, e.g. *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; Munsky et al., 2015). 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 (Dobrzyński & Bruggeman, 2009; 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 (Jones et al., 2014; Sanchez et al., 2013; Sepúlveda et al., 2016; Silander et al., 2012). 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 (Lim et al., 2003; Mitarai et al., 2008; Murugan, 2006). 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, DNA) to interact by random collisions is less frequent and this contributes to rare events of gene activation and therefore noisy expression (Becskei et al., 2005; Roberfroid et al., 2016). Recent studies have shown that bacteria capitalise 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; Kearns & Losick, 2005; Ojima et al., 2012; Saini et al., 2010).

Negative auto-regulation 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 (Ferrell, 2002; 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; Dubnau & Losick, 2006). If auto-activation 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 3 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 enterohaemorrhagic *E. coli* (EHEC) since Shiga toxin-encoding genes are located within lambdoid prophages. Expression of *stx* genes is therefore dependant 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 (V 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 characterised 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; Grundy et al., 2003; Grundy & Henkin, 1998; Huang et al., 2012; Mironov et al., 2002; Nahvi et al., 2002; Serganov, 2009; Winkler et al., 2002), 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; Suddala & Zhang, 2019).

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 non-coding RNAs (ncRNAs), which hybridise with mRNA to block the binding of the ribosome in a mechanism analogous to RNA interference (RNAi) in eukaryotic cells (Fischer, 2015), namely the anti-sense 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; Marincola et al., 2019). 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 (Engl, 2018; 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 (Elowitz et al., 2002; Rosenfeld et al., 2005). Modelling 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., 2012, 2014). Such asymmetric partitioning can affect the distribution and localisation 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 modelling 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 behaviour in modelling

5.1.1. The challenge of data acquisition about pathogen behaviour 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 behaviour 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 behaviour 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 (Chen et al., 2016; de Oliveira Elias et al., 2019; 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 (Laurent Guillier & Augustin, 2006). The challenge is thus to consider pathogen behaviour at the single-cell level so as to consider the heterogeneity of both the population and the food environment.

To characterise 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 (Lund et al., 2021).

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 (Gnanou Besse et al., 2006). Figure 4 illustrates the impact of using enumerations of samples inoculated with single cells to construct growth kinetics. Each of these enumerations along the kinetics (Figure 4B) has as its origin a different cell with a specific lag time (Figure 4A). Using common exponential growth models at the population level is thus not relevant for such situations.

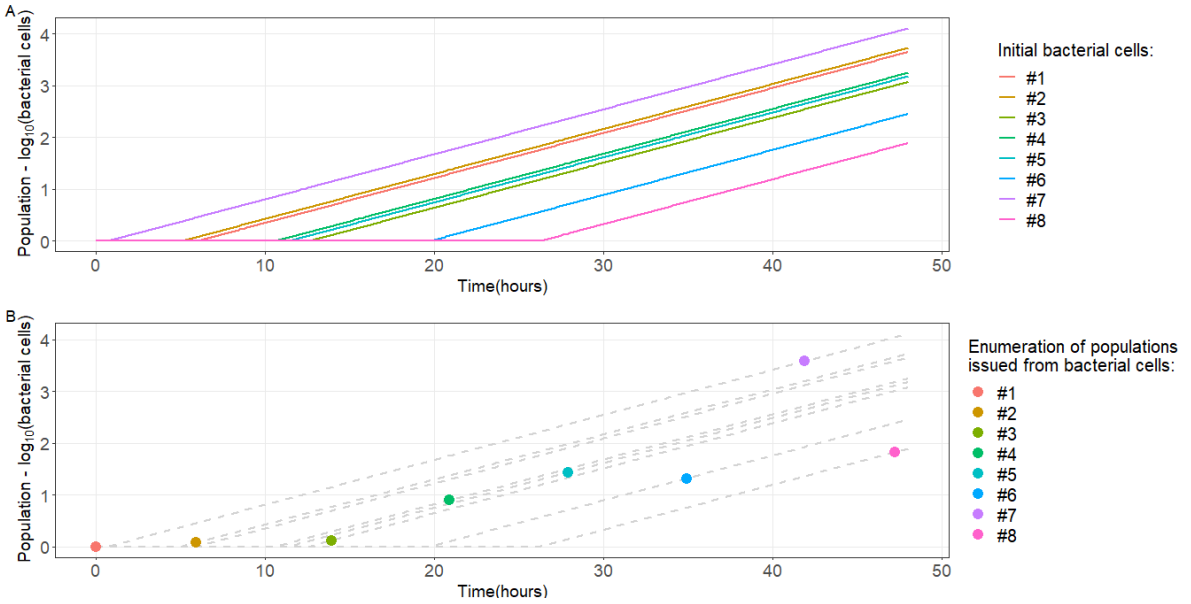

Figure 4. A. Growth kinetics in environments inoculated with a single bacterial cell that present an exponential growth rate of 0.2h-1 and a variable individual lag time (values were randomly drawn from exponential distribution of mean 10h). B. Enumerations carried in each of the environments at different sampling 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 "vertical" method and the "detection time" method, respectively.

The determination of individual cell growth probability is even more challenging in foods. When using a large inoculum, the presence of non-growing 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 non-growing cells (Aguirre & Koutsoumanis, 2016). Characterising cellular growth probability in foods involves the inoculation of numerous samples with a few cells per sample. The samples should then be enumerated after an incubation period long enough to

distinguish cells that are still in lag times and cells that will never initiate growth (Augustin et al., 2015; Couvert et al., 2010).

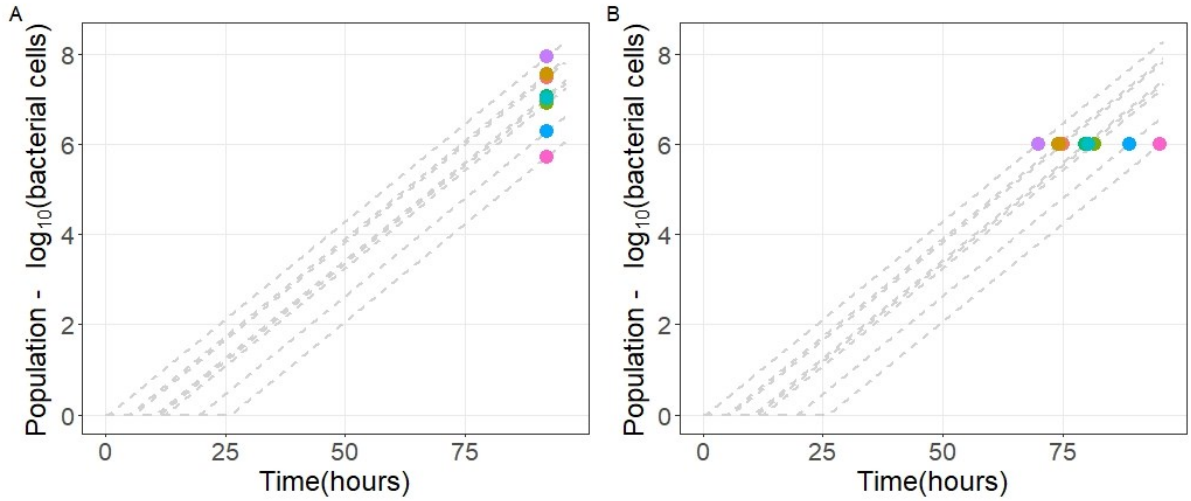

Figure 5. Schematic representation of the methods to estimate individual lag times through enumerations A. In the "vertical" method, a large number of samples are enumerated at one time. B. In the "detection time" method, times to reach a given threshold are used. In both case, the estimation of individual 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.

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 behaviour 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.

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 (L. 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 (Nd), (ii) insurance that the probability of having a single cell is high and (iii) precise knowledge of the growth rate (μmax) (Baranyi et al., 2009; L. Guillier et al., 2006; Lund et al., 2021; Métris et al., 2003). 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; Niven et al., 2006). 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; Niven et al., 2006). 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}).

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 non-optimal conditions, is equal to 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., 2018; Koutsoumanis & Lianou, 2013). The principle relies on observing more than one hundred cells immobilised 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 non-growing 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 (Laurent Guillier & Augustin, 2006).

As there are many models for predicting the growth rate according to environmental conditions (temperature, pH, water activity, …), 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 (Baranyi & Pin, 1999; Laurent Guillier et al., 2005).

So far, very few papers have considered modelling cell growth probability according to environmental conditions (Augustin & Czarnecka-Kwasiborski, 2012).

5.2. Risk assessment and behaviour 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 characterisation, which is the characterisation of the dose-response relationship to estimate the probability and severity of adverse effects based on the ingested dose. Finally, risk is estimated and characterised 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 modelling 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 behaviour, which is likely to impact the microbial response within the food.

An individual-based modelling 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 behaviour 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 behaviour 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 modelling. The main reason for the incapacity of the population-based approach to predict observed experimental behaviour 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 behaviour (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 behaviour 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; Ellouze et al., 2010; Pouillot & Lubran, 2011). The decision support tool Sym'Previus is a software which has integrated

a single-cell growth probability module (Tenenhaus-Aziza & Ellouze, 2015). For all these studies, considering individual cell behaviour 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 behaviour 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). Moving from a hazard-based and final product testing approach to a risk-based management approach requires specific statistical and mathematical tools able to predict microbial behaviour throughout the food chain (Codex alimentarius, n.d.). 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 programmes in a quality assurance context. Yet, early applications of next-generation sequencing emphasised its great potential to complete characterisation 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 behaviour, 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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2) Les techniques d'analyse et d'imagerie de fluorescence en cellule unique

La plupart des techniques utilisées en microbiologie permettent d'appréhender la physiologie microbienne au niveau populationnel. Ces approches "*whole population*" permettent d'alimenter des modèles décrivant la croissance des cellules dans des conditions de croissance favorables en matrice alimentaire, mais deviennent biaisées quand ces dernières sont défavorables en sous estimant les capacités d'adaptation des bactéries (Pin et al., 1999; Ross and McMeekin, 2003). L'acquisition des données doit pouvoir prendre en compte l'hétérogénéité physiologique des sous-populations, et ces sous-populations sont elles-mêmes la conséquence de variations phénotypiques au niveau de la cellule individuelle par adaptation physiologique ou changement stochastique de l'expression des gènes. Depuis plus d'une décennie, les techniques permettant de caractériser les propriétés d'un micro-organisme à l'échelle de la cellule unique sont en plein essor.

Certaines de ces techniques renseignent sur des aspects moléculaires ou testent les interactions de la membrane et la turgescence des cellules e.g. spectrométrie Raman, microscopie à sonde locale (Arnold and Bailey, 2000; Arnoldi et al., 2000; Benoit and Gaub, 2002; Wang et al., 2020), mais ne sont pas adaptées à une observation de la physiologie de la cellule et sont souvent destructrices. Ce sont les études en fluorescence qui composent la majorité des techniques dédiées au suivi du phénotype des bactéries.

2.1) Les sondes fluorescentes pour l'étude des micro-organismes

Une grande diversité de sondes fluorescentes est utilisée en biologie cellulaire et en microbiologie pour contraster certaines propriétés des cellules e.g. structures, interactions entre protéines, transcription, pH, intégrité de la membrane… En 1852, Stokes (Stokes, 1852) découvre que les molécules fluorescentes possèdent la propriété

d'entrer dans un état excité quand elles sont frappées par un photon présentant une longueur d'onde définie ($\lambda_{excitation}$) et sortent de cet état par émission d'un photon de plus faible énergie ($\lambda_{émission}$). Les organismes procaryotes non photosynthétiques sont le plus souvent très peu auto-fluorescents et sont donc compatibles avec une large panoplie de fluorophores. Le marquage fluorescent des bactéries a été en particulier façonné par la découverte de la "*green fluorescent protein*" (*GFP*) de la méduse *Aequorea victoria* par Osamu Shimomura en 1962 et les travaux de Roger Tsien qui obtint par mutation des protéines de couleurs bleu/cyan et jaune (Shimomura et al, 1962). La protéine fluorescente rouge DsRed est quant à elle identifiée dans le corail. Ces marqueurs n'étant pas cytotoxiques et produits naturellement par des organismes vivants, le clonage du gène codant le fluorophore dans le chromosome bactérien constitue une évolution naturelle de la technique qui sera présentée dans *E. coli* par Martin Chalfie en 1994 (Chalfie et al, 1994). L'étude de fluorescence en cellule unique prendra son essor au début du millénaire (Barer and Harwood, 1999; Joux and Lebaron, 2000) et est depuis devenue un outil ubiquitaire dans les études avec une multitude de sondes spécifiques permettant le suivi des différentes propriétés de la cellule (Bridier et al., 2015).

La première utilisation des fluorophores en bactériologie est la géolocalisation cellulaire qui peut être réalisée par un marqueur chimique des acides nucléiques tel que le SYTO9 (Bridier et al., 2010; López-Amorós et al., 1997) ou par une protéine fluorescente telle que la GFP constitutivement exprimée par la cellule (Ma et al., 2011). Ce type de marquage fluorescent permet, par analyse d'images, l'extraction de paramètres tels que le nombre et la position des cellules. Dans un second temps, il est possible de déterminer l'espèce étudiée. Il est possible d'utiliser lorsqu'ils sont disponibles des anticorps couplés à un fluorophore marquant certains antigènes de l'enveloppe externe des bactéries (Davey and Kell, 1996; Rockabrand et al., 1999). Une approche plus versatile est l'utilisation de la reconnaissance de marqueurs génomiques détectables par la technique de "*Fluorescence In Situ Hybridization*" (FISH) où une

séquence nucléotidique couplée à un fluorophore est complémentaire de la séquence recherchée, qui est-elle même spécifique de la cellule recherchée.

Du côté de la physiologie des cellules, en utilisant des marqueurs d'acides nucléiques présentant différentes perméabilités membranaires, il est possible de visualiser et de quantifier la perte d'intégrité membranaire des bactéries, associée à une perte de viabilité. Le couple de fluorophores le plus utilisé dans ce cadre (kit *live/dead*) est un mélange de Syto 9, capable de pénétrer dans toutes les cellules quel que soit leur état physiologique, et l'iodure de propidium (IP), molécule qui ne pourra se lier aux acides nucléiques intracellulaires qu'en cas de perte d'intégrité de la membrane. La fluorescence permet également d'étudier certains aspects du métabolisme d'une cellule. Pour ce faire, des marqueurs spécifiques, dont la fluorescence dépend de leur environnement physico-chimique, permettent par exemple de mesurer le pH local (Han et al., 2009; Lin et al., 2003) ou le potentiel électrochimique de membrane des bactéries (Franke et al., 2019; González and Tsien, 1997). Il est également possible d'évaluer certaines activités métaboliques telles que l'activité estérasique (Bhargava et al., 2021; Gao et al., 2014) ou la respiration (Altman, 1976; Rodriguez et al., 1992; Smith and McFeters, 1997). Il est aussi possible de réaliser une fusion transcriptionnelle pour suivre un processus métabolique. Le gène codant la protéine fluorescente est cloné après le codon stop d'un gène d'intérêt mais avant le terminateur (Figure 2). La transcription du fluorophore et par conséquent la fluorescence est ainsi dépendante de l'activité du promoteur du gène choisi pour être représentatif de l'activation/inhibition d'une voie métabolique. Une fusion traductionnelle permet de suivre la position d'une protéine. La fusion du gène étudié avec le gène du fluorophore tout en supprimant le codon stop entre les deux, permet l'obtention d'une protéine hybride fluorescente (Figure 2). Le marquage de deux protéines par deux fluorophores différents permet l'étude de l'interaction physique directe. Pour ce faire, le premier fluorophore doit avoir une bande d'émission correspondant à la bande d'excitation du second. Cela crée un phénomène de transfert d'énergie par résonance de fluorescence

(FRET) qui fait apparaître la seconde raie d'émission si les fluorophores sont à moins de 60 angstroms.

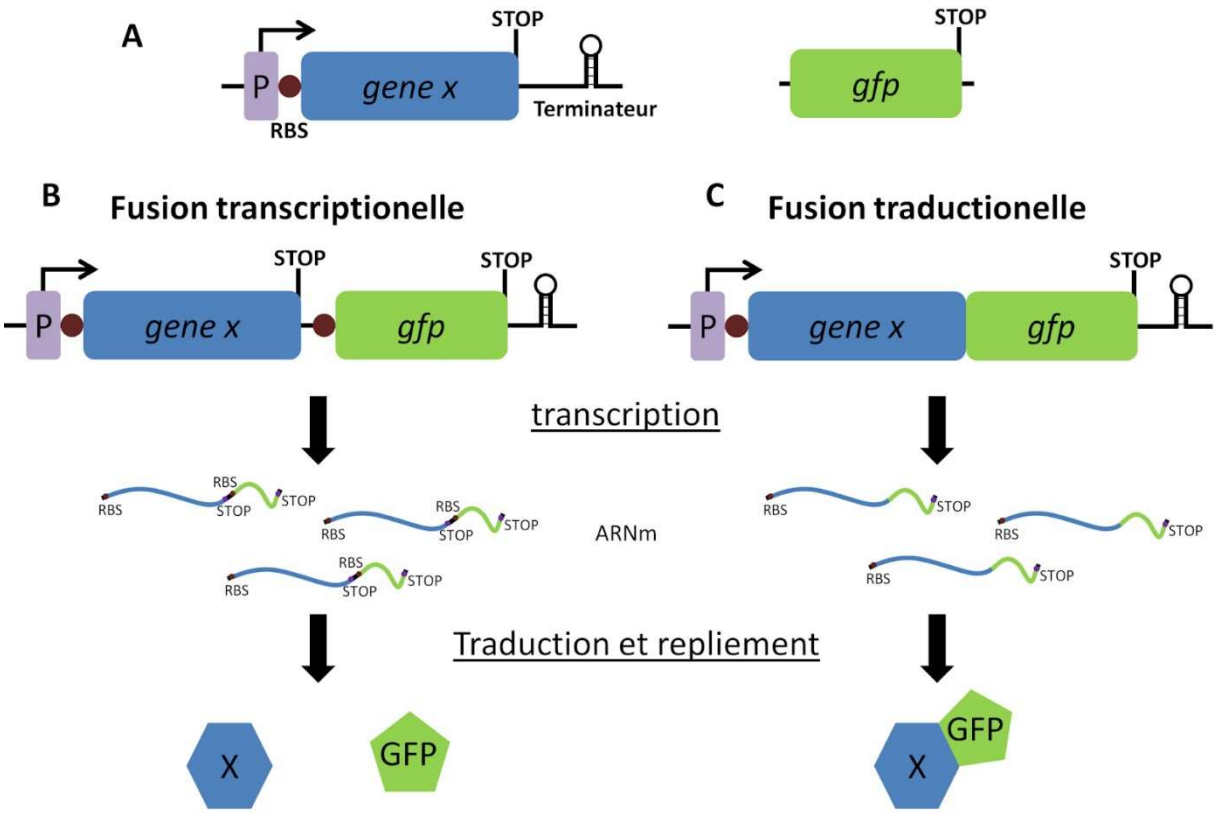


Figure 2 : Schéma des fusions transcriptionnelles et traductionnelles par intégration chromosomique. A) Initialement sur le chromosome, le gène d'intérêt (bleu) est précédé de son promoteur (violet), d'un site de liaison du ribosome (RBS, rouge), et suivi par un terminateur de transcription. Le gène codant la fluorescence est représenté en vert. B) La fusion transcriptionnelle est réalisée par clonage de la cassette entre le terminateur et le codon STOP du gène. Un ARN messager polycistronique (avec deux RBS, deux séquences codantes et deux codons STOP) produit deux protéines distinctes. C) La fusion traductionnelle est réalisée par clonage de la cassette en amont du codon STOP du gène d'intérêt. L'ARNm produit est monocistronique (possède un RBS unique et un seul codon STOP) et code une chaine peptidique unique. La protéine hybride obtenue est la protéine X marquée par la GFP.

Enfin, il est possible d'étudier le microenvironnement local en fluorescence avec par exemple le rouge de Nile qui marque les gouttelettes lipidiques (Lopez et al., 2010), le DDAO ou le TOTO-3 marque l'ADN extracellulaire (Conover et al., 2011) et les lectines ont une affinité pour des familles de polysaccharides (Guasch et al., 1995; Zippel and

Neu, 2011). Quant aux protéines, de nombreux marqueurs non spécifiques existent (Haugland et al., 2005), mais un marquage spécifique nécessite des anticorps.

Les études basées sur la fluorescence sont limitées par un certain nombre de points devant être pris en considération. En premier lieu, certains fluorophores peuvent avoir un effet toxique sur les cellules, ce qui empêche une étude dans le temps. De manière plus générale, l'activité métabolique, le pH intracellulaire, les molécules chargées et les phénomènes d'extinction moléculaire (*quenching*) peuvent altérer la fluorescence en inhibant ou augmentant son intensité, voire décaler la valeur d'émission (Kasten, 1993; Shapiro, 2000; Sjöback et al., 1998). Le besoin en oxygène est particulièrement limitant pour les techniques de fluorescence, celui-ci étant nécessaire par exemple à la maturation de la GFP, les milieux anaérobies demandent l'utilisation de fluorophores particuliers. Une limite plus technique de ces expériences est que chaque caractère étudié nécessite son propre fluorochrome avec ses propres plages de longueur d'onde pour l'excitation et l'émission. Il devient rapidement difficile de concilier les capacités de détection du matériel avec plus de deux ou trois molécules, tout en conservant une séparation suffisamment contrastée entre les pics d'émissions. Finalement, dans le cas des constructions génétiques, la production d'un fluorophore représente un coût métabolique pour la bactérie qui peut biaiser le comportement de la cellule.

Une technique récente et indépendante du taux d'oxygène fait appel à l'activation de fluorescence par glissement de conformation lors de l'absorption d'un tag (Fluorescence Activating and absorption Shifting Tag ou FAST) (Plamont et al., 2016). La molécule FAST n'est pas fluorescente en elle-même et il en va de même pour le fluorogène HBR/HMBR nécessaire à son activation. Le fluorogène possède une bande d'absorption et peut être introduit dans le milieu à tout moment pour activer la FAST (Figure 3). Lors de la liaison à la FAST, la $\lambda_{excitation}$ du fluorogène glisse vers le rouge et le rendement quantique de la fluorescence augmente fortement ce qui permet l'émission et la détection du signal (Plamont et al., 2016). Ce nouveau système est avantageux car il

est relativement petit (la moitié du poids moléculaire de la GFP) tout en conservant une fluorescence d'intensité comparable. Il est également facilement inactivable par retrait du fluorogène et hautement dynamique pour son activation/répression. Enfin, comme chaque fluorogène est spécifique d'une FAST, l'activation séquentielle de différents fluorophores est réalisable et permet l'utilisation de combinaisons de fluorophores dans le même échantillon. Une version plus avancée de la FAST pour tester l'interaction protéine-protéine est la splitFAST qui emploie deux fusions traductionnelles portant chacune une partie de la FAST. Si les protéines interagissent, la FAST peut se former et seule la forme complète peut lier le fluorogène et produire de la fluorescence témoin de l'interaction directe (Tebo and Gautier, 2019).

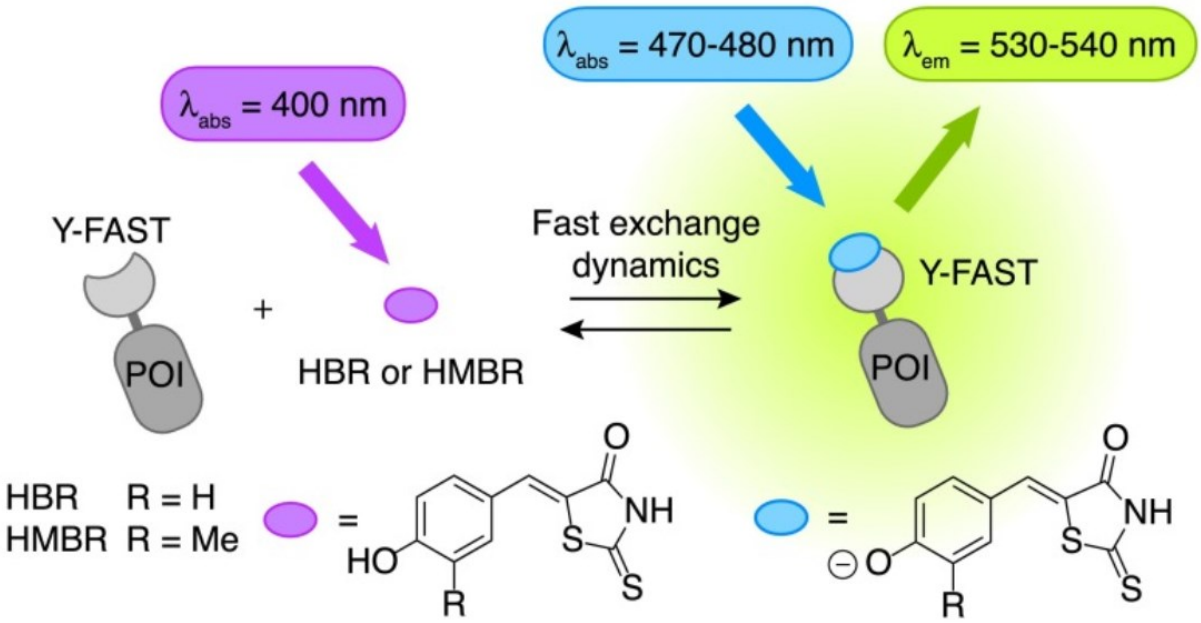


Figure 3 : Yellow-FAST permet le marquage de protéines par fusion traductionnelle *in vivo*. Le fluorogène HBR/HMBR se lie à Y-FAST et permet l'activation de l'expression de fluorescence marquant la protéine d'intérêt (POI). Le décalage vers le rouge (*red shift*) de l'absorbance et l'émission de lumière est dû à un changement de conformation et l'ionisation de la molécule.
D'après (Plamont et al., 2016).

Parmi les études en cellules uniques par fluorescence, il est nécessaire de faire une distinction entre, d'un côté les techniques qui permettent d'étudier des bactéries individuelles suspendues dans un milieu liquide (cultures planctoniques), de l'autre les

techniques qui permettent ces analyses pour des bactéries dans des biotopes structurés (matrice alimentaire ou synthétique, hôte animal ou végétal…).

2.2) La fluorescence pour analyser les bactéries planctoniques à l'échelle de la cellule unique

Les études classiques de fluorescence en cultures planctoniques ne permettent pas le suivi en cellule unique (lecteur de plaque) ou ne sont pas adaptées à une étude dynamique (microscopie classique). Les techniques de microfluidique où un faible volume de réactif peut être manipulé plus finement, permettent une meilleure approche de la physiologie cellulaire.

La cytométrie en flux

En 1934, Moldovan invente le comptage capillaire qui sera popularisé une fois les marquages fluorescents devenus plus facilement disponibles dans les années 1950 (Moldovan, 1934). Le comptage évolue en 1972 avec le "*Fluorescence activated cell sorter*" (FACS) qui permet de trier les cellules. La cytométrie en flux consiste en un comptage d'une suspension bactérienne dans un capillaire où une série de microgouttelettes qui passent devant un laser permet de déterminer par la diffraction de la source lumineuse la présence des cellules. De nombreux paramètres peuvent être déterminés par la perturbation du trajet lumineux comme la taille (FSC , *Forward Scatter*), la granularité (SSC, *Side Scatter*), la forme ou même la viabilité de la bactérie (Álvarez-Barrientos et al., 2000; Vives-Rego et al., 2000). L'analyse de la fluorescence est également possible, permettant ainsi de trier les phénotypes. Mais l'avantage principal de la technique repose sur sa rapidité d'exécution avec pour les machines les plus récentes, la possibilité de traiter plus de 100 000 évènements par seconde, et l'automatisation de la collecte des données (Hammes et al., 2012). L'étude statistique des cellules uniques peut ainsi être rapportée à des populations entières. Les limitations de la cytométrie se trouvent avant tout dans le stress mécanique infligé aux cellules qui sont fragilisées, ce qui peut avoir des conséquences sur les expériences ultérieures. Il

est également impossible de suivre le devenir d'une cellule donnée, seulement l'évolution de la population. Ces points sont en particulier problématiques pour l'observation dans le temps de la croissance et réponse cellulaire sur plusieurs générations. Il existe cependant trois autres techniques de microfluidique permettant la culture de la cellule unique (Figure 4).

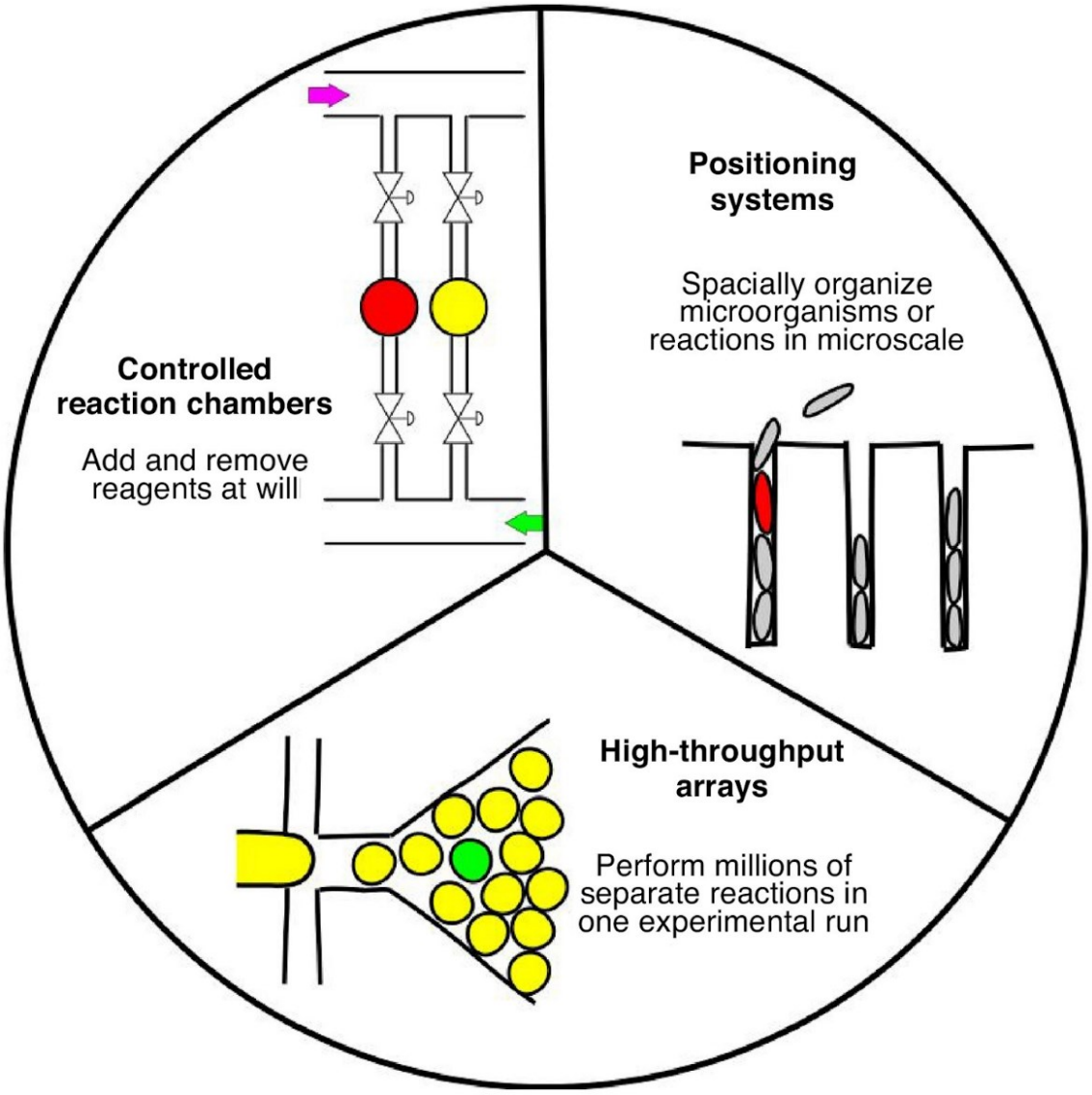


Figure 4 : Trois techniques de microfluidique avec croissance en milieu contrôlé. Quadrant à gauche, les chambres à réaction sont contrôlées avec un flux réglable de milieu frais. Quandrant à droite, culture en micro-puits permet un suivi générationnel. Quadrant du bas, l'encapsulation permet une étude physiologique unicellulaire pouvant être adaptée à une grande population.
D'après (Scheler et al., 2019).

Les chambres à réactions contrôlées (micro-canaux) et l'étude temporelle en cellule unique

Souvent comparé à un système d'expérimentation miniature : "*microfluidic lab-on-chip*" (MLOC), cette technique permet une observation de longue durée en cellule unique et consiste en une série de canaux et de chambres avec un passage des fluides contrôlé soit par des valves pneumatiques (*Quakes valves*), des gradients thermiques (Karbalaei et al., 2016; Sammarco and Burns, 1999), des valves mécaniques (Brett et al., 2011), des champs magnétiques (Qian and Bau, 2009) ou des forces acoustiques (Wiklund et al., 2012). Il est ainsi possible de régler précisément la composition et la quantité de produits dans l'environnement de la cellule. La technique accommode les dispositifs en parallèle sur des centaines de canaux (Watterson et al., 2020), ce qui permet l'étude des interactions au niveau populationnel. Le système est hautement modulable et de nombreux articles décrivent l'utilisation de senseurs, filtres, séparateurs, mixeurs, dispenseurs et pompes pour une étude spécifique (Juncker et al., 2002; Lee et al., 2005; Zhang et al., 2017).

Observation de la diversification génique et phénotype des lignées

La "*mother machine*" mise au point par l'équipe de Wang *et al.* en 2010 représente une avancée obtenue en combinant la microfluidique et l'imagerie cellulaire pour suivre l'évolution au cours du temps du devenir d'une lignée issue d'une cellule unique. Un canal principal par lequel circule en permanence un afflux de nutriments est bordé de puits. Au sein de ceux-ci a été isolé une cellule mère dont la largeur est équivalente à celle du puits pour diriger l'élongation et la septation de telle manière que les cellules fille soient poussées vers l'extérieur du puits où le surplus de bactéries sera évacué par le courant. Avec ce type de système, il est possible de suivre en parallèle l'arbre généalogique de plusieurs groupes de cellules restreintes dans leur puits. La position des cellules dans le puit renseignent sur leur position dans l'arbre, ce qui centre l'étude sur l'analyse de l'hétérogénéité intrinsèque (Araci and Brisk, 2014; Kiviet et al., 2014). Cependant, ces mêmes conditions signifient aussi que les interactions de populations

sont limitées, que des gradients de nutriments/métabolites peuvent s'installer dans le puits et que la binarisation des cellules pour le tracking temporel est complexe, même avec des logiciels spécialisés comme ImageJ (Arnoldini et al., 2014).

L'encapsulation pour tester en série des réactions cellulaires

Cette technique consiste en un flux de culture auquel on joint un flux laminaire contenant les réactifs avant la ségrégation des cellules par segmentation du flux principal. Cette étape d'encapsulation est réalisée par deux flux perpendiculaires contenant un liquide lipidique et fluoré, à la fois hydrophobe et lipophobe, ce qui garantit que les molécules organiques ne diffuseront pas, comme décrit par Scott en 1948 et Simons en 1952 (Scott, 1948 ; Simons et al, 1952). La répartition des cellules suit une loi de poisson: $P(X=K)=[(e^{-\lambda})/(K!)]\lambda^{K}$, où P(X=k) est la probabilité d'avoir k cellules par gouttelettes et λ est le nombre moyen de cellules par gouttelettes (Clausell-Tormos et al., 2008; Huebner et al., 2007). Chaque gouttelette est son propre espace scellé, l'équivalent d'un puits de microplaque. Les micro-gouttelettes permettent de limiter au µL, voire quelques pL, le milieu dans lequel la réaction test a lieu, l'utilisation des réactifs et échantillons cellulaires est de fait très efficace. Une fois l'émulsion eau dans huile réalisée, le mélange des deux milieux aqueux dans chaque gouttelette permet de révéler le caractère étudié par microscopie classique. L'analyse des caractéristiques d'une bactérie (génomique, épigénétique, niveau de transcription, protéomique et métabolique) à l'échelle de la cellule unique peut ainsi être réalisée sur une culture entière (Beer et al., 2007; Frenz et al., 2008; Wang et al., 2010; Wheeler et al., 2005). L'utilisation d'un champ magnétique lié à un détecteur de fluorescence permet également de réaliser un tri cellulaire comme pour un FACS (Mazutis et al., 2013).

Malgré les attraits dus à la possibilité de réaliser des études en parallèle de manière massive et la capacité de suivre dans le temps les résultats de chaque cellule unique, l'encapsulation possède l'inconvénient majeur de nécessiter une observation dans un

milieu très homogène et éloigné des conditions rencontrées dans l'environnement. L'isolement dans un compartiment ne permet pas de tester les réactions dues à un mode de vie en communauté de la bactérie tel que le biofilm.

2.3) L'imagerie de fluorescence de bactéries en milieux structurés

Les matrices alimentaires sont des milieux complexes, leur rhéologie et texture sont impactées par les interactions de nombreux composés. Identifier quelles molécules participent à la structure et décrire leurs rôles dans l'émulsion, la viscoélasticité ou la gélification est un exercice difficile. Par exemple, l'avancée de la protéolyse des acides aminés prend part à l'élasticité d'un fromage (Lacou et al., 2016). Maintenir une telle complexité sur un milieu modèle nuirait aux expériences en raison de la difficulté à reproduire la balance exacte des composés et les différentes propriétés optiques/fluorescentes de ces derniers. Ainsi, lors de la formulation des milieux synthétiques il est préférable de se limiter à un ou deux agents gélifiants pour réduire la complexité de l'écosystème, mais l'éloigne également de la matrice alimentaire que l'on souhaite modéliser.

L'agent responsable de la structure du gel va définir la dynamique des interactions entre population bactérienne et les forces visco-élastiques (Zhang et al., 2021). La morphodynamique des microcolonies est donc liée au choix fait en amont de la préparation de l'hydrogel.

Au niveau de la littérature des hydrogels à vocation alimentaire, la gélification par des protéines, qui donnent des gels granuleux (Aguilera et al, 2018), est généralement évitée et on leur préfère les polysaccharides d'origine naturelle comme l'agarose (Alehosseini et al., 2019; Yin et al., 2020), la gélatine (Antwi et al., 2006; Król and Jarmoluk, 2016), les pectines (Dafe et al., 2017; Tarifa et al., 2021), le chitosane (Qu and Luo, 2020), les alginates (Oberoi et al., 2021) et plusieurs types de gommes comme le xanthane (Verheyen et al., 2018), la guar et la gomme d'*Acacia arabica* (Clark et al.,

1993). L'amidon est aussi parfois utilisé en association avec un autre élément gélifiant pour modifier la texture du gel (Dafe et al., 2017).

La microscopie photonique conventionnelle n'est pas adaptée aux études dans ces milieux structurés en raison de la fluorescence brouillée qui apparaît dans les échantillons 3D. Des techniques adaptées ont dû être mises au point par sectionnement optique, excitation non linéaire du plan et déplacement de l'objectif hors de l'alignement axial.

La microscopie confocale laser à balayage (MCLB)

La microscopie conventionnelle dite à champ large utilise un éclairage de fond pour illuminer toute la hauteur de l'échantillon. Ainsi, même s'il existe un plan focal de l'objectif observé avec un maximum d'intensité, il reste toujours un bruit de fond venant des couches en dehors du focus. Pour observer en détail un plan dans un volume, il devient nécessaire de préparer des coupes fines au microtome, une préparation restrictive empêchant l'étude en 3D et ayant un impact lourd sur le devenir des cellules. Une solution non-invasive est finalement présentée en 1955 par Marvin Minsky (Minsky M, 1957 patent), qui consiste à répartir le biovolume en un empilement (*stack*) virtuel de plans en 2 dimensions, appelées sections optiques, qui pourront être recomposées en une image en 3 dimensions (Lichtman and Conchello, 2005). Pour ce faire, une membrane est placée sur un plan conjugué du plan focal de l'objectif, d'où le terme confocal, et dispose d'un orifice de très faible diamètre (pinhole) placé à la verticale du plan focal illuminé.

La configuration classique d'un microscope confocal moderne (Figure 5) inclut un laser qui remplace les anciennes lampes à mercure, un filtre acousto-optique adaptable (AOTF) qui module l'intensité et la longueur d'onde du faisceau et un "*Acousto-Optical Beam Splitter*" (AOBS) qui affine la séparation de deux longueurs d'onde d'excitation proche. Cet assemblage est suivi d'un miroir dichromatique qui reflète une longueur

d'onde courte (haut niveau d'énergie, valeur d'excitation) mais laisse passer une longueur d'onde longue (bas niveau d'énergie, valeur d'émission) et d'un jeu de quatre miroirs formant un scanner en tandem (FOV-*Resonant*) pour orienter le faisceau et lui permettre de balayer l'échantillon. Un jeu de lentilles dans l'objectif concentre les rayons émanant de la source vers l'échantillon au premier passage et concentre la fluorescence de l'échantillon vers la tête d'épingle (*pinhole*) au second passage. Enfin, en raison de la faible intensité de signaux reçus, un tube photomultiplicateur (PMT) est nécessaire pour amplifier la force du signal qui sera reconstitué par ordinateur sur un écran.

En raison de l'angle d'approche différent des signaux en fonction de leur profondeur Z dans l'échantillon, le chemin optique des plans hors focus est bloqué et seul le plan observé est visible. Comparé au champ large, la technique confocale permet également d'augmenter la netteté des axes XY, la faisant passer de 0,5 µm à 0,2 µm (Pawley, 2006) et de 1,6 µm à 0,7 µm en Z (Paddock and Eliceiri, 2014). La microscopie confocale n'illumine pas le plan horizontal dans sa totalité, il est nécessaire de balayer la surface du plan avec le point focal pour construire une image. Historiquement, le premier choix fut de bouger l'échantillon et de garder le faisceau lumineux stable (*Stage Scanning*) pour éviter des effets indésirables dus à des défauts dans la lentille. Le second choix consistait à faire bouger le faisceau sur l'échantillon (*Beam Scanning*) et fut préféré en recherche biologique, où bouger l'échantillon risquerait de le décaler et de réduire la résolution finale. Cette technologie était cependant encore lente, coûteuse et peu sensible. C'est au début des années 90 que l'invention de la MCLB (White et al., 1987), qui remplaça la source lumineuse par un laser, et utilisant des miroirs contrôlés par un galvanomètre et l'informatique pour reconstituer une image, que la technique se popularisa.

La microscopie confocale a des avantages certains pour l'étude des structures en 3D (Figure 6) et en particulier les agrégats de cellules uniques (figure 7). La microscopie

confocale pose cependant un dilemme du fait que plus le pinhole est étroit, plus la section optique est fine, ce qui donne une meilleure résolution verticale mais moins de photons détectés donc un signal plus faible. Le photomultiplicateur compense cet effet jusqu'à un certain point. La puissance du laser est quant à elle limitée par le photobleaching et la mort des cellules exposées à des radiations lumineuses trop fortes, en particulier celles tirant vers le bleu.

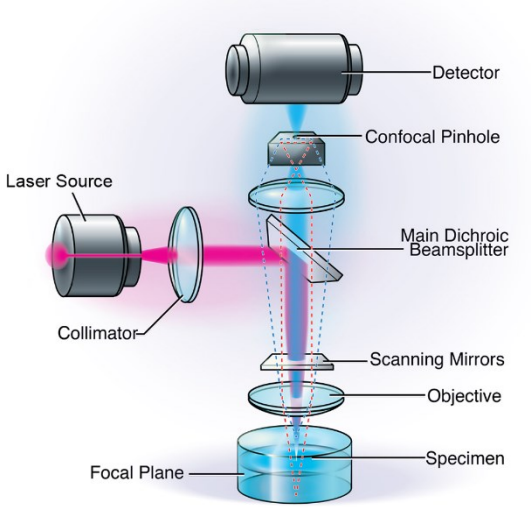


Figure 5 : Schéma général d'un microscope confocal laser à balayage. Le laser émis par la source lumineuse est concentré en un point sur le plan de l'échantillon observé (chemin rose). La fluorescence réémise (chemin bleu) est concentrée par une lentille de telle manière que la lumière provenant de plans sous le spécimen (pointillés rouge) ou d'au-dessus du spécimen (pointillés bleu) soit interceptée par la membrane du pinhole avant d'atteindre le détecteur (principe du confocal). Le miroir de scan permet de diriger le laser pour balayer l'échantillon de ligne en ligne et former une image.
D'après (Riddle et al., 2017).

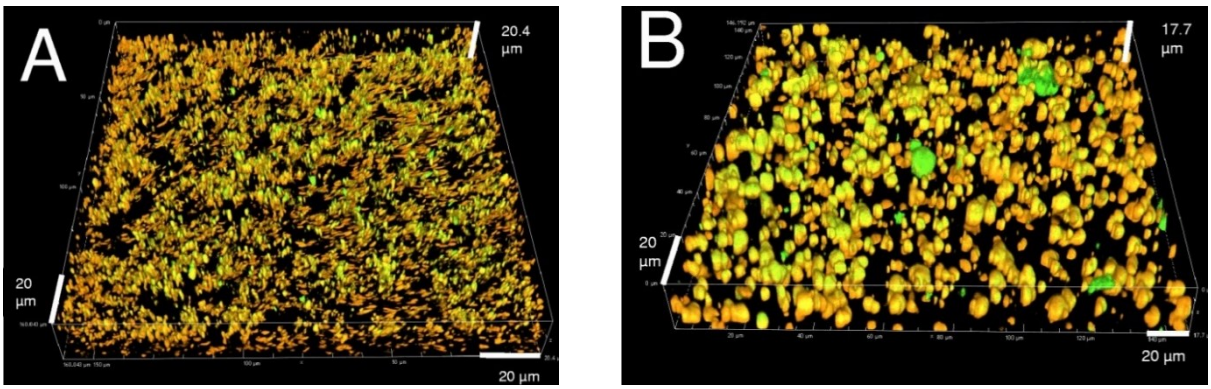


Figure 6 : Structures bactériennes observables en 3D par MCLB. *L. monocytogenes* est cultivée dans une émulsion (A) ou la même émulsion gélifiée (B) contenant 1% de lipides. Les gouttelettes lipidiques

sont colorées en orange, les cellules de *L. monocytogenes* sont vertes et les régions jaunes indiquent une croissance de la bactérie à l'interface entre les phases aqueuse et lipidiques.
D'après (Verheyen et al., 2019).

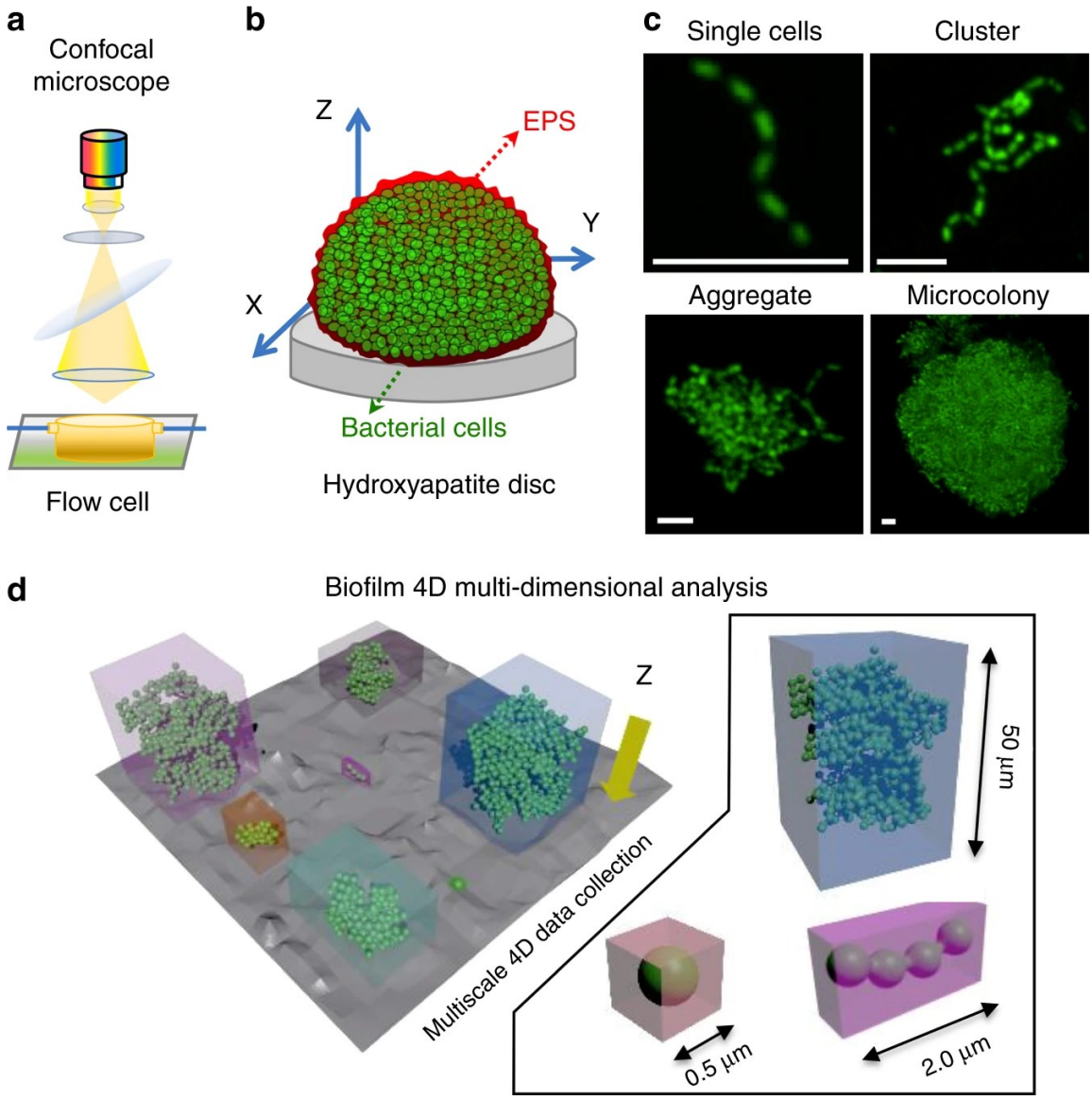


Figure 7 : Exemple d'une étude en cellule unique par MCLB. a) Montage du microscope pour l'étude en cellule d'écoulement. b) Représentation schématique d'un biofilm avec les cellules (vert) contenu dans un exopolysaccharide (rouge) sur la surface d'hydroxyapatite du puits (gris). c) Projection en Z des étapes de la formation du biofilm vue en MCLB depuis des "colonisers" (cellules uniques, cluster, agglomérat) jusqu'à la structuration en une microcolonie de surface. Les échelles sont fixées à 5µm.
D'après (Paula et al., 2020).

L'imagerie multiphotonique

La capacité à exciter un fluorophore par absorption simultanée de deux (voire trois) photons de longueur d'onde inférieure à $\lambda_{excitation}$ fut théorisée en 1931 par Goppert-Mayer en 1931 et démontrée formellement en 1961 par Kaiser et Garret (Figure 8) (Goppert-Mayer, 1931 ; Kaiser et Garret, 1961). Il faut cependant attendre le début des années 90 pour voir les premiers usages de cette activation par multiphoton dans un cadre de recherche (Denk et al., 1990). L'inconvénient d'utiliser un seul laser est que les photons émis doivent être dans la gamme d'excitation du fluorophore utilisé, c'est à dire entre 400 et 600 nm, ce qui est sub-optimal en terme de pénétration des surfaces en raison des phénomènes d'absorption et de diffraction (Ntziachristos, 2010), de photobleaching de la protéine et de toxicité cellulaire par irradiation (Chen et al., 2002). Le concept du multiphoton ouvre une nouvelle plage dans l'infrarouge proche (Near Infra-Red ou NIR) de 700-1100 nm où les tissus vivants sont perméables à la lumière (Kobat et al., 2009; Theer et al., 2003). La mortalité cellulaire est fortement réduite grâce à l'utilisation d'une longueur d'onde plus faible et le photobleaching est plus lent à apparaître grâce au meilleur contrôle de la région excitée (Helmchen and Denk, 2005). Cela implique aussi une meilleure résolution en Z puisqu'il n'y a plus de fluorescence provenant d'autre plan que celui illuminé simultanément par les deux rayons, le multiphoton est même caractérisé d'être "*point to point*" en raison du volume inférieur au fL lluminé (Figure 9). Ceci rend la technique particulièrement intéressante pour une observation au niveau de la cellule unique dans des populations denses comme les biofilms et microcolonies. Le laser du multiphoton ne peut pas être continu pour des raisons thermiques et doit donc être pulsé, ce qui permet d'opérer à faible puissance de l'ordre du mV malgré une forte demande en énergie (10 gigawatts). La polarisation temporaire de l'échantillon à chaque cycle du laser permet d'ailleurs l'emploi de techniques indirectes d'exploration de la matrice (Yue et al., 2011). En contrepartie, le multiphoton est moins efficace qu'avec un laser unique pour ce qui est de la vitesse d'acquisition. En effet, il est difficile d'obtenir un point focal dans l'espace et le temps en raison de l'atténuation dans le milieu et l'émission asymétrique des photons. Ces

deux paramètres sont également vitaux pour maximiser le contraste. A haute vitesse d'acquisition cela pose problème car le nombre de photons à exploiter n'est plus suffisant pour la détection quand on passe sous la barre de 1 µS par pixel. Cela rend le suivi (*tracking*) de cellules mobiles plus difficile en multiphoton. La technique n'est également pas exploitable pour tous les fluorophores avec en particulier GFP qui doit être adapté pour ce rôle et DsRed dont l'émission passe du rouge au vert (Drobizhev et al., 2011; Marchant et al., 2001).

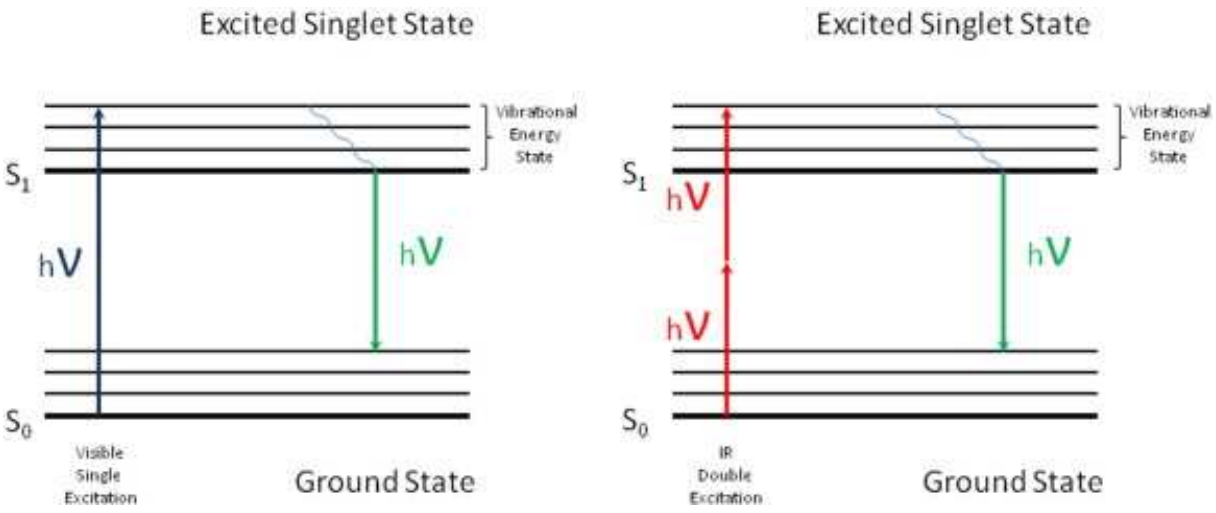


Figure 8 : Principe de base de l'excitation par multiphotons. Il est possible d'amener une molécule à un état d'énergie supérieur (dit excité) soit par irradiation avec un photon unique dans la gamme visible du spectre lumineux (phénomène linéaire utilisé en *Wildfield* et MCLB), soit par stimulation simultanée avec plusieurs photons de plus faible énergie (infrarouge) mais dont la somme réunit une énergie équivalente au photon du spectre visible (non-linéaire 2P et 3P-LSM).
D'après (Bridier et al., 2015).

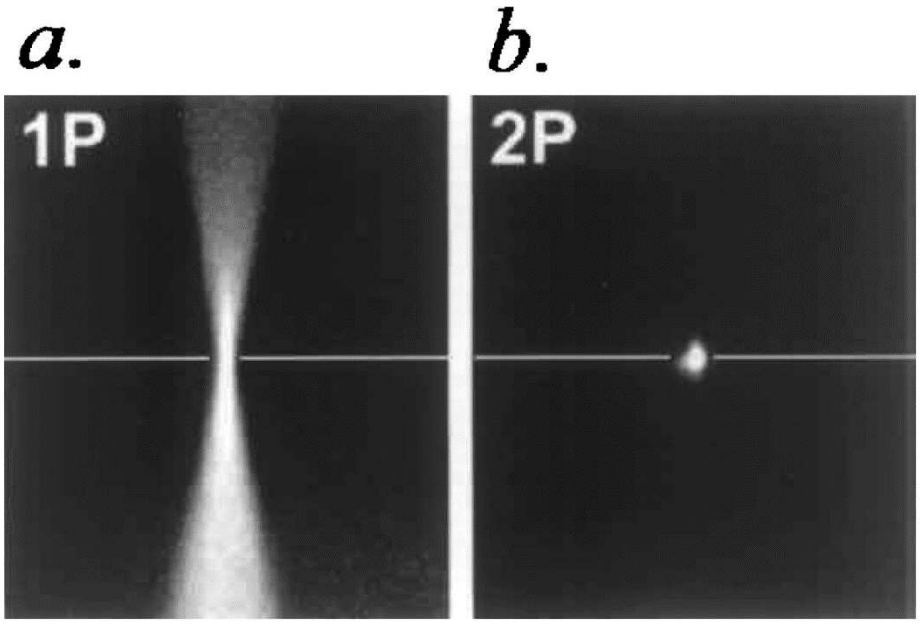


Figure 9 : Visualisation du trajet de l'activation de la fluorescéine sur les axes X et Z. Localisation de l'expression de fluorescence en X et Z lors d'une activation par un seul laser (photon unique en a) ou par plusieurs lasers plus faibles s'entrecroisant (multiphoton en b). La ligne blanche indique le plan focal que l'on souhaite observer.
D'après (Soeller and Cannell, 1999).

La microscopie à feuille de lumière ou «*LightSheet Fluorescence Microscopy*»

Un écueil récurrent de la microscopie est l'atténuation du signal en fonction de la hauteur de l'échantillon observé. Une solution permettant de fournir une intensité d'excitation stable sur l'axe Z est de ne pas être dépendant de la distance de Rayleigh pour la résolution. Placer l'objectif perpendiculairement à un axe illuminé par le laser atteint cet objectif car l'épaisseur du faisceau devient le point déterminant, d'où le terme de feuille de lumière (*lightsheet fluorescence microscopy* ou LSFM) (Figure 10). La première description de cette disposition date de 1903 par Siedentopf et Zsigmondy et il faut attendre 1993 pour un le développement d'un appareil moderne (Voie et al., 1993; Siedentopf et Zsigmondy, 1903). Contrairement aux appareils coaxiaux, la LSFM dispose de nombreux avantages, le photobleaching et la toxicité sont beaucoup plus faibles, même en comparaison au multiphoton. Ceci est dû au fait que seule une section

est exposée au laser et ce dernier n'a pas besoin d'être de forte intensité. Un autre avantage est que la disposition du matériel se prête parfaitement à l'analyse 3D en matrice solide, l'échantillon devant être maintenu dans l'espace (Reynaud et al., 2014) et pouvant être tourné pour présenter différentes facettes devant le détecteur. La LSFM fonctionne comme un appareil à champ large sans besoin de balayer l'échantillon mais n'a pas à se soucier de la fluorescence incidente des autres plans (Ahrens et al., 2013) et est donc plus rapide que les techniques confocales avec un meilleur contraste. Il est également possible d'utiliser deux objectifs se faisant face pour doubler le signal recueilli. Grâce à la géométrie particulière du chemin optique, il est possible de faire des observations linéaires et non linéaires en LSFM (Olarte et al., 2018) ce qui permet de mieux étudier les structures 3D car l'analyse non linéaire élimine la majorité de l'excitation due à la diffraction.

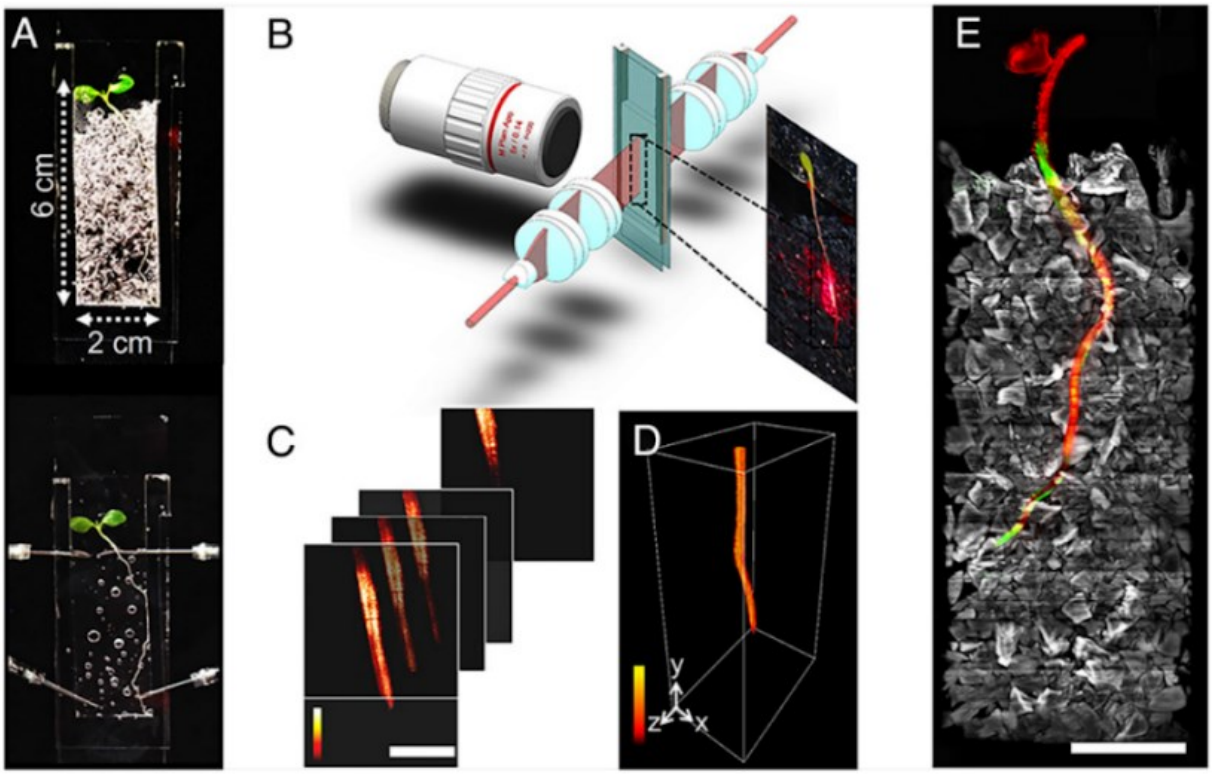


Figure 10 : Microscopie à feuille de lumière sur un rhizome de plantes. A) En haut, échantillon de mésocosme avec un terreau transparent contenant *B. subtilis*. En bas, échantillon saturé en liquide réfractant d'indice constant. B) principe du microscope avec une feuille de lumière générée par le passage du laser dans un Powell lens et deux lentilles cylindriques. Le signal de fluorescence diffracté

par les structures est capté par un objectif non co-axial. C) Les données sont obtenues par translation verticale et horizontale de l'échantillon devant l'objectif. Échelle 2 mm. D) Reconstitution 3D à partir de scans verticaux. E) Volume 3 × 60 × 20 mm^3 contenant une pousse de plantes (rouge) et des particules de terreau (gris), ainsi que *B. subtilis* qui est marqué par la GFP (vert). Échelle 5 mm.
D'après (Liu et al., 2021).

L'analyse d'image multidimensionnelle permettant de décrire les populations microbiennes spatialisées

Avec les progrès de la capture d'image, il est rapidement apparu qu'une étape importante et souvent limitante de l'analyse spatiale des communautés microbienne est l'analyse d'images et l'extraction des paramètres d'intérêt (e.g., position, sphéricité, intensité lumineuse des différents canaux, nombre, sillage, vitesse, aire, volume, diamètre). Même assisté par ordinateur, un traitement demandant des réglages manuels pour chaque prise de vue devient difficilement réalisable quand une seule campagne d'étude peut générer plusieurs milliers d'images. L'analyse d'images à haut débit nécessite une détection automatique d'objets à l'aide d'algorithmes adhoc. La méthode classique est l'attribution au bruit de fond de chaque pixel ne passant pas un seuil d'intensité, suivi d'une reconnaissance morphologique sur les pixels restant par correspondance avec une formule mathématique décrivant la forme d'une cellule (Gonzalez and Woods, 2018). Un outil plus puissant et plus adaptable à différents objectifs est l'architecture en réseau de neurones convolutifs où plusieurs formules mathématiques organisées par niveau forment une série de filtres pour l'attribution des données (Stringer et al., 2020). Ce système est également plus long à mettre en place car il nécessite d'entraîner le logiciel avec des images "types" sélectionées par l'utilisateur qui fixe également quelle réponse est attendu pour chaque exemple. Ceci crée ainsi le risque de causer une reconnaissance spécifique de paramètres non pertinents si ceux si sont présents dans les images utilisées comme exemples, en particulier si celles ci ne sont pas assez nombreuses et variées (Hinton et al., 2012; Jeckel and Drescher, 2021).

Un point important pour l'étude des biofilms et colonies est que la segmentation devient rapidement un challenge lorsque les cellules sont jointives ou font partie d'une communauté dense (Vicar et al., 2019). Il peut être alors avantageux de passer à une segmentation sémantique (les individus d'une même classe ne sont pas départagés) et réaliser une analyse sur des volumes prédéfinis (cube cytométrie) plutôt que sur chaque instance (Figure 11).

De nombreux logiciels de traitement d'image existent, et alors que certains programmes ne font que des segmentations, ou uniquement de l'analyse de paramètres, d'autres font les deux e.g. Image J et son plug-in microbe J (Ducret et al., 2016; Gómez-De-Mariscal et al., 2019; Schneider et al., 2012). Il est parfois possible de devoir coder à un niveau plus ou moins élevé e.g. BactMAP (van Raaphorst et al., 2020). Le type de données recherché détermine aussi quel logiciel spécialisé est le plus à même de convenir aux besoins e.g. COMSTAT 1&2 (Heydorn et al., 2000; Vorregaard, 2008) permettent la segmentation et l'analyse de surface du biofilm mais ne permettent pas l'analyse des propriétés internes ou la visualisation de données (Hartmann et al., 2021).

Dans le cadre de ce travail, IMARIS (Oxford Instruments) et Biofilm Q v0.2.2 (Hartmann et al., 2021) ont été retenus. Ces deux logiciels sont complémentaires dans leurs capacités, IMARIS dispose d'une segmentation par instance, permet la visualisation en 3D des stacks et la récupération des propriétés morphologiques générales des microcolonies de microcolonies ainsi que la traque et quantification de la motilité des cellules uniques. Biofilm Q est de son côté plus avantageux pour une segmentation sémantique et l'étude des paramètres internes de la microcolonie et en particulier du ratio de fluorescence en fonction d'une répartition axiale.

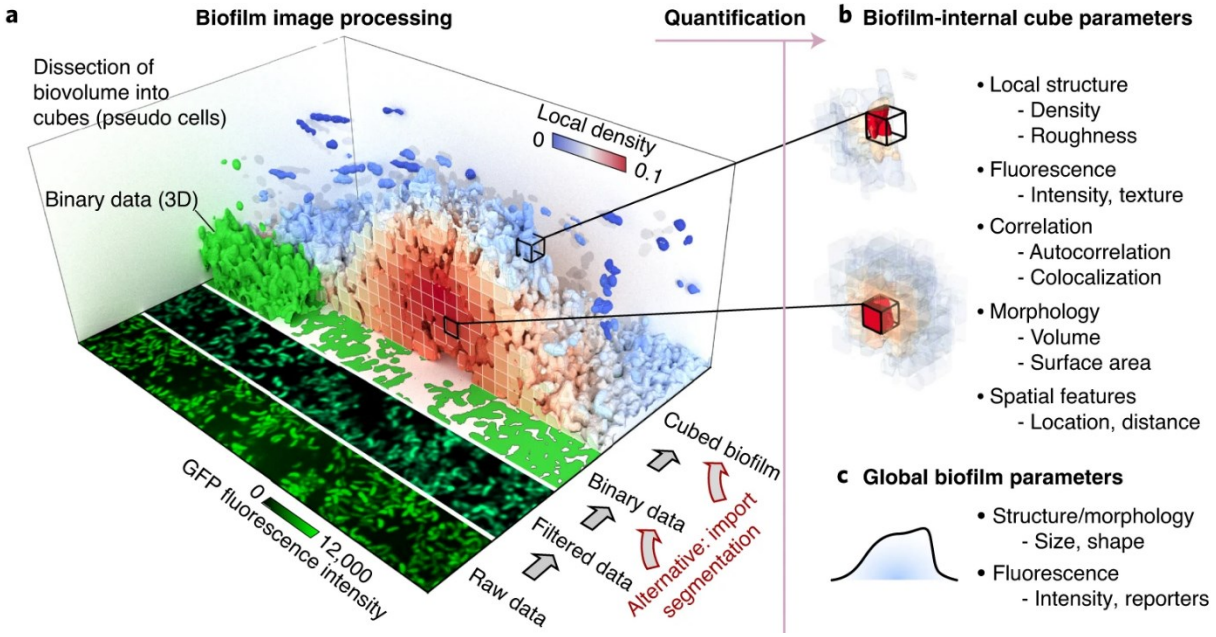


Figure 11 : Quantification des communautés microbiennes par segmentation sémantique et cube cytométrique dans Biofilm Q. a) pipeline du processus d'analyse de l'image, l'intensité de fluorescence est filtrée à partir d'un seuil et la binarisation des cellules qui en résulte permet de créer une représentation sémantique du biofilm. Celui-ci est ensuite segmenté en cube de taille prédéfinie et une analyse des paramètres b) est réalisée sur les propriétés du biofilm dans chaque cube. Alternativement l'étude peut être réalisée sur le biofilm tel quel sans segmentation supplémentaire (c)
Adapté de (Hartmann et al., 2021).

3) Les microorganismes pathogènes d'origine alimentaire et les fonctions d'intérêt pour la croissance en matrice alimentaire

La croissance des micro-organismes en matrice alimentaire est complexe et encore mal comprise. Les impératifs et les enjeux de suivi des populations sont cependant très différents en fonctions des micro-organismes e.g. souches d'intérêt technologiques, probiotiques, d'altération ou pathogènes. Ces derniers en particulier sont très surveillés en raison du risque sanitaire qu'ils représentent.

3.1) Situation épidémiologique des bactéries pathogènes d'origine alimentaire

A l'échelle mondiale, l'Organisation mondiale de la santé (OMS) maintient une veille de l'épidémiologie des zoonoses alimentaires par le biais du "*Foodborne Disease Burden Epidemiology Reference Group*" (FERG). En 2010, l'OMS estimait à 600 millions le nombre d'infections dues à des contamination alimentaires (Chlebicz and Śliżewska, 2018). Cependant, au vu du peu de moyens disponibles, de la qualité de suivi très différente selon les pays et des problèmes d'identification de la source infectieuse pour certaines maladies, l'OMS juge que ces informations sont parcellaires et non comparables (Grace, 2015; Hald et al., 2016). Cette veille sanitaire est en particulier faible dans les pays pauvres et en voie de développement qui sont pourtant les plus touchés, ce qui rend difficile l'appréhension de l'incidence réelle (Grace, 2015; Käferstein, 2003).

La surveillance épidémiologique des États-Unis d'Amérique est gérée par le "Center of Disease Control and Prevention" (CDC) et en particulier par le "*Foodborne Diseases Active Surveillance Network*" ainsi que "*The National Institutes of Health*" (NIH). Aux USA, l'incidence de *Campylobacter* est de 20 cas pour 100 000 habitants, 8,4 cas pour 100 000 habitants pour *Salmonella*, suivi par *C. perfringens* avec 7 cas pour 100 000 habitants puis ce sont les STEC qui causent 1,8 cas pour 100 000 habitants avec O157:H7 responsable de 36 % des infections, et *L. monocytogenes* présente 0,24 cas pour 100 000 habitants (Batz et al., 2012; CDC, 2011; Sanyaolu, 2016). Les pertes économiques dues à ces différentes infections alimentaires sont estimées à 77 milliards de dollars chaque année (Scharff, 2012). Les USA sont l'équivalent le plus proche en termes de population, de type de produits consommés et de technologies de l'Union européenne. Cependant, ces données ne sont pas comparables avec les incidences européennes en raison de la divergence des sources et de la méthodologie entre les entités de surveillance (Glass et al., 2014; Scallan et al., 2011). La hiérarchie de l'incidence des infections est cependant comparable à ce qui se voit en Europe.

En Asie, et au Japon en particulier, les infections à *Campylobacter* sont en forte progression (Kaakoush et al., 2015). En plus des très nombreux cas de *Salmonella enterica,* le sérovar *Salmonella* Weltervreden y est également très commun bien que pratiquement inexistant ailleurs (Jain et al., 2015). Enfin, la consommation de poissons tropicaux est beaucoup plus élevée, ce qui explique la hausse du nombre d'infections dues à des pathogènes présents dans les estuaires tels que *Vibrio parahaemolyticus* (Alam et al., 2002; Wong et al., 2000).

Dans l'Union Européenne, le processus d'harmonisation de la surveillance des incidents fut amorcé avec la directive 2003/99/EC qui obligea les États membres de l'Union Européenne à collecter et transmettre à la Commission Européenne les données pertinentes sur les agents zoonotiques, le nombre d'infections par ces agents, les résistances aux antibiotiques observées et les épidémies qui surviennent sur leur territoire. La Commission Européenne transmet ces informations à l'Autorité européenne de sécurité des aliments (*European Food Safety Agency* ou EFSA) qui publie un rapport annuel sur la situation de la sécurité sanitaire en UE. La collecte des données sur les maladies fut renforcée avec la décision 1082/2013/EU qui permit la construction d'un réseau de surveillance des maladies transmissibles au niveau européen. En particulier, huit agents zoonotiques sont étroitement surveillés pour leur fréquence chez les animaux d'élevage et leur présence dans les produits destinés à la consommation : *Brucella, Campylobacter, Echinococcus, E. coli* productrices de shigatoxines (STEC), *L. monocytogenes, Mycobacterium bovis, Salmonella et Trichinella* (Figure 12).

Sur la période 2014 à 2019, le nombre de cas annuels d'infections est dominé par deux pathogènes, *Campylobacter* et *Salmonella* avec respectivement 220 000 à 250 000 et 85 000 à 95 000 infections annuelles (soit 40 et 20 cas pour 100 000 habitants). En troisième position, on retrouve les cas d'infection par les STEC avec 6 000 à 8 000 cas annuels (2,2 cas pour 100 000 habitants), dont l'incidence est proche de celle des

yersinioses avec 6 000 à 7 000 cas annuels. *L. monocytogenes* est en cinquième position avec 2 200 à 2 700 contaminations annuels (0,42 cas pour 100 000 habitants) et est suivi par *Echinococcus, Q fever, Tularaemia, Brucella, Trichinella* qui ne dépassent pas de manière régulière le cap des 1 000 cas par an et dont l'incidence varie fortement d'année en année (EFSA, 2019, 2018b, 2017, 2016, 2015).

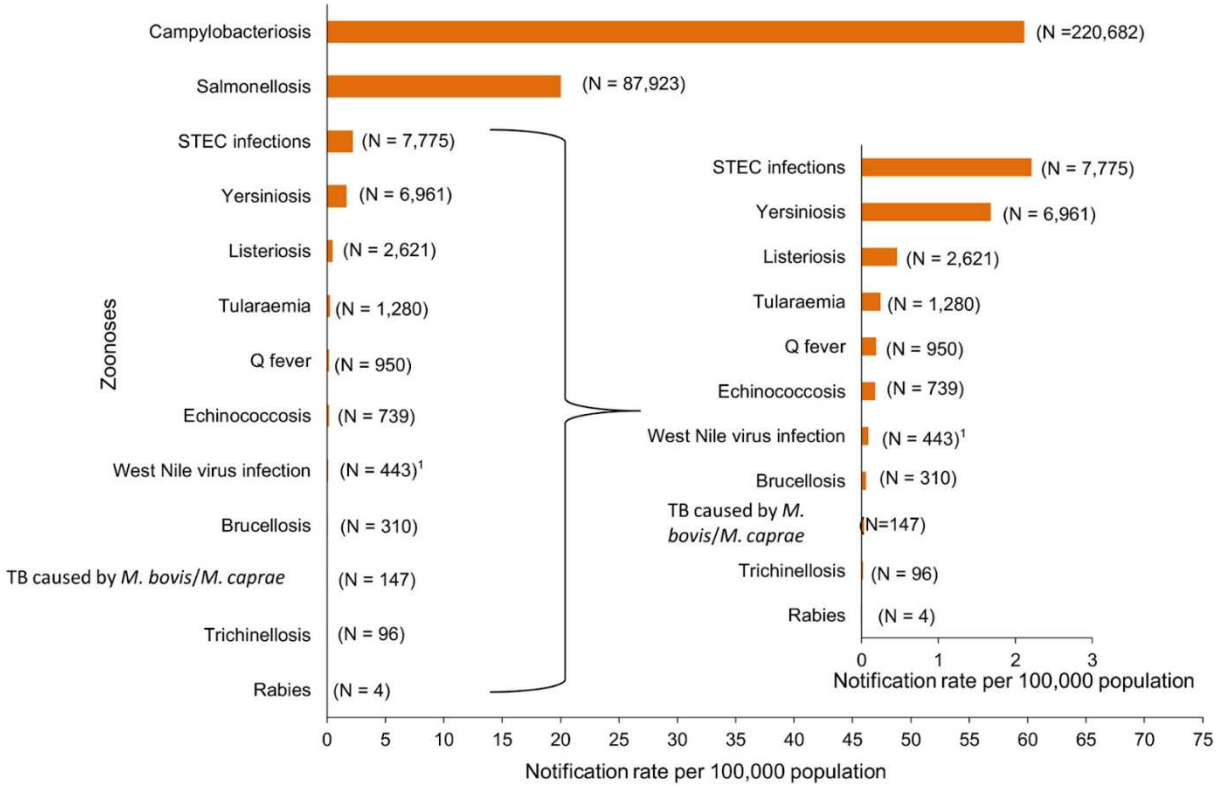


Figure 12 : Nombre de cas signalés (N) et taux de notification des zoonoses humaines confirmées dans l'UE en 2019. Le nombre total de cas confirmés est indiqué entre parenthèses en face de chaque barre, sauf pour le virus du Nil occidental pour lequel le nombre total de cas apparaît.
D'après (EFSA, 2021a).

Deux points importants viennent modifier la modélisation du risque posé par ces pathogènes alimentaires. En premier lieu vient la sévérité de la maladie. En 2019, *L. monocytogenes* ne représente qu'une petite fraction du nombre d'infections alimentaires mais nécessite une hospitalisation dans 92,1 % des cas et l'issue peut être fatale pour 17,6 % des patients. Moins alarmants mais tout aussi sérieux *Brucella* et les STEC recensent 71 % et 38 % d'hospitalisation pour une léthalité dans 1,75 % et 0,21 %

des cas. Les STEC en particulier peuvent provoquer des séquelles lourdes au niveau des reins avec l'apparition de syndrome d'hémolyse rénale (SHU-STEC) en particulier chez les enfants. Ces troubles rénaux sont accompagnés de troubles neurologiques majeurs dans 25 % des cas (Loos et al., 2012) et engendrent des séquelles neuro-motrices permanentes dans 4% des cas (Loos et al., 2017; Rosales et al., 2012).

Le second point qui vient compléter l'analyse du risque est la tendance de progression du nombre d'infections au cours du temps. Si *Campylobacter* et *Salmonella* sont stables sur la période 2014 à 2018 et le nombre de cas de *Brucella* en baisse, *L. monocytogenes* et les STEC poursuivent une lente progression du nombre d'infections qui s'est confirmée sur une période de plusieurs années. Les infections aux STEC sont d'ailleurs passées devant celles dues au *Yersinia* pour le nombre de cas sur la période 2014 à 2018.

L'EFSA est une des sources les plus complètes et précises au monde en termes de données épidémiologiques. Cependant, les données dont dépend l'EFSA viennent d'entités étatiques et organisations européennes avec leurs propres critères et capacités de suivi. L'EFSA met ainsi en garde sur le fait que suivant l'origine, les chiffres peuvent ne pas être comparables pour certaines infections. En particulier, seuls les cas de salmonellose dans les volailles de chair, tuberculose et bruceliose bovine, trichinellose porcine *et echinococcose* sont interprétables entre les membres de l'UE et font l'objet d'un modèle prédictif par l'EFSA au niveau européen. Les *Salmonella* venant d'autres sources, *E. coli* et *L. monocytogenes* ne sont pas suivies avec les mêmes critères par tous les États européens. Enfin pour *Campylobacter, Yersinia,* la fièvre *Q, Francisella tularensis* et *Taenia spp., les* analyses ne sont pas comparables entre les pays.

Au niveau national, la France enregistre 350-400 cas de listériose par an, principalement sporadiques avec au moins une épidémie (2-20 cas) chaque année. Le nombre d'infections est en augmentation depuis 2006 et atteint 0,56 incidents par 100 000

consommateurs en 2019 (ANSES, 2016). Concernant *E. coli*, le sérotype O157 est le plus identifié dans les infections par des EHEC (37 % en 2011, 17% en 2015). Les cas sporadiques sont majoritaires à l'exception de cinq épidémies recensées entre 2011 et 2018. Les cas de SHU sont suivis, en particulier chez les moins de quinze ans, et le pays enregistre 0,74 SHU pour 100 000 habitants en moyenne (ANSES, 2011a).

D'autres pathogènes comme *Brucella* ont vu le nombre de cas chuter de plus de 800 en 1978 à 28 en 2012 et six cas possibles en 2013. Par la suite tous les cas sont liés à une contamination hors du territoire (ANSES, 2014). *Campylobacter* est à 11,8 cas pour 100 000 habitants (2018) mais l'ANSES reconnaît que la surveillance est limitée avec une estimation à 842 cas pour 100 000 habitants (ANSES, 2011b). *Salmonella* spp reste très présent avec 40 % des toxi-infections alimentaires reliés à sa présence (ANSES, 2011c).

3.2) *E. coli* O157:H7 et *Listeria monocytogenes,* deux pathogènes majeurs d'origine alimentaire

Au vu de différent paramètres épidémiologiques, tels que le nombre d'infections zoonotiques, la sévérité de la maladie et la progression de l'incidence dans le temps, *E. coli* O157:H7 (STEC/EHEC), et *L. monocytogenes* se démarquent des autres pathogènes et sont étudiés plus en détails.

Escherichia coli O157:H7

Escherichia coli furent nommés ainsi en l'honneur de Theodor Escherich qui en fit la découverte en 1919. *E. coli* sont des protéobactéries à Gram négatif aéro-anaérobies facultatives en forme de bacilles retrouvées dans tous les animaux à sang chaud où ils sont considérés comme étant un acteur majeur du microbiote intestinal. La bactérie est positive à la catalase mais ne possède pas d'activité oxydative, réduit les nitrates (Singh and Prakash, 2008), et fermente le glucose, ainsi que le lactose dans la majorité des cas. *E. coli* est aussi caractérisé par une motilité péritriche et une grande résistance aux stress acides. Du fait de son abondance, sa résilience et sa facilité de manipulation,

cette bactérie est l'une des plus étudiées et correspond à un des modèles d'étude les plus aboutis.

La plupart des souches *E. coli* sont commensales et considérées sans danger pour la santé et nécessaires au bon fonctionnement du système digestif. Cependant, en cas de troubles du microbiote intestinal ou d'infection des intestins, cette bactérie peut être sur-représentée. *E. coli* est une bactérie naturellement compétente, ce qui lui donne un avantage compétitif sur le long terme, mais permet aussi de récupérer plus facilement des gènes de virulence sous la forme de plasmides ou d'inserts chromosomiques d'origine virale et ainsi de devenir un pathogène infectieux pour l'Homme (Kaper et al., 2004; Palchevskiy and Finkel, 2006). Les *E. coli* pathogéniques peuvent être différenciés entre pathogènes intestinaux et extra-intestinaux. Concernant les *E. coli* pathogéniques de l'intestin, 7 pathotypes peuvent être différenciés (Croxen et al., 2013): les *E. coli* Entéropathogénique (EPEC), Entérohémorragique (EHEC), Entérotoxigénique (ETEC), Entéroinvasive (EIEC), Entéroaggrégative (EAEC), Diffusive Adhérente (DAEC) et Adhérente Invasive (AIEC) (Strober, 2011). Parmi ceux-ci, les EHEC constituent le problème de santé publique le plus sérieux en raison de multiples épidémies avec un pourcentage relativement élevé de cas mortels, tels que les incidents « Jack in the box » de 1993 aux USA où 732 contaminations dans 73 restaurants résultèrent en 173 conséquences graves et 4 morts ou encore la contamination des radis noirs au Japon en 1996 où 9578 personnes furent infectées (Kalgi and Martens, 2015; Pennington, 2010).

Un classement sérotypique permet une meilleure représentation des EHEC avec les cinq sérogroupes les plus communs étant O26, O103, O111, O145, O157 (Karmali, 2004). Cette structuration n'est pas parfaite comme l'atteste le cas de O104:H4 qui est une souche hybride Entéroaggrégative mais n'est pas AEEC ; et toutes les O26, O103, O111, O145, O157 ne sont pas forcément des EHEC ou pathogéniques, mais ce classement permet une gestion du risque plus simple et rapide.

Les EHEC sont commensales dans le tractus digestif de la plupart des ruminants et du gibier comme les cervidés, les lapins et les sangliers (Ferens and Hovde, 2011; Gyles, 2007), à l'exception de O104:H4 qui est principalement d'origine humaine. En conséquence, la viande rouge est un vecteur important de ces infections (Figure 13). Cela se répercute sur les études de contamination de la viande de bœuf qui sont dominées par les EHEC (Tesson et al., 2020). Le sérogroupe O157 est le plus représenté des EHEC chez les bovins avec une prévalence de 0,2% à 48,8% selon les élevages contre 0,4% à 75% pour toutes les autres souches confondues (Hussein, 2007). En outre, parmi les O157, le sérotype O157:H7 est le plus retrouvé chez les patients lors de cas d'épidémies (comme aux USA et Japon mentionnés auparavant) et environ 0,5% des viandes et 0,9% des fromages au lait cru portent O157:H7 qui peut aussi être trouvé dans le lait et les végétaux (Caprioli et al., 2005; Franz and Van Bruggen, 2008; Miszczycha et al., 2012).

Les EHEC font partie de la famille des STEC pour shigatoxine *E. coli*, et sont parfois dénommées VTEC pour *E. coli producteurs de* vérotoxines. Les STEC sont caractérisées par la présence du gène *stx,* codant la Shigatoxine. La présence de ce gène a longtemps été considérée comme nécessaire mais pas suffisante pour la pathogénicité (Caprioli et al., 2005). L'Etat français a récemment publié un arrêté fixant la liste des agents biologiques pathogènes, mettant en application les recommandations du rapport de l'EFSA (EFSA, 2021b) et reclassant ainsi des souches STEC en pathogènes de classe 3* (Arrêté du 06/11/2021; legifrance). La shigatoxine (Stx), également identifiée chez *Shigella dysenteriae 1* et confirmée chez *Shigella flexneri, Enterobacter cloacae and Citrobacter freundii* (Herold et al., 2004), est codée par deux gènes portés par des prophages, potentiellement présents dans les matrices alimentaires (Herold et al., 2004; Yan et al., 2011). Ceci rend l'évaluation du nombre de souches possédant *stx* dans un échantillon difficile par une simple PCR car le gène peut aussi être présent dans des bactériophages et les cellules capables de l'expression de s*tx* dans le tube digestif

peuvent ne pas la produire au moment du test (Caprioli et al., 2005). Enfin, les STEC ont démontré une capacité de croissance en dessous de 15°C, ce qui est peu commun chez *E. coli* (Vidovic et al., 2011). *E. coli* est également moins sensible à la congélation si la croissance a eu lieu à 37°C au lieu de 10°C, ce qui favorise la survie des souches d'origine animale (Semanchek and Golden, 1998).

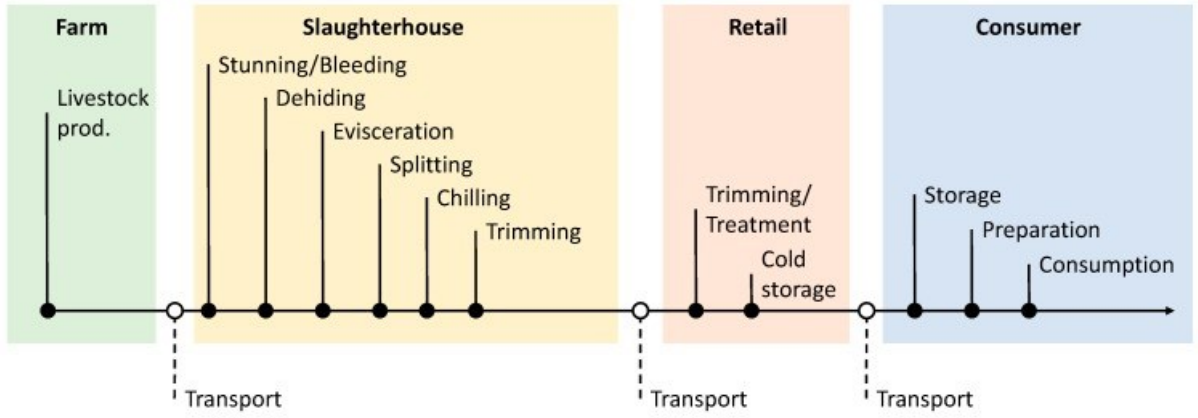


Figure 13 : Étapes de la production de viande bovine où l'évaluation quantitative du risque microbiologique (QMRA) est réalisée. Les études QMRA sont le plus souvent réalisées au niveau du dépeçage des carcasses et de la préparation des plats (19 articles sur 27 considérés par Tesson et al, 2020). Les étapes d'éviscération, réfrigération, élagage, conservation réfrigérée et conservation chez le consommateur sont également souvent au centre de QMRA (15 articles sur 27).
D'après (Tesson et al., 2020).

Lors de l'infection par les EHEC, la bactérie adhère à un entérocyte et promeut la disparition des microvillosités pour permettre à Stx de se regrouper pour former un endosome. Une fois à l'intérieur de la cellule, Stx bloque la synthèse des protéines et conduit à l'apoptose (Valério et al., 2010). Ceci crée une rupture de la barrière intestinale avec pour conséquence l'apparition de diarrhée sanglante. Stx peut également passer dans le sang, par translocation à travers les cellules polarisées de l'épithélium intestinal (Acheson et al., 1996; Hurley et al., 1999), ou en profitant de l'extravasation de neutrophiles recrutés sur le site de l'infection (Hurley et al., 2001) et provoquer des anémies et de la thrombocytopénie. Dans les cas graves, les cellules rénales peuvent être affectées et provoquer ainsi un Syndrome d'Urémie hémolytique

(SHU). Ce dernier conduit dans 4% des cas à des séquelles neuromotrices lourdes et est mortel dans 3 à 5% des cas, en particulier chez les enfants de moins de 5 ans (Delignette-Muller and Cornu, 2008; Goldwater and Bettelheim, 2012; Kaper et al., 2004).

Listeria monocytogenes

Isolée en 1911 en Suède et décrite en 1924 à l'université de Cambridge, *Listeria monocytogenes* est ainsi nommée en l'honneur du père de l'asepsie moderne, Joseph Lister. *L. monocytogenes* est un bacille tellurique ubiquitaire des sols (10^4 CFU/g), des eaux (usée, épurée, boues et eau de surface) et est trouvé au contact des racines des plantes (Jablasone et al., 2005; Kutter et al., 2006; Locatelli et al., 2013; Piveteau et al., 2011; Vivant et al., 2013). Il s'agit d'un firmicute à Gram positif, aérobie facultatif. En condition anaérobie, on observe la production d'acide lactique et en milieu microaérobie ce sont les acides lactique/acétique/butyrique et isovalérique qui sont produits. La capacité de *L. monocytogenes* à digérer l'esculine est souvent utilisée pour une identification rapide de *Listeria* spp et permet une croissance forte dans le lait (Fraser and Sperber, 1988). Le genre *Listeria* contient 17 membres dont *L. aquatica, L. booriae, L. cornellensis, L. fleiscmannii, L. floridensis, L. grandensis, L. grayi, L. innocua, L. ivanovii , L. marthii, L. monocytogenes, L. newyorkensis, L. riparia, L. roucourtiae, L. seeligeri, L. weihenstephanensis et L. welshimeri* (Leclercq et al., 2010; Orsi and Wiedmann, 2016; Weller et al., 2015). *L. monocytogenes* est la seule espèce pathogène pour l'Homme et les infections à *L. ivanovii* impliquent principalement les bovins (Cummins et al., 1994; Lessing et al., 1994; Snapir et al., 2006). Connue pour être pathogène dès 1929, il faut attendre les années 80 pour que soit apportée la preuve que *L. monocytogenes* est transmissible par les aliments. En tant que bactérie psychrotrophe, *L. monocytogenes* est caractérisée par une température minimale de croissance de -2°C (Vos et al., 2009), avec un optimum de croissance situé à 37°C (Augustin et al., 2005). *L. monocytogenes* présente des flagelles sur toute la surface (péritriche) mais n'est motile qu'en dessous de 37°C. La bactérie forme des biofilms

très résilients et peut survivre sur les surfaces dans un environnement en Industrie AgroAlimentaire (IAA) pendant des années malgré les basses températures et les processus répétés de Nettoyage et Désinfection (N&D) (Carpentier and Cerf, 2011; Ortiz et al., 2014; Vongkamjan et al., 2013). Ainsi en 2013, l'EFSA a montré une prévalence de *L. monocytogenes* dans 10% des poissons et 0,9% des fromages à pâtes molles.

Il existe 4 lignées de *L. monocytogenes* (Orsi et al., 2011), qui étaient historiquement divisées en treize sérotypes en fonction des antigènes et flagelles détectés : les sérotypes I (1/2b, 3b, 4b, 4d, 4e), II (1/2a, 1/2c, 3a, 3c) et III (4a and 4c) (Nadon et al., 2001; Roberts et al., 2006). Toutes les souches étaient alors classées au même niveau de virulence malgré des contre-exemples (Gray et al., 2004; Orsi et al., 2011) comme le sérotype 4b, rare mais responsable d'épidémies (Roberts et al, 2006), alors que les sérotypes 1/2a et 1/2b étaient responsables d'un nombre élevé de cas sporadiques. Des caractéristiques de 4b étaient aussi parfois observées chez des membres du sérotype III (Nightingale et al., 2005; Ward et al., 2004).

Cette classification a évolué grâce à une nouvelle répartition basée sur les complexes clonaux (CC) (Maury et al., 2016). Celle-ci gagne en clarté et signification par l'utilisation de la biodiversité des *L. monocytogenes* permettant de répartir les clones selon leur degré de virulence, le tropisme de l'infection et l'origine du pathogène. En particulier, Maury *et al.* ont identifié que les clones les plus communément retrouvés dans les matrices alimentaires ne sont pas ceux majoritairement détectés en milieu clinique. Les CC1, CC2, CC4 et CC6 sont les agents les plus communs de la listériose, alors que le CC9 (dont EDG-e) et le CC121 qui dominent dans les échantillons alimentaires sont beaucoup moins représentés dans les infections (Figure 14). Cette spécificité entre les complexes clonaux se poursuit dans le diagnostic des listérioses avec le CC1 fortement associé aux infections du système nerveux central (SNC) qui passent les barrières intestinale et hémato-encéphalique, et également aux infections Maternelle-Néonatale (MN) qui passent les barrières intestinale et placentaire, avec les CC2 et CC4, tandis que

les CC9 et CC121 sont principalement des bactériémies qui ne passent que la barrière intestinale. Enfin les clones hypervirulents sont associés aux vecteurs plus classiques comme les fromages et le CC1 tandis que les clones moins virulents comme les CC9 et CC121 ont une plus grande persistance sur les plans industriels et sont retrouvés dans la viande (Figure 14) (Maury et al., 2019).

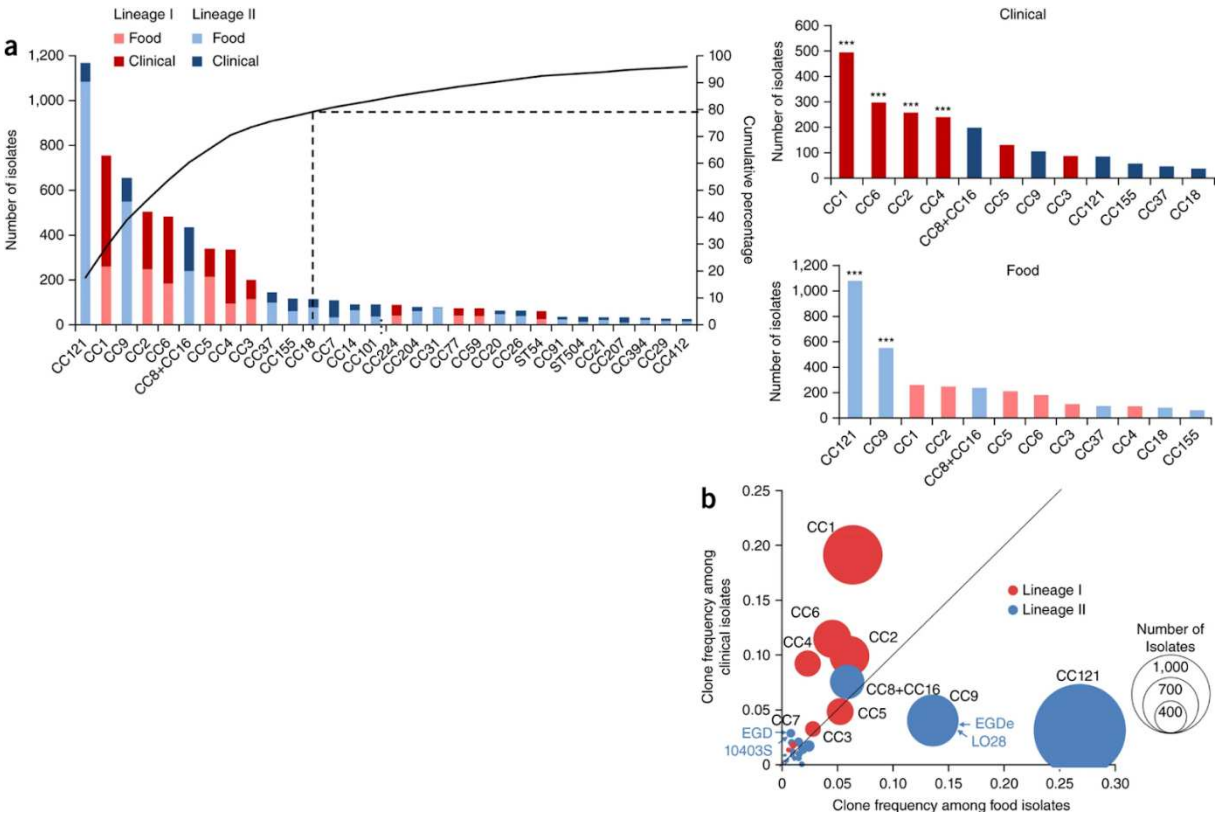


Figure 14 : Prévalence et distribution des clones MLST dans des sources cliniques et alimentaires.
Étude réalisée sur 6 633 isolats cliniques et alimentaires. a) la prévalence des clones MLST, la courbe est le pourcentage cumulatif du nombre d'isolats qui sont présentés en barres. Sur la droite est représentée la distribution de 12 clones, correspondant à 79,2 % des isolats, en fonction du nombre d'isolats quand on cherche une origine clinique (haut) ou alimentaire (bas). b) Fréquence des clones en fonction des isolats cliniques (axe Y) ou alimentaire (axe X). La taille du cercle est proportionnelle au nombre d'isolats. Dans a et b, seuls les clones avec plus de 10 isolats sont montrés.
D'après (Maury et al., 2016).

Les cas de zoonoses alimentaires impliquant *L. monocytogenes* sont relativement rares en France avec quelques centaines de cas par an (Van Cauteren et al., 2017). Il s'agit principalement de personnes immunodéprimées, de personnes âgées ou de femmes enceintes qui sont à risque avec un taux d'incidence très supérieur à la population générale. La virulence de *L. monocytogenes* est portée par deux îlots de pathogénicité (désignés par *Listeria* pathogenecity Islands 1 et 2), l'un portant les gènes *prfA, plcA, hly, mlp, ActA* et *plcB* et et l'autre portant les gènes *inlA* et *inlB,* codant les deux principales internalines A et B de la bactérie. Une vingtaine d'autres internalines existent et jouent des rôles de support lors de l'effacement ou l'infection de nouveaux hôtes sans être nécessaires pour l'expression de la virulence (Dortet et al., 2011; Rajabian et al., 2009).

La listériose est une maladie grave avec un taux d'hospitalisation estimé à 92% et environ 25% à 30% de mortalité (1 mort pour 6 cas) (EFSA, 2021a; Lomonaco et al., 2015). L'infection est caractérisée par une longue période d'incubation de 18 jours liée au processus d'infection intracellulaire de ce pathogène. Le processus d'infection est amorcé par l'internalisation de *L. monocytogenes* par les internalines InlA et InlB qui se lient respectivement la E-cadherine et la Met par l'intermédiaire de répétitions riches en Leucine (LRR). Une cascade de signalisations est alors enclenchée et provoque un réarrangement du cytosquelette d'actine pour aboutir à la formation d'un endosome internalisant la cellule bactérienne (Bierne et al., 2007; Camejo et al., 2011; Pizarro-Cerdá and Cossart, 2018).

Une fois dans l'hôte, le système LLO (Lysteriolysin O, PlcA, PlcB, ActA) perfore la vacuole avant la formation du lysosome (Dramsi and Cossart, 2003; Mengaud et al., 1991; Vazquez-Boland et al., 1992). Plus rarement, la cellule empêche la formation du phagosome et persiste sous forme vacuolaire (Kortebi et al., 2017). Une fois dans le cytoplasme, le glucose-1-phosphate et le glucose-6-phosphate de l'hôte deviennent les sources d'énergie de *L. monocytogenes* (Chico-Calero et al., 2002; Ripio et al., 1997). La motilité intracellulaire est dépendante de ActA par l'intermédiaire de la création par

polymérisation d'une queue d'actine (*commet tail*), qui masque également la cellule face aux systèmes d'autophagie intracellulaire (Birmingham et al., 2007; Perrin et al., 2004; Yoshikawa et al., 2009). C'est également un moyen de poursuivre le cycle infectieux, la queue d'actine finit par percuter la membrane et, avec l'aide de InC qui favorise le relâchement de la tension du cytosquelette, crée un pseudopode dans une cellule jointive (Gianfelice et al., 2015; Ireton et al., 2014; Rajabian et al., 2009). A la rupture de ce dernier, la bactérie se retrouve entourée d'une double vacuole dans la cellule jointive, sans avoir besoin de sortir de l'hôte et de s'exposer au système immunitaire (Alberti-Segui et al., 2007; Cossart and Sansonetti, 2004; Kuehl et al., 2015). *L. monocytogenes* se reproduit principalement dans les hépatocytes qui expriment fortement MET (le récepteur de l'hormone de croissance hépatocytaire HGH). La E-cadherin, molécule clé des liaisons intercellulaires, permet à la bactérie de passer les barrières hémato-encéphalique et placentaires (Dramsi et al., 1995; Lecuit et al., 2004, 2001). La listériose peut ainsi prendre la forme de septicémie, méningite, méningo-encéphalite, et peut causer des avortements spontanés chez la femme enceinte.

3.3) Mécanismes d'adaptation et de résistance aux milieux acides développés par *E. coli* O157:H7 et *L. monocytogenes*

Les capacités de colonisation et de résistance aux conditions de stress des bactéries pathogènes contribuent à leur persistance et capacité de contamination de la chaîne alimentaire. De la production primaire au consommateur, en passant par l'atelier de production, les pathogènes alimentaires rencontrent des conditions hostiles à leur croissance, et en particulier deux paramètres physico-chimique sont souvent exploités par l'industrie agroalimentaire à ces fins : la salinité du produit par l'ajout de NaCl pour réduire l'Aw et l'acidification du milieu qui nécessite une présentation plus complète.

L'acidification des matrices alimentaires

La valeur de pH d'un aliment est un paramètre physico-chimique qui va avoir un impact majeur dans la description de caractères comme la tendreté, la saveur, ainsi que la

croissance bactérienne et donc le temps de conservation. L'analyse de plus de deux mille aliments montre une majorité de pH modérément acides e.g. légumes, lait, poissons (pH 5-6,5), voire très acides e.g. fruits, certains légumes (pH 2-4), et rarement des pH neutres ou alcalins e.g. certains crustacés, blanc d'œuf (pH 7,3-9) (Bridges and Mattice, 1939). Avant l'invention de la réfrigération, le besoin de mieux contrôler la durée de vie des aliments a donné naissance à des techniques de conservation comme la salaison, le séchage ou le fumage. En particulier, la fermentation, qui renforce le caractère acide des produits, (e.g. sauces, légumes, fruits) est devenue une technique majeure de conservation, et les bactéries responsables du processus de fermentation ont été longuement sélectionnées pour créer une standardisation de l'acidification (George et al., 2018).

La viande rouge, le vecteur principal de *E. coli* O157:H7

Chez les mammifères, les différents tissus et en particulier les muscles, présentent un pH neutre (7,0-7,2) nécessaire au maintien des réactions enzymatiques et structures de la cellule. Le processus de transformation du muscle en viande est amorcé dès l'abattage des animaux avec l'arrêt de la respiration. Le déficit en oxygène du muscle empêchant les réactions oxydatives, les tissus basculent sur la respiration anaérobie et consomment le glycogène intracellulaire, produisant ainsi de l'acide lactique et des ions H^+ (Honikel, 2004). La réaction s'arrête une fois les réserves de glycogène épuisées, le glucose de la cellule n'est lui pas utilisé (Honikel, 2004). Il est à noter que c'est à ce stade où le pH est encore neutre que la contamination par *E. coli* O157:H7 est la plus élevée, de par le risque de perforation accidentelle des viscères et d'outils mal stérilisés. L'acidification de la viande est un processus rapide mais déterminant pour les caractéristiques organoleptiques et les paramètres technologiques qui définissent la qualité du produit fini (White et al., 1984). Il faut environ 6 à 8h pour que la viande de porc atteigne un pH de 5,5 à5,7; 16h pour l'agneau et 18 à 22h pour le bœuf (Honikel, 2004). Les points critiques de cette évolution sont le pH de la viande lorsque le muscle est encore chaud (15-45 minutes post mortem) et le pH final (Figure 15). Un pH trop

bas (<5,8) lorsque le muscle est chaud, affecte la tenue des protéines et la rétention des fluides, ce qui donne une apparence pâle, molle et exsudative (*Pale Soft Exudative* "PSE") (Honikel, 2004). L'industrie se base sur la valeur de ce pH précoce pour déterminer la qualité de la pièce (Chmiel et al., 2014; Vermeulen et al., 2015). Par contraste, le pH final de la viande commerciale devrait se situer entre des valeurs de 5,6-5,9 avec un risque au-dessus de pH=6,0 d'avoir une rétention trop importante de liquide qui conduit à un produit sombre, dur et sec (*Dark Firm Dark* "DFD") qui est de plus sensible à la croissance bactérienne (Bidner et al., 2004; Bocian et al., 2018). En effet, à un pH de 5,5 certaines bactéries d'altération ne peuvent plus croître et la diminution de l'Aw indirectement causée par cette baisse de pH est également un facteur d'allongement de la durée de vie. Enfin, la baisse du pH dans la viande ne suit pas une décroissance linéaire monophasique mais sigmoïdale et polyphasique e.g. La viande de bœuf présente un plateau pour des valeurs de pH 6,8 à 6,2 en maturation précoce (< 8-9 heures post mortem) avant que l'acidification ne reprenne (Boudjellal et al., 2008).

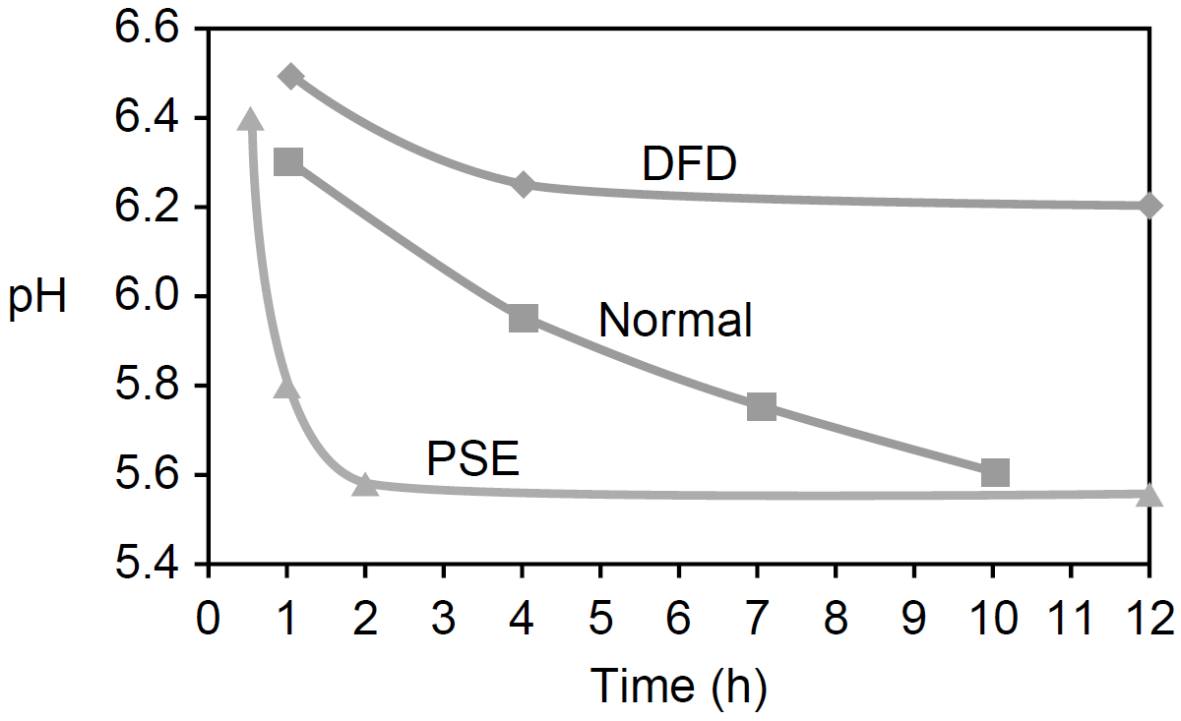


Figure 15 : Evolution post mortem du pH dans la viande de porc. L'acidification post mortem des muscles définit la qualité de la viande. Une évolution normale est définie par un pH de 6 quand la viande

est encore chaude (1 h) et de 5,5 après une demi-journée. Une acidification trop rapide avec un pH sous 5,8 à 1h donne une viande pâle, molle et exsudative (PSE). Au contraire, une acidification faible avec un pH de 6 après 12 heures conduit à un phénotype dur, sec et sombre (DFD) plus sensible à la contamination bactérienne.

D'après (Honikel, 2004).

Les fromages, sources de contamination par *L. monocytogenes*

Le lait est par essence un produit dont le pH est légèrement acide de 6,7. Lors de la fabrication de fromage, la première étape dite de coagulation ou caillage requiert l'ajout d'un coagulant d'origine animale/végétale appelé "présure" ainsi que d'une acidification substantielle du milieu pour assurer la bonne gélification du lait en "caillé". Cette acidification du lait est réalisée de deux manières différentes selon le type de fromage que l'on souhaite obtenir. Un composant acide peut être directement ajouté pour une baisse immédiate du pH e.g. vinaigre (acide acétique), jus de citron (acide citrique) ou acide tartrique selon les lieux et traditions. La seconde option est l'inoculation du lait (volontaire ou occurrent naturellement) par des bactéries lactiques, telles que *Lactoccocus lactis,* qui consomment le lactose du lait et produisent de l'acide lactique en retour, provoquant une baisse progressive du pH (10-20h). Ces bactéries sont souvent nécessaires à l'expression de la saveur pour certains types de fromages de par l'impact de leurs enzymes sur les protéines du lait (Khalid and Marth, 1990).

Après les étapes d'égouttage et de moulage qui retirent la majorité du liquide (lactosérum) et l'étape de salage qui fait monter l'osmolarité, les fromages sont entreposés pour affinage sur une durée variant de quelques semaines (croûte "fleuri", 2 à 4 mois) à plusieurs mois (pâtes "persillé", 3 à 6 mois) voire des années (pâtes "pressées cuites", 9 à 24 mois). Le pH peut varier de manière significative lors de ces périodes avec pour cas extrême le Bleu qui débute comme une pâte très acide (pH=4,5) et finit sa maturation à un pH presque neutre (pH=6,5).

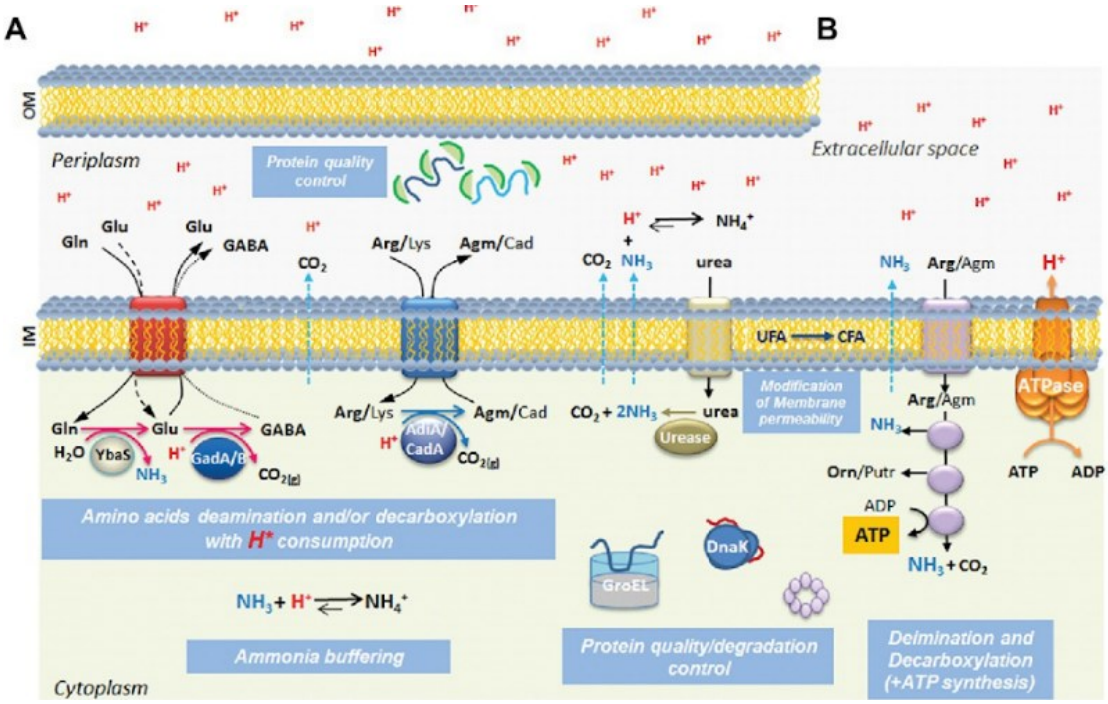
Les fromages présentent tous des caractères acides de par le procédé de fabrication, cependant la gamme de valeurs de pH est étendue entre les milieux avec des pâtes légèrement acides pour un pH compris entre 6,5 et 6,2 (e.g. Limburger, Bleu, Brie, Quesco Fresco …), puis des conditions modérément acides entre 6,0 et 5,1 de pH (e.g. Ricotta, Brick, Gruyère suisse, mozzarella, gouda, parmesan, cheddar…) voire très acides avec un pH aux alentours de 4,9-4,6 (e.g feta, crèmes de fromages…). L'acidité de la pâte est également liée à des paramètres de qualité du fromage comme la consistance de la pâte. Les fromages sont de manière générale "durs" entre des valeurs 6,5 et 5,6, "coulants" pour un pH de 5,4-5,0 et "crémeux" pour des valeurs inférieures à 4,9. Cet effet est en relation directe avec l'effet du pH sur la concentration micellaire / libre de calcium qui est un déterminant majeur de la stabilité de la pâte (Thybo et al., 2020).

La réponse des bactéries au stress acide

La présence d'une concentration élevée d'ions H^+ dans l'environnement est un des stress les plus communément rencontrés par les organismes unicellulaires. Cette condition peut venir de réactions chimiques inorganiques dans les sols/solutions concentrées ou de l'activité cellulaire la dégradation de tissus morts générant des acides organiques. Les acides peuvent modifier des réactions enzymatiques ou dénaturer les protéines et ils favorisent l'apparition d'espèces réactives de l'oxygène qui peuvent causer des dommages à l'ADN. Afin de survivre, la réponse engagée par les micro-organismes aura pour objectif de maintenir et/ou restaurer leur pH intracellulaire (pHi). (Figure 16) (Foster, 2004; Lund et al., 2014).

La paroi des cellules est très imperméable aux protons et permet l'instauration d'un gradient fort entre les bicouches lipidiques (Mitchell, 1961). En fonction des composants du milieu, il est également possible de modifier la composition de la membrane pour renforcer cet effet (Brown et al., 1997; Fozo and Quivey, 2004; Kim et al., 2005) e.g. par ajout de cyclopropanes trouvés dans les produits laitiers et la viande, pour *E. coli* (Lolli et al., 2018; Shabala and Ross, 2008) ou par la diminution d'acide gras

saturés à chaînes ramifiées pour *L. monocytogenes* (Giotis et al., 2007). Les cellules maintiennent également leur pH intracellulaire par différents mécanismes. Ceux-ci regroupent l'extrusion des protons par activation de la pompe à protons F1F0-ATPase (Sanders et al., 1981), et la production d'enzymes favorisant une réaction avec un accepteur de proton (ammoniac) qui sera expulsé de la cellule par la suite (glutamate/GABA, arginine/agmatine)(Krulwich et al., 2011; Pennacchietti et al., 2018). Enfin, si le pH intracellulaire baisse trop, la cellule peut compenser les effets néfastes par la production de protéines chaperonnes qui vont aider au maintien du repliement des protéines essentielles à la survie.



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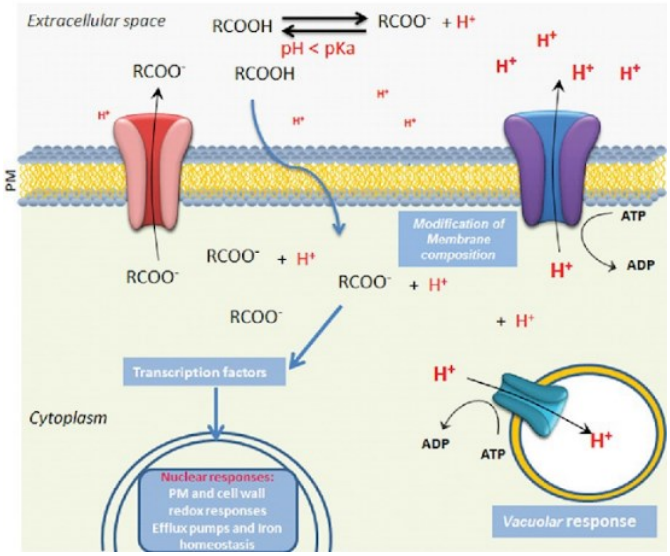

Figure 16 : Schéma récapitulatif de la réponse microbienne aux pH acides. Mécanismes de contrôle du pH chez les bactéries à Gram négatif (A) et chez les bactéries à Gram positif (B). De gauche à droite, à la membrane les systèmes YbaS et GAD, la désamination et la décarboxylation des acides aminés , la dénaturation de l'urée, chez les Gram positifs et l'activation des pompes à protons. Sur la ligne du bas, l'effet tampon de l'ammoniac et le contrôle du repliement cellulaire. En (C) est présenté le cas des acides faibles qui pénètrent la membrane avant de se dissocier.
D'après (Lund et al., 2020).

Gènes d'intérêt et correction du pH chez *E. coli* et *L. monocytogenes*

Chez *E. coli*, GAD (glutamate décarboxylase) est le principal système impliqué dans la résistance aux milieux acides (Blankenhorn et al., 1999; De Biase et al., 1999; Smith et al., 1992). Parmi les trois types de système de résistance à l'acide qu'*E. coli* possède, il s'agit de celui dont la délétion ou l'inhibition a le plus d'impact sur la survie (Lin et al., 1995). GAD est constitué de trois protéines, les glutamate décarboxylase GadA et GadB ainsi que de l'antiport glutamate/GABA GadC.

GadA et GadB sont des enzymes aux séquences protéiques très similaires (Smith et al., 1992), catalysant la réaction de dégradation du L-glutamate en γ-aminobutyrate (GABA) et CO_2, ce qui consomme un ion H^+ et augmente ainsi le pH cytoplasmique de la bactérie (Figure 17). Le co-facteur phosphate de pyridoxal (PLP, vitamine B6) est

également nécessaire à l'activité catalytique. Le gène *gadA* est codé 2100Kb en amont de *gadB* et *gadC* (Blattner et al., 1997) et est exprimé séparément de *gadBC*. L'expression du système GAD s'effectue en phase stationnaire et ne nécessite pas la présence d'acide extracellulaire pour être initiée (De Biase et al., 1993; Lin et al., 1995). La présence de stress acide ou osmotique entraîne cependant une élévation du taux de transcription qui peut aller jusqu'à 10 fois le niveau de base (De Biase et al., 1999).

GadB est un hexamère de 330 KDa, si le pH intracellulaire est supérieur à 5,6, la protéine est présente sous une forme entièrement cytoplasmique (forme C), il faut que le pH intracellulaire descende sous 5,6 pour qu'un changement de conformation (forme A) cause la migration à la membrane et l'activité décarboxylase (Capitani et al., 2003) avec un optimum enzymatique pour un pH de 3,8-4,6 (Sukhareva et al., 1994). La synergie entre GadB et GadC permet un afflux constant de L-glutamate pour maintenir la réaction ainsi que la sécrétion de GABA dans le milieu qui stabilise le pH de l'environnement immédiat de la cellule. La régulation de l'expression de *gadB* est complexe, l'AMP cyclique et H-NS sont des répresseurs de la transcription de GAD. H-NS en particulier, est responsable de l'inhibition d'expression en phase exponentielle de croissance. GadX (autrefois YhiX) est un promoteur de l'expression de *gadB* si le milieu est acide et GadW est un inhibiteur de cette activation GadX dépendante (Seo et al., 2015; Tucker et al., 2003).
Il a été confirmé, au moins chez les EPEC, que *gadX* est aussi lié à l'expression de la virulence par régulation de la production de PerA avec une inhibition du gène de virulence en condition acide (Shin et al., 2001).

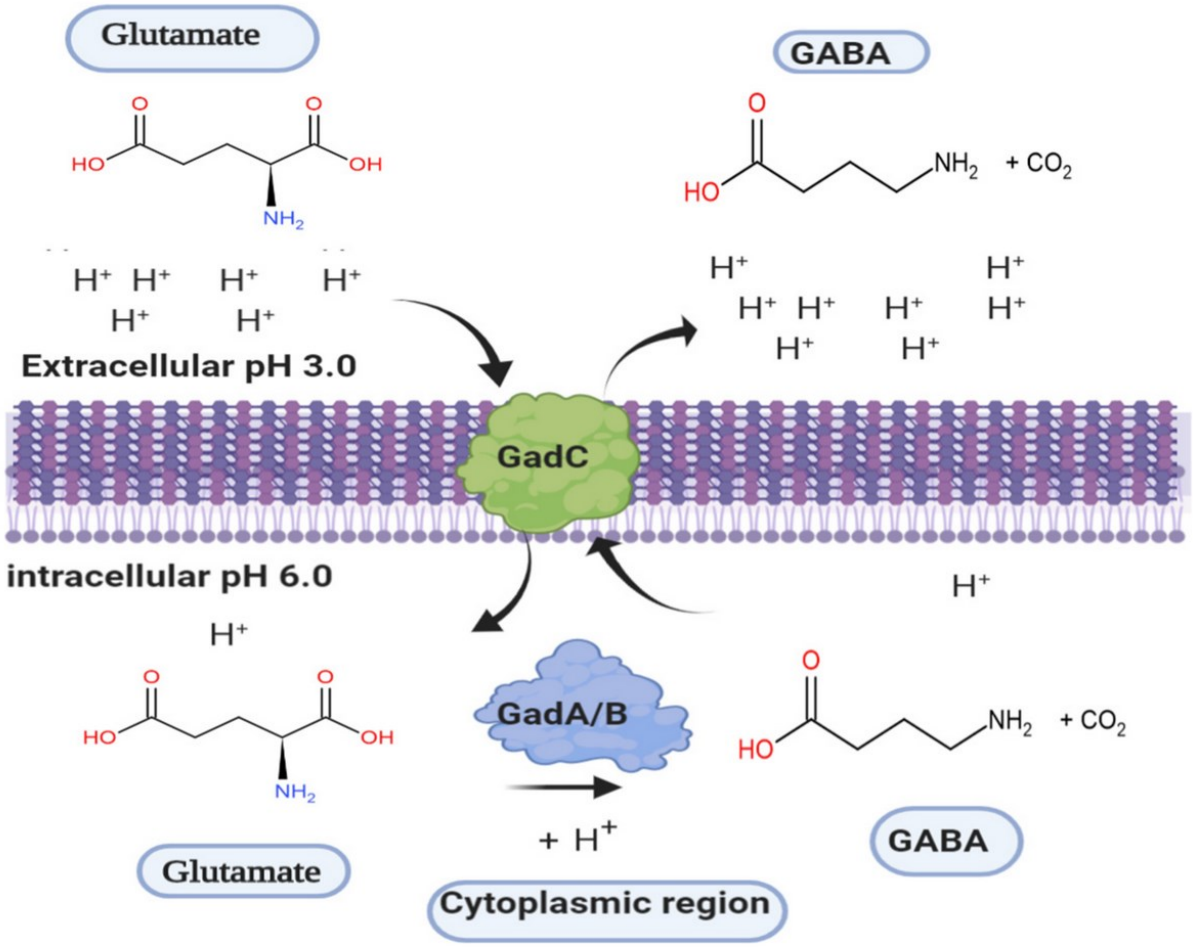

Figure 17 : Schéma de l'action générale du système GAD pour le maintien du pH intracellulaire. L'enzyme glutamate décarboxylase GadB catalyse la transformation de glutamate en GABA au prix d'un proton intracellulaire. En second temps, l'antiport GadC est capable d'utiliser l'énergie de passage de GABA pour permettre le passage d'une autre molécule de glutamate dans le cytoplasme.
D'après (Yogeswara et al., 2020).

Si *L. monocytogenes* vit principalement dans les sols, il s'agit également d'un pathogène dont la voie d'entrée est intestinale et nécessite une bonne résistance aux acides. *L. monocytogenes* dispose de fait du système GAD (Cotter et al., 2005, 2001), mais il s'agit d'une version plus complexe avec un système enzyme/antiporter *gadD1/T1* en charge des stress acides faibles, un autre *gadD2/T2* pour les valeurs plus faibles de pH et un troisième *gadD3* surexprimé en milieu acide mais également actif en condition neutre de pH pour la formation de GABA (Cotter et al., 2005; Karatzas et al., 2010). Un autre système prévalant dans la résistance au stress chez *L. monocytogenes* est le système ADI (Arginine déaminase). L'opéron ADI contient les gènes *arcABC* qui codent pour

l'arginine déaminase (ArcA), l'ornithine carbamoyl-transferase (ArcB) et la carbamate kinase (ArcC) qui ensemble convertissent l'arginine en ornithine et ammoniac ainsi que du CO_2. L'ammoniac va ensuite lier un proton pour devenir de l'ammonium avec pour conséquence une élévation du pH intracellulaire. L'antiport ArcD expulse l'ornithine hors de la cellule et importe de l'arginine du milieu externe, maintenant l'apport en substrat nécessaire à la transformation.

ArgR est un régulateur inhibiteur de ADI par l'intermédiaire de la répression des gènes *arcA* et σ^B lorsque l'arginine est rare dans le milieu (Figure 18), mais ArgR devient un activateur de la transcription de ces mêmes gènes dès que le cofacteur arginine est présent (Cheng et al., 2017). Cela signifie que la présence d'ArgR peut significativement affecter la survie de *L. monocytogenes* en milieu acide en fonction de la source d'arginine. Si *argR* est inactivé, la levée de l'effet inhibiteur n'est plus visible au niveau de la population totale (Cheng et al., 2017). Cette dépendance à l'arginine est due au fait que les régions promotrices d'*argR* forment des trimères ou des hexamères en fonction de la présence du cofacteur arginine (Cherney et al., 2009; Ni et al., 1999). ArgR est également un inhibiteur de la transcription de *argX*, un smallRNA qui possède un effet interfèrent avec arcC (van der Meulen et al., 2019).

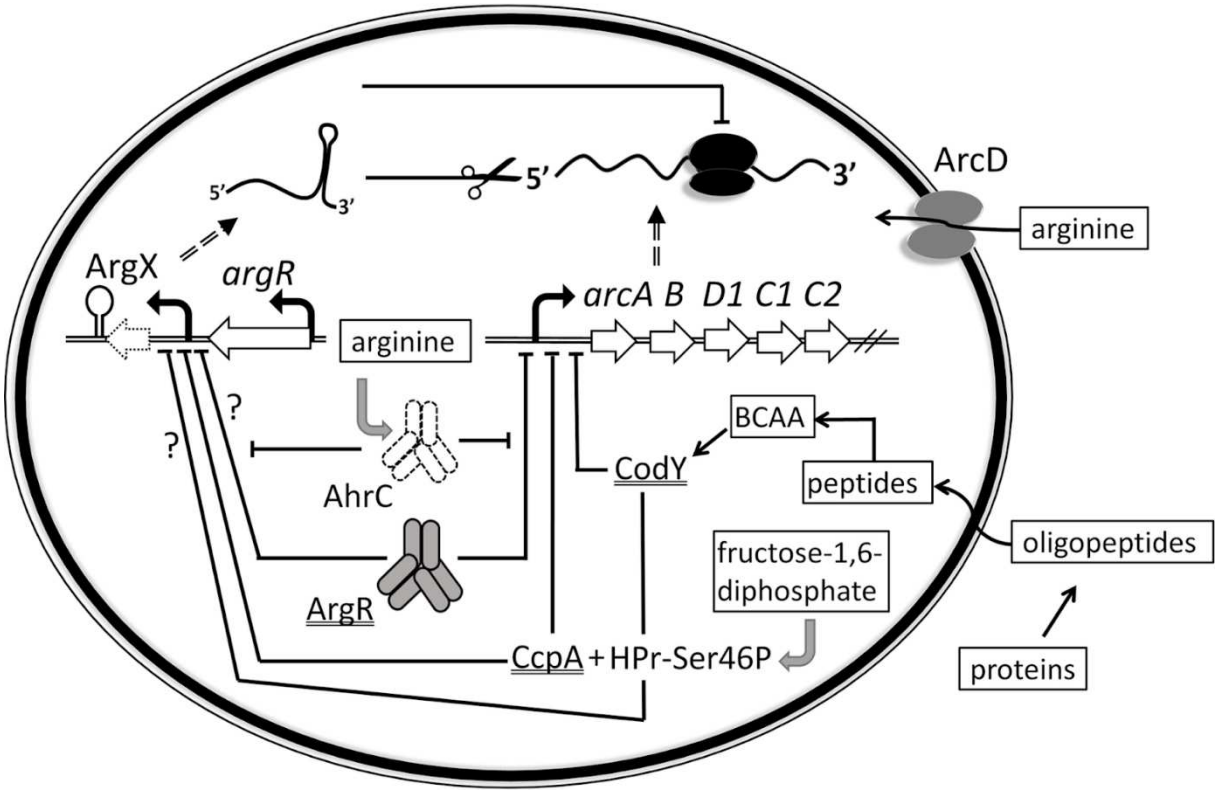

Figure 18 : Modèle du métabolisme de l'arginine et sa régulation par ArgR tel que décrit chez *Lactococcus lactis*. Schéma du système ADI (arginine déaminase) dans une cellule. Les gènes de l'opéron arcABCD codent pour l'arginine déaminase (ArcA), l'ornithine carbamoyl-transferase (ArcB) et la carbamate kinase (ArcC) qui permettent de maintenir le pH intracellulaire par production à partir d'arginine de NH_3 qui lie un proton avant d'être évacués hors de la cellule. ArgR est un régulateur de ce système qui inhibe les gènes arc en l'absence d'arginine mais qui promeut le système en présence d'arginine, en particulier par inhibition du sARN ArgX qui interfère avec *arcC*.
D'après (van der Meulen et al., 2019).

Le système à deux composants LisRK possède un rôle dans la modification des membranes face aux stress acide et osmotique, ce qui fait de lui un composant vital pour la survie de *L. monocytogenes,* aussi bien dans les sols que dans les aliments (Cotter et al., 1999; Nielsen et al., 2012). LisRK est aussi impliqué dans la résistance à plusieurs antibiotiques et les β-lactames en particulier (Collins et al., 2012; Cotter et al., 2002, 1999). La virulence de *L. monocytogenes* est indirectement liée à l'expression du système *lisRK* par l'effet du système à deux composants sur la transcription des gènes *virR* et *phoP* (Arenas et al., 2013; Mandin et al., 2005). Cependant d'autres gènes liés à la virulence sont réprimés par l'intermédiaire du smallRNA LhrC qui inhibe *oppA*, *tcsA*

et *lapB et* l'absence de ces protéines transmembranaires permet une meilleure évasion du système immunitaire (Ross et al., 2019; Sievers et al., 2015, 2014). Dans l'opéron *lisRK*, *lisK* code l'histidine kinase transmembranaire qui sert de senseur et s'autophosphoryle tandis que *LisR* code pour le second messager LisR qui est phosphorylé par LisK et possède la fonction de régulateur de la transcription (Figure 19) (Arenas et al., 2013). En plus d'une meilleure tolérance au stress, les changements dans la membrane de *L. monocytogenes* dus à LisRK sont également responsables d'une meilleure capacité d'adhésion et font donc partie de manière périphérique du procédé de colonisation des surfaces (Aslan et al., 2021). Une dernière participation de LisRK semble être dans l'apparition de cellules persistantes, l'activation forcée de *lisRK* produit des populations présentant un niveau de résistance à *l'ampicilline* équivalent à celui des persisteurs (Aslan et al., 2021).

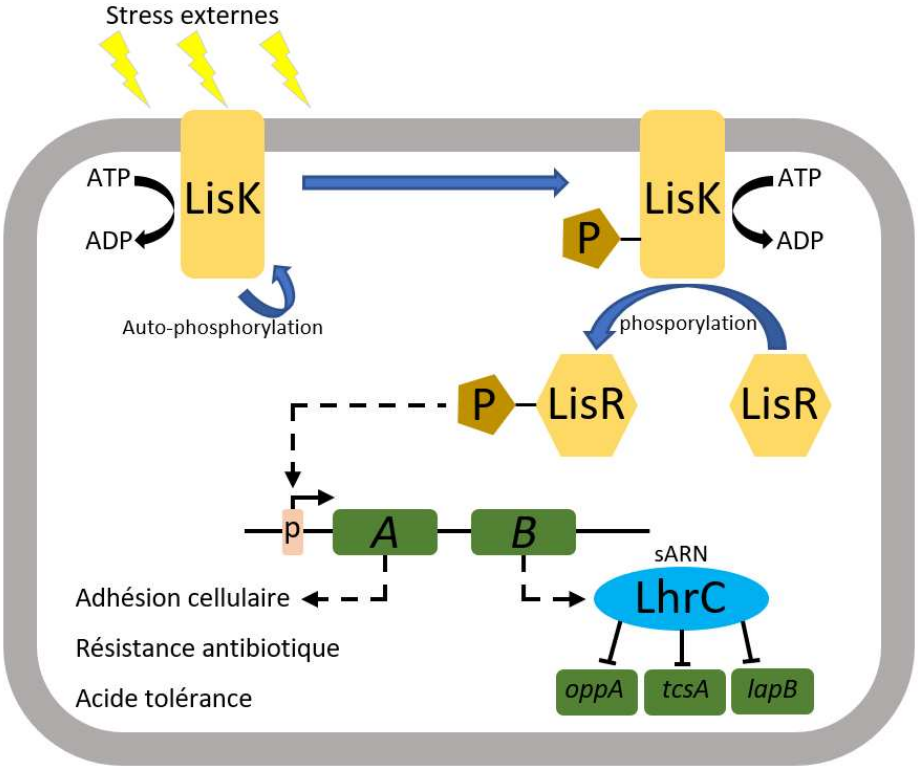

Figure 19 : Régulation du stress et de la virulence par activation de LisRK. Les stress à la membrane sont détectés par le système à deux composants LisRK. Le senseur histidine kinase LisK s'auto-phosphoryle et phosphoryle le facteur de régulation LisR qui va modifier l'activité de transcription de gènes liés à la résistance à l'acidité et certains antibiotiques ainsi que l'adhésion cellulaire. LisRK cause aussi la production du sARN LhrC qui inhibe l'expression de gènes de la virulence.

4) Récapitulatif des points principaux de la bibliographie

-L'organisation spatiale et l'expression génique des bactéries dans les matrices alimentaires sont mal définies. La modélisation de la croissance prend rarement en compte les conditions du micro-environnement ou l'hétérogénéité de phénotype à l'échelle cellulaire. Cela mène à des prédictions de croissance erronées dans les aliments.

-Les matrices alimentaires sont des milieux complexes avec de multiples gradients physico-chimiques entraînant l'apparition de différents micro-environnements au contact des cellules.

-Les bactéries disposent de nombreux moyens de réguler leur expression par des moyens stochastique (variation de phase), transcriptionnel et post-traductionnel, ce qui peut entraîner une diversité de phénotypes importante.

-Un milieu synthétique simplifié constitue un écosystème où les caractéristiques de la croissance et diversité de phénotypes d'une bactérie pathogène peuvent être étudiées en fonction de modulations précises du milieu.

-Les pathogènes alimentaires *E. coli* O157:H7 et *L. monocytogenes* représentent un risque important pour la santé du fait de leur relative abondance et de la sévérité de l'infection qu'ils provoquent. Leur tolérance au milieu acide serait particulièrement intéressante à explorer au vu du pH des aliments dans lesquels ils sont communément isolés.

Partie II : Résultats

1) L'organisation spatiale de *Listeria monocytogenes* et *Escherichia coli* O157:H7 cultivés dans des matrices gélifiées

Impact du milieu et de la répartition spatiale cellulaire sur la croissance

L'organisation spatiale des bactéries pathogènes dans les matrices alimentaires reste une question mal comprise. En comprendre mieux les principes permettrait d'améliorer l'évaluation du risque pour le consommateur et ainsi de réduire le nombre d'infections par ingestion d'aliments contaminés. En combinant la microscopie confocale laser à balayage (MCLB) avec le marquage cellulaire par fluorescence, exprimé constitutivement par *L. monocytogenes* et *E. coli*, il a été possible d'observer les motifs de la colonisation dans l'espace de ces deux pathogènes alimentaires dans des matrices gélosées en mono- et co-cultures et ce dans des conditions environnementales différentes. Des valeurs incrémentales de l'agent gélifiant, l'agarose à faible point de fusion (LMPA), déclenchent la transition entre une population de cellules isolées et mobiles dispersées dans la matrice à une population de bactéries sessiles spatialement organisées en microcolonies. La taille, le nombre et la morphologie des microcolonies sont fortement affectés par la supplémentation du milieu en NaCl et acide lactique, deux composants fréquemment trouvés ou utilisés dans les produits alimentaires. De manière frappante, la motilité des cellules uniques est partiellement restaurée dans les concentrations élevées de LMPA par l'ajout de NaCl pour *L. monocytogenes* ou d'acide lactique pour *E. coli* O157:H7. Une co-culture des deux espèces dans le même gel affecte le "pattern" de la colonisation : *L. monocytogenes* est plus à même de coloniser des matrices acides en présence de *E. coli*. De manière générale, cette investigation apporte un aperçu de la distribution spatiale et de la dynamique des structures de ces bactéries pathogéniques dans les matrices gélifiées. Leur impact potentiel sur la sécurité alimentaire est discuté par la suite.

Le choix de l'agent gélifiant de la matrice modèle

L'utilisation d'acide lactique et de NaCl est justifiée par la composition des vecteurs principaux de ces pathogènes : la viande et le fromage. L'étude de l'organisation spatiale ne peut pas être réalisée en matrice alimentaire à cause de l'opacité et de la complexité de ces milieux. Un milieu synthétique simplifié permettrait la modélisation précise du comportement d'une population bactérienne à un changement de paramètre physico-chimique ou l'introduction d'un composé dans le milieu. Cependant, avant de poursuivre sur des tests plus avancés, une bonne compréhension de la croissance dans le gel de base est requise, d'où une première étude sur la variation de paramètres plus communéments étudiés e.g. pH, Aw, texture. Comme agent gélifiant pour ce nouveau modèle d'hydrogel, on recherche un composé utilisé dans l'industrie alimentaire, non cytotoxique, formant une structure stable dans le temps et avec un point de solidification le plus proche possible de 37°C. L'agarose, la gélatine et le guar présentant ces caractéristiques, le meilleur candidat fut déterminé par une étude sur la texture du gel obtenu (Figure 20).

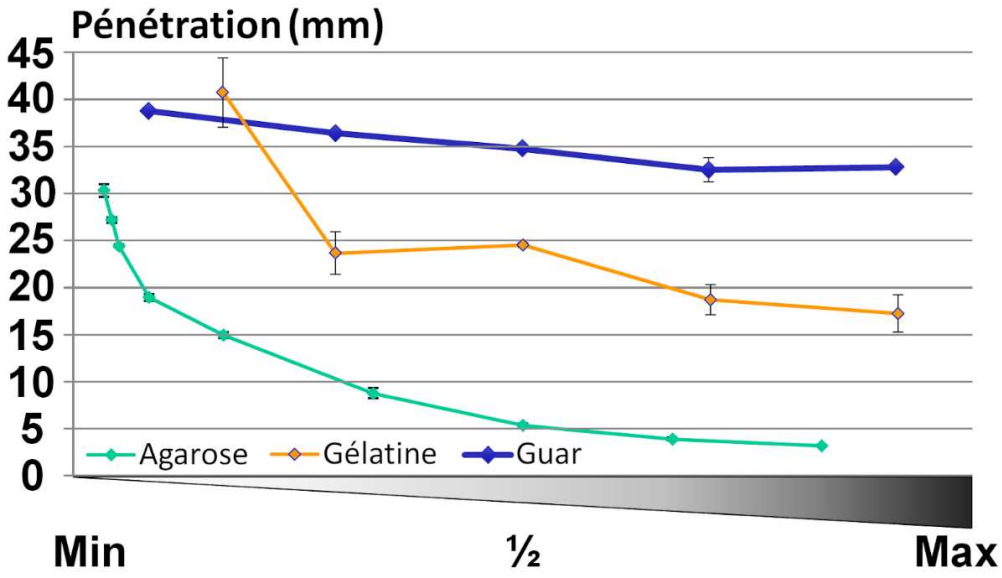

Figure 20 : Étude de pénétrométrie sur les agents gélifiants. La texture des hydrogels est testée par impact avec une sonde dont la profondeur finale est relative à l'inverse de la viscosité du milieu. Les gels sont semi-solides aux alentours d'une valeur Y de 40 et plus cette valeur diminue, plus le milieu est solide. Chacun des trois agents est testé sur une gamme de pourcentages dans le milieu compatible avec les produits agroalimentaires, agarose de 0% à 6% ; gélatine de 4% à 16%, guar de 1,4% à 2,6%.

Au vu des études mettant en évidence l'impact de la structure du milieu sur le comportement des microcolonies, il est préférable que l'agent gélifiant apporte une modification élevée de la texture du gel sur une faible plage de concentration. Il s'est révélé difficile de modifier la texture du gel par variation de la quantité de guar introduite dans le milieu, et au-delà de 2,6% de guar la formation d'un milieu homogène n'était plus possible. La gélatine présente de meilleures caractéristiques, en particulier entre les valeurs de concentrations 6% et 7%, mais des propriétés élastiques sont notées et pourraient jouer un rôle dans la plus grande variabilité des résultats de pénétrométrie dans ce milieu. Enfin, l'agarose présente le profil recherché avec une augmentation logarithmique de la rigidité du milieu. La gamme 0,25%-1% est en particulier intéressante car on retrouve des textures proches de matrices alimentaires comme les jelly cakes (0.25 % agarose), la viande hachée (0,5 % agarose) et les fromages comme le Gouda (1,0 % agarose). Cet agarose particulier *i.e.* "Low Melting Point Agarose" (LMPA) est retenu pour l'élaboration de tous les hydrogels présentés dans les résultats ci-dessous, en commençant par l'étude de la répartition spatiale des populations.

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Spatial organisation of *Listeria monocytogenes* and *Escherichia coli* O157:H7 cultivated in gel matrices

Cédric SAINT MARTIN[1,2], Maud DARSONVAL[1], Marina GRÉGOIRE[1], Nelly CACCIA[2], Lucas MIDOUX[1], Sophie BERLAND[3], Sabine LEROY[2], Florence DUBOIS-BRISSONNET[1], Mickaël DESVAUX[2]* & Romain BRIANDET[1]*

[1] Université Paris-Saclay, INRAE, AgroParisTech, MICALIS Institute, 78350 Jouy-en-Josas France,

[2] Université Clermont Auvergne, INRAE, UMR454 MEDIS, 63000 Clermont-Ferrand, France,

[3] Université Paris-Saclay, INRAE, AgroParisTech, SayFood, 91300 Massy, France.

*Corresponding authors: M. Desvaux (mickael.desvaux@inrae.fr) & R. Briandet (romain.briandet@inrae.fr)

Abstract

The spatial organisation of bacterial pathogens in food matrices remains poorly understood, but is important in improving risk assessment and preventing infection of consumers by contaminated foodstuff. By combining confocal laser scanning microscopy with genetic fluorescent labelling of *Listeria monocytogenes* and *Escherichia coli* O157:H7, it was possible to investigate the spatial patterns of colonisation of both foodborne pathogens in gel matrices, alone or in combination, in various environmental conditions. Increasing low melting point agarose (LMPA) concentrations triggers the transition between a motile single-cell lifestyle to a sessile population spatially organised as microcolonies. The size, number and morphology of microcolonies were highly affected by supplementations in NaCl or lactic acid, two compounds frequently used in food products. Strikingly, single-cell motility was partially restored at higher LMPA concentration in the presence of lactic acid for *Escherichia coli* O157:H7 and in the presence of NaCl for *Listeria monocytogenes*. Co-culture of both species in the hydrogel affected pathogen colonisation features; *Listeria*

monocytogenes was better able to colonise gel matrices containing lactic acid in the presence of *Escherichia coli* O157:H7. Altogether, this investigation provides insights into the spatial distribution and structural dynamics of bacterial pathogens in gel matrices. Potential impacts on food safety are discussed.

Keywords

Listeria monocytogenes, Escherichia coli O157:H7, food matrix, hydrogel, spatial organisation, bacterial motility, confocal laser scanning microscopy

Abbreviations

CLSM: confocal laser scanning microscopy

LMPA: low melting point agarose

L. monocytogenes: *Listeria monocytogenes*

E. coli: *Escherichia coli*

1) Introduction

In Europe, *Listeria monocytogenes* (*L. monocytogenes*) and *Escherichia coli* (*E. coli*) O157:H7 are ranked as the third and fifth most common foodborne zoonotic agents, respectively, with a number of related cases that has been rising slowly for over 8 years (EFSA and ECDC; 2021). *E. coli* O157:H7 is generally associated with meat contamination because of its presence in the digestive tract of livestock, but contamination of other products such as raw-milk products or vegetables are also reported (Luna-Guevara et al., 2019). As a food contaminant, *L. monocytogenes* is mostly found in vegetables, fish and cheese, but being a saprophyte and ubiquitous microorganism, the panel of possible vectors is quite wide, including soil, water or animals (Kallipolitis et al., 2020). Detection and control of bacterial loads in food matrices is hindered by the fact that current predictive models are derived from planktonic bacterial growth, which is poorly accurate when applied to bacterial growth in solid and semi-solid food matrices (Pin et

al., 1999; Skandamis and Jeanson, 2015; Smith and Schaffner, 2004). Food matrices are spatially organised environments with heterogeneous microstructures harbouring multiple micro-gradients that evolve with time and microbial activity, *e.g.* oxygen concentration, pH, temperature, nutrient consumption or metabolite accumulation (Lobete et al., 2015). There is no generic model to describe substrate diffusion limitation effects in such food matrices, and the existing models can be contradictory (Cussler, 1997; Ribeiro et al., 2005). As a general rule, small molecules less reactive with the matrix (neutral charge or same charge as the reticulating agent) will diffuse faster, and large polymers with flexible chains diffuse more easily than rigid molecules of the same radius (Silva et al., 2013). Local heterogeneities and their dynamics can lead to differentiation in microbial populations as cells can undergo adaptive responses to a given environment, such as genotypic variation and stochastic gene expression (Stewart and Franklin, 2008). This heterogeneity of expression can lead to phenotypic diversity of cell subpopulations, expanding the possibilities of multiple stress tolerance or virulence levels for a given species in the same environment (Caniça et al., 2019; Nielsen et al., 2013).

The distribution and physiology of bacterial cells in a food matrix are also dependent on the texture of the media. Matrix hardness alters bacterial motility both on surfaces and inside the matrix itself (Yang et al., 2017). The decrease in bacterial speed is also coupled with the selection of swimming behaviour between "run'' and "tumbles" as meshes of reticulating agents create traps that limit diffusion through straight trajectories (Licata et al., 2016). Studies on the bacterial growth inside food matrices are still sparse. Most concern predictive modelling and population survival under stress exposure (Antwi et al., 2006; Costello et al., 2018; Jeanson et al., 2015; Kabanova et al., 2012). While the behaviour of cheese starters such as *Lactococcus lactis* has been described (Theys et al., 2009, 2008), few investigations have been dedicated to pathogens such as *L. monocytogenes* (Baka et al., 2017, 2016; Uyttendaele et al., 2009; Verheyen et al., 2019) and, to our knowledge, none to *E. coli* O157:H7. As a first step to gain information and explore the behaviour of foodborne pathogens in semi-solid food

matrices, *L. monocytogenes* and *E. coli* O157:H7 were grown in synthetic gel media mimicking food matrices such as ground meat or soft cheese. Using fluorescent bacterial strains, the spatial organisation and dynamics of the pathogens were investigated *in situ* by confocal laser scanning microscopy (CLSM). This experimental setup allowed the extraction and analysis of the microcolonies size and morphological parameters as well as single-cell motility in various environmental conditions. The gel matrix was tuned with different concentrations of low melting point agarose (LMPA) to control the hydrogel texture. Besides, different NaCl and lactic acid concentrations were considered respective to conditions encountered by these pathogens in food.

2) Materials and methods

Bacterial strains and culture conditions

From cryotubes stored at -80 °C, bacterial strains were plated on Petri dishes with TSA (Tryptone Soya Agar, Oxoid, reference CM0129) and incubated overnight at 37 °C. One bacterial colony was picked up and inoculated in TSA (Tryptone Soya Agar, Oxoid, reference CM0129) broth prior to overnight incubation with orbital shaking (150 rpm). When required, growth media were supplemented either with tetracycline (10 µg/mL; ALFA AESAR, reference B21408) or chloramphenicol (7 µg/mL; EUROMEDEX, reference 3886-B). *L. monocytogenes* EGD-e (Glaser et al., 2001) was transformed by electroporation with the plasmid pAD_{1}-cGFP (Balestrino et al., 2010) constitutively expressing the *gfpmut2* gene, which encodes the corresponding green fluorescent protein (Cormack et al., 1996). Non-cytotoxic *E. coli* O157:H7 CM454 (Gobert et al., 2007; Renier et al., 2014) was transformed with the plasmid pVaAg-Red constitutively expressing the red fluorescent protein mRuby2 (Ageorges et al., 2019).

Gel matrices

To mimic semi-solid food matrices, such as minced meat and soft cheese, gel matrices of different textures were obtained using low melting point agarose (LMPA; UltraPure Agarose, Invitrogen, reference 16500-500) at three concentrations (0.25%, 0.50% and

1.00%). From overnight pre-cultures and following serial dilutions, the bacterial inoculum was adjusted to attain the desired initial concentration. After boiling to ensure complete melting, the TSB-LMPA was cooled to 40 °C to prevent heat stress prior to adding the bacterial inoculum. Medium could be supplemented with lactic acid (Sigma Aldrich, 27715-1L-R) to adjust media to pH=5 (0.8 µM) and/or 25 g/L NaCl (AnalaR NORMAPUR, VWR chemicals 27810.295). During meat maturation, pH=5 is generally found as a result of lactic acid release (Honikel, 2004), whereas 25 g/L NaCl is classically found in some cheese products, such as feta (Hashem et al., 2014).

After homogenising and gentle stirring to avoid bubble formation, the inoculated gel matrix was immediately distributed in each well of a 96-well microtiter plate of microscopic grade (Microclear, Greiner Bio-one, France). The microtiter plates were then incubated at 20 °C and observed under the microscope at different time points. At the end points, bacteria were enumerated as Colony-Forming Units (CFU) by recovering the gel matrix from a well with a microstreaker and transferring it into a tube with 10 mL of sterile saline solution (9 g/L NaCl). The hydrogel was disrupted and homogenised by mechanical grinding with an Ika-Ultra-Turrax T25 (8000 rpm) to fragment the microcolonies into dispersed cells. Following serial dilutions, samples were spread on Petri dishes and incubated overnight and the results were expressed as CFU/mL.

Confocal Laser Scanning Microscopy (CLSM)

All microscopic observations were performed with a Leica HCS-SP8 confocal laser scanning microscope at the INRAE MIMA2 imaging platform (www6.jouy.inrae.fr/mima2). GFPmut2 (λ_{ex}485; λ_{em}508) and mRuby2 (λ_{ex}559; λ_{em}600) were excited respectively with laser bands at 488 nm and 561 nm. A 40x water long-range objective (numerical aperture = 0.8) was used with a 512 x 512 pixel definition (387 µm x 387 µm). A bidirectional acquisition speed of 600 Hz allowed a frame rate of 2.3 images per second. For 3D stack analysis, a 1 µm step between z levels was used. For each condition, a minimum of 2 stacks in 4 different wells were acquired (8 z-

stacks), except for co-cultures in 1.00% LMPA where only 2 wells were analysed (4 z-stacks). For the tracking of swimming cells, a 2-minute time lapse with one image every 459 ms was used, three times for each condition. Each microscopic image shown in the following results has a side (X/Y) of 387 µm and a height Z of 300 µm for a total volume of 4.5 10^6 μm^3. For image analysis, all microscopic pictures were treated on IMARIS 9.64 (Bitplane, Switzerland). The raw fluorescence of each image was first binarised using the automated Imaris isosurface function. From binarised objects, it is possible to extract geometric parameters (*e.g.* biovolume, position, intensity, sphericity, speed) as raw data for further treatments in a data spreadsheet. For single-cell tracking in LMPA, observations in control, lactic acid and NaCl conditions were performed for both pathogens during 2 minutes to evaluate the motility radius. The effective radius of motility corresponds to a circle around a cell at a given time T_0, with the periphery of said circle being an estimate of the position of the bacterium at time T_1.

Gel textural measurements

To assay the texture of gel matrices, 30 mL of each hydrogel was prepared in 40 mL plastic cups (weight about 30 g, internal diameter 28 mm) and a TA.XT2 texture analyser (Stable Micro System) equipped with a 5 Kg load cell. Hydrogels were compressed using a flat disc (25 mm diameter, height 2 mm) pushing at a crosshead speed of 1 $mm.s^{-1}$ until a depth of 40 mm was reached (1.5 cm gap with bottom of cup). During compression, the normal force (N) increased linearly at a fast pace until it reached a plateau. The value of the average force of the plateau (FP, N) was used to calculate the rupture point (in weight, g) to represent the hardness of the sample.

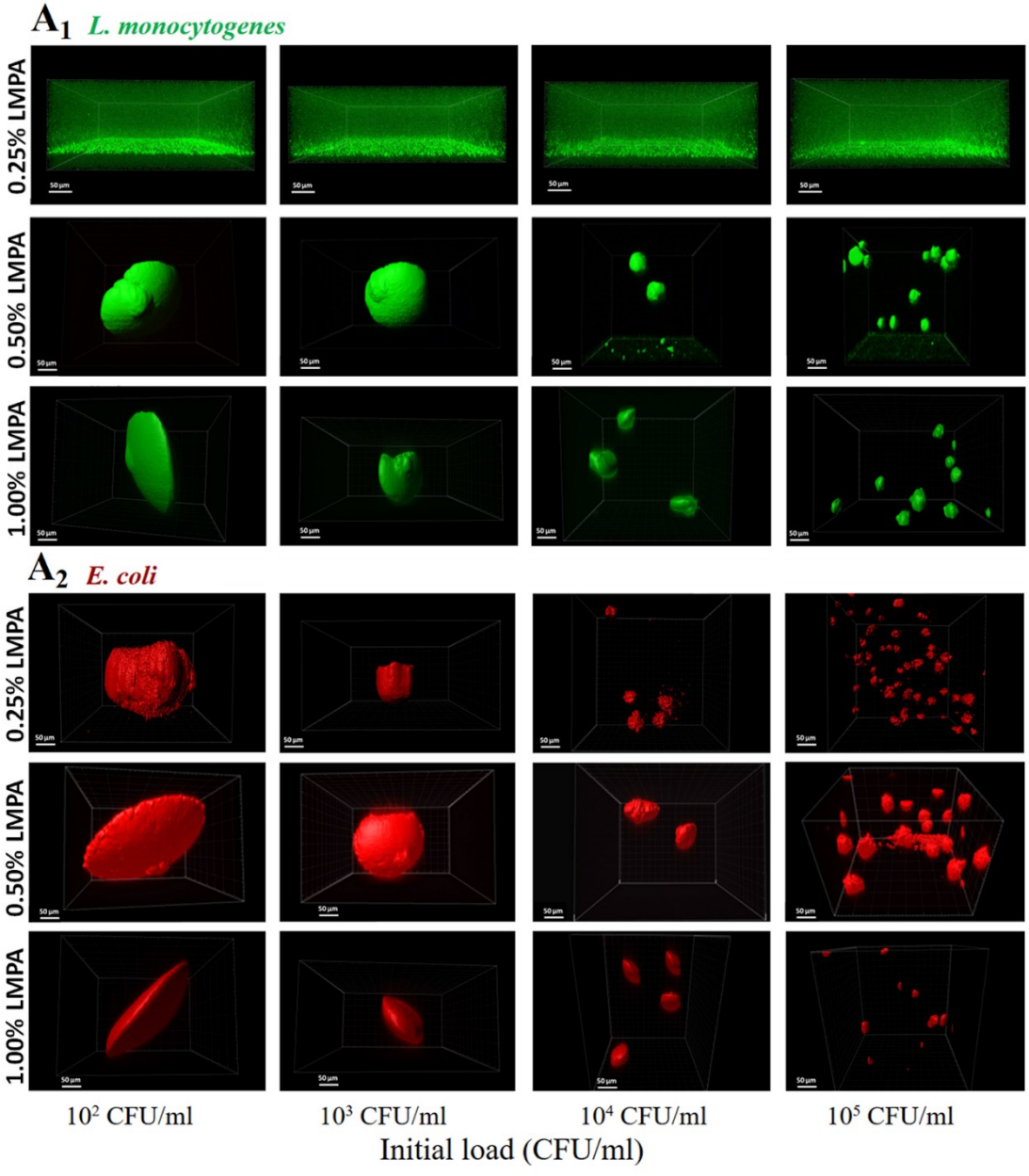
Statistics

ANOVA variance analysis was done using Statgraphics centurion (Statgraphics Technologies, Inc.) from 4 to 8 independent replicates. Differences were considered significant when $P<0.05$, with P being the critical probability associated with the Fisher test. Graphs and heatmaps were performed with Prism 9 (Graphpad; CA, USA).

3) Results

3.1) Microcolony size and morphology are influenced by the initial load of the inoculum and LMPA concentration of the gel matrices

To evaluate how initial bacterial load could affect the spatial organisation and distribution of bacteria in gel matrices, the size and morphology of in-gel bacterial microcolonies were assessed for initial concentrations ranging from 10^2 to 10^5 CFU/mL at three different LMPA concentrations: 0.25%, 0.50% and 1.00% (Figure 1).



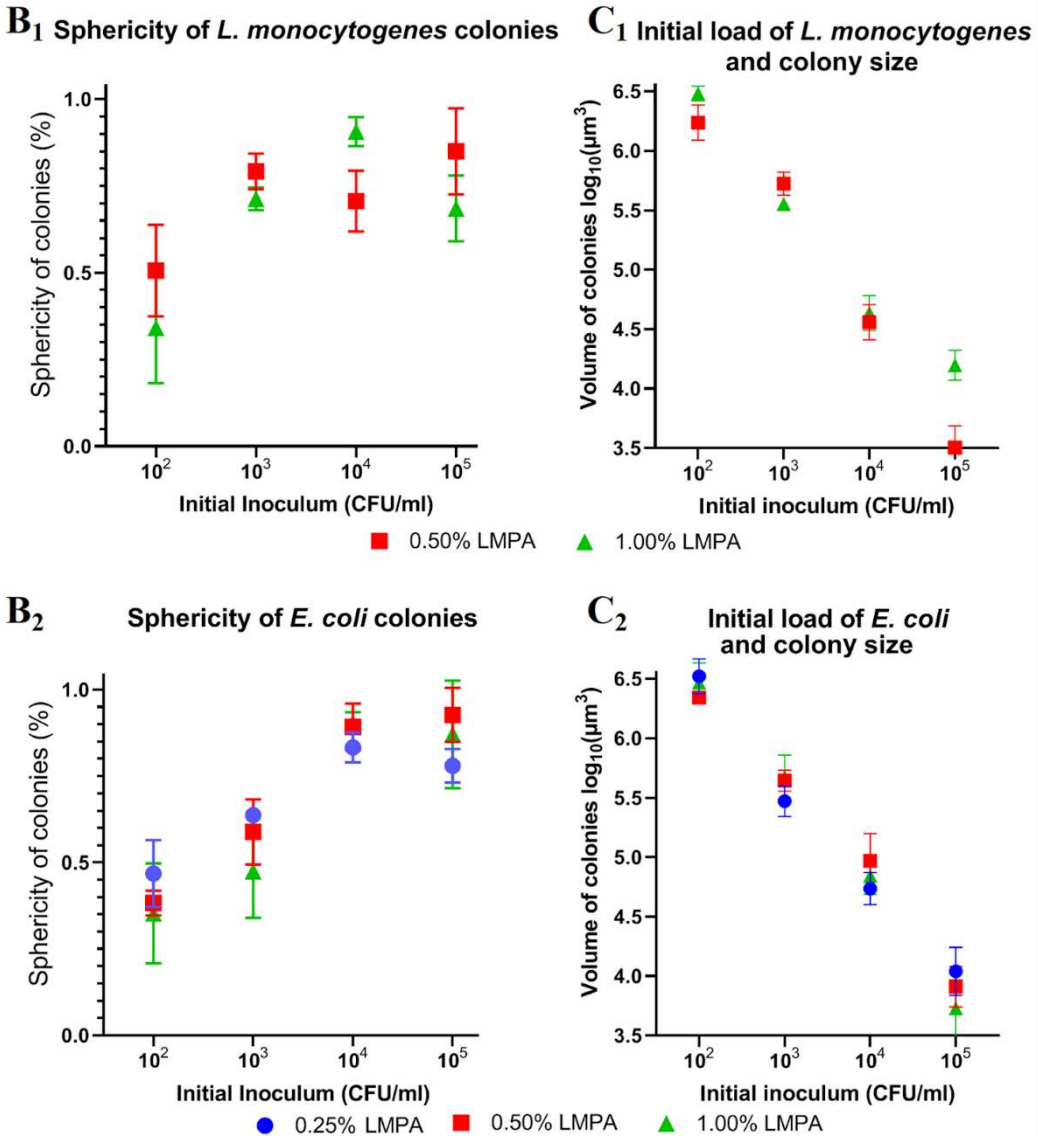


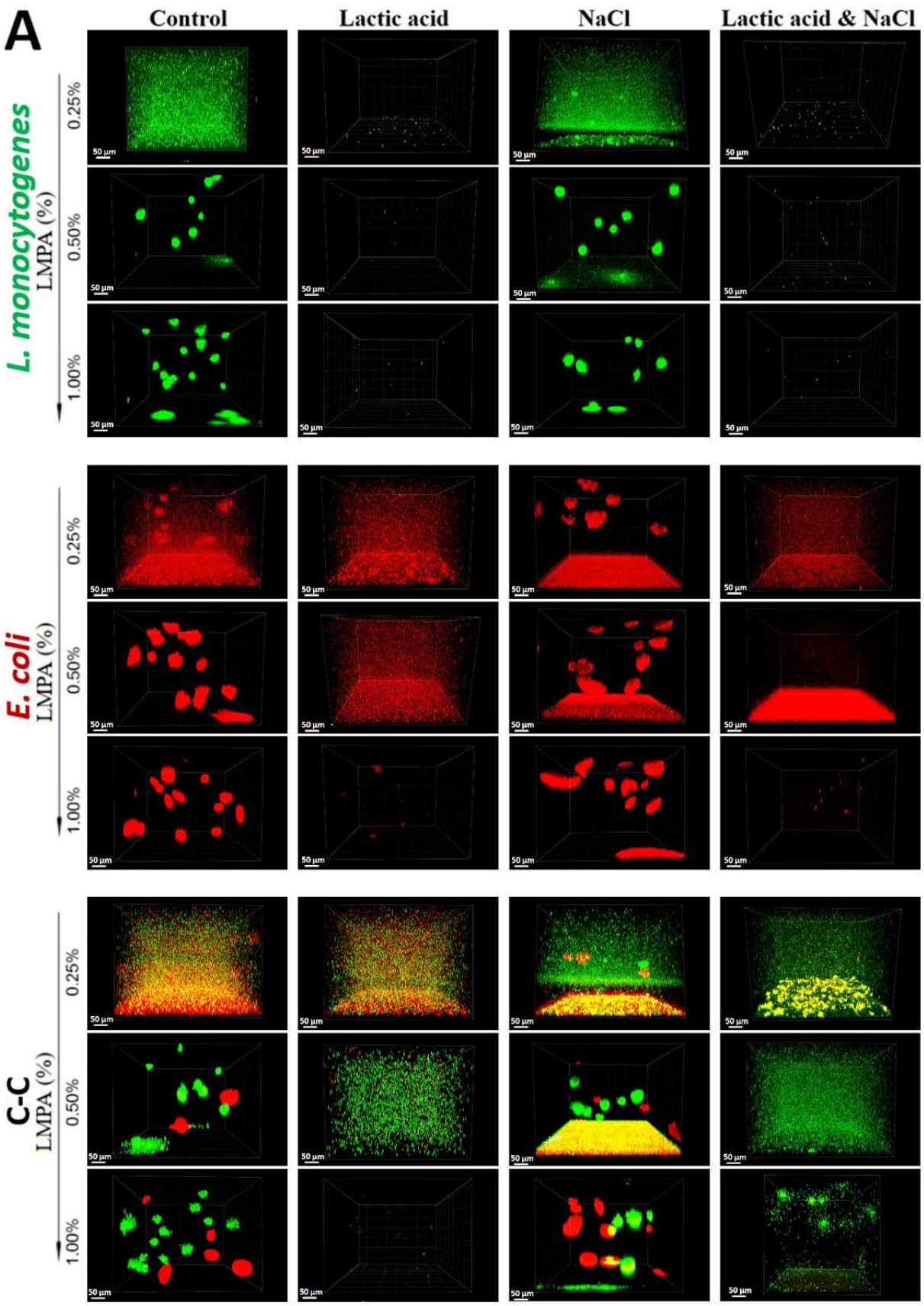
Figure 1: Effect of the bacterial initial load on the spatial distribution, biovolume and morphology of microcolonies of *L. monocytogenes* and *E. coli* O157:H7 in gel matrices. A: CLSM observations of *L. monocytogenes* (A_1, green) and *E. coli* O157:H7 (A_2, red) at 0.25%, 0.50% and 1.00% LMPA for different bacterial initial loads after 96 h of growth at 20°C. Representative pictures correspond to microcolony isosurfaces after fluorescence segmentation. B: Mean sphericity of microcolonies of *L. monocytogenes* (B_1) and *E. coli* (B_2) at different bacterial initial loads. Error bars indicate the mean standard deviation. C: Mean biovolume in $\log_{10}$ (μm^3) of microcolonies of *L. monocytogenes* (B_1) and *E. coli* (B_2) at 96 h at different bacterial initial loads. For B_1 and C_1, as there are no microcolonies at 0.25% no data are shown for this value. Each value presented is the average of 5 to 8 independent replicates.

Microcolonies in the gel matrices generally appeared with spherical/ovoid shapes with a rougher surface for *E. coli* O157:H7 compared to *L. monocytogenes* (Figures 1 A_1/A_2). At low initial loads, scarce but large microcolonies were observed, whereas microcolonies became smaller but more numerous with higher initial loads (Figure 1 A_1/A_2). Quantitative analysis demonstrated similar trends for both pathogens (Figure 1B). For initial loads from 10^3 to 10^5 CFU/mL in all LMPA concentrations, the mean biovolumes of microcolonies were 6.3 10^5 μm^3 and 1.5 10^4 μm^3, respectively, and inversely proportional to the initial load. The change in LMPA concentration did not significantly modify the biovolume of microcolonies except for *L. monocytogenes* at 0.25% LMPA where no microcolonies were observed. Below an initial load of 10^3 CFU/mL (*L. monocytogenes*, Figure 1 B_1) or 10^4 CFU/mL (*E. coli*, Figure 1 B_2), both pathogen microcolonies tended to adopt a lenticular form associated with a reduced sphericity (sphericity <0.7). These results indicate that, in this experimental model, both the density of the initial inoculum and the LMPA concentration are driving colony morphology. A last point of interest here was that, while the morphology and distribution of bacterial cells in the hydrogel changed significantly over the different concentrations tested, the average number of cells per given volume (CFU/mL) was not significantly different (supplementary material Figure S1).

3.2) Bacterial growth in gel matrices supplemented with lactic acid and NaCl

Considering that lactic acid and/or NaCl can be found in food matrices which are subject to contamination by *L. monocytogenes* or *E. coli* O157:H7 (*e.g.* some meat or cheese products), their effect on bacterial colonisation was further considered. Besides CLSM examinations in these different conditions with various concentrations of LMPA, lactic acid and/or NaCl (Figure 2A), microcolonies were further quantitatively analysed regarding their bacterial biovolumes (Figure 2B). In this study, bacterial microcolonies were defined as detected objects larger than 10^3 μm^3. This threshold volume

corresponds to a spherical object with a diameter of 20 µm, which was the lowest measurement for microcolonies in the control conditions.



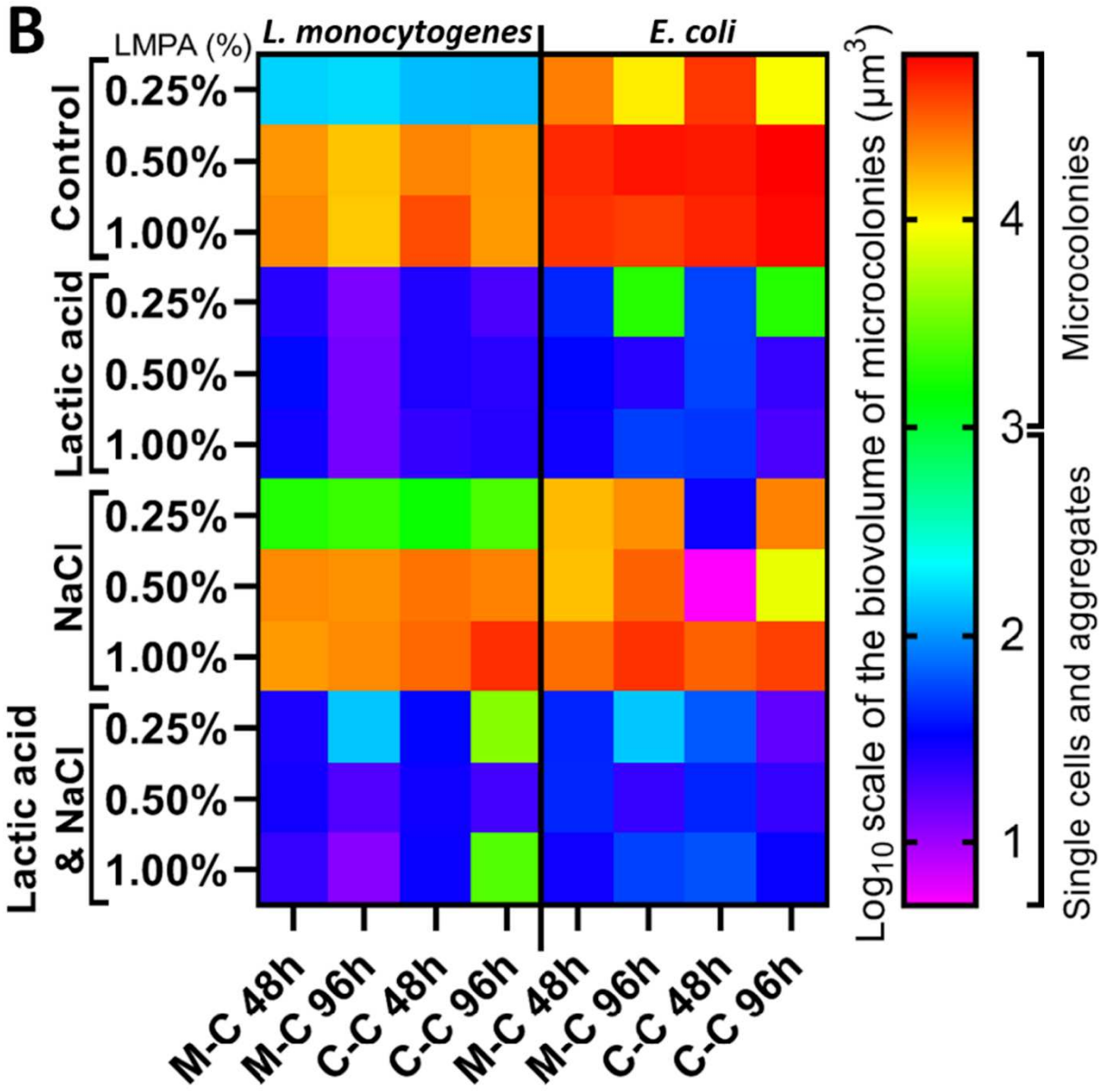


Figure 2: Impact of NaCl and/or lactic acid supplementation on microcolonies of *L. monocytogenes* and *E. coli* O157:H7 in gel matrices. A: CLSM observations of genetically engineered *L. monocytogenes* (green) and *E. coli* O157:H7 (red) cells in gel matrices with different LMPA concentrations (0.25, 0.50 or 1.00%) *(i)* without the addition of lactic acid or NaCl (control condition), *(ii)* with the addition of lactic acid, *(iii)* with supplementation NaCl, or *(iv)* with both lactic acid and NaCl, in mono- and co-cultures at 96 h. B: Heatmap of the mean biovolume of microcolonies in the different gel matrix conditions. The colour scale is expressed in $\log_{10}$ (μm^3) with cooler tones indicating small microcolonies and warmer colours indicating large microcolonies. Data originate from CSLM observations performed at 48 h and 96 h in mono-culture (M-C) and co-culture (C-C) conditions. A threshold of 10^3 μm^3 was set as the limit between single cells or small aggregates and microcolonies. Each value presented is the average of 5 to 8 independent replicates.

With increasing LMPA concentrations, a shift from single cells to microcolonies was observed from and above 0.50% LMPA for *L. monocytogenes*, while *E. coli* were observed as microcolonies from 0.25% LMPA (Figure 2A). Between 0.50% and 1.00% LMPA, the biovolumes of microcolonies were significantly different and ranged from 1.0 10^2 µm^3 to 7.0 10^4 µm^3 (Figure 2B).

Considering the lactic acid effect, quantitative analysis yielded a drop in mean biovolume from 10^4 µm^3 in gels without lactic acid to below 10^2 µm^3 with lactic acid for both *L. monocytogenes* and *E. coli* O157:H7, whatever the LPMA concentration (Figure 2). While *E. coli* O157:H7 was more resilient to lactic acid with a final population density of around 10^7 CFU/mL (from 10^9 CFU/mL control), it was significantly lower for *L. monocytogenes* with a decrease from 10^9 CFU/mL (control) to 10^5 CFU/mL (Figure 2, supplementary material Table S1). Interestingly, in the presence of *E. coli* O157:H7, *L. monocytogenes* exhibited greater resilience to the effect of lactic acid (Figures 2) and reaches a final density of 10^7 CFU/mL. On the contrary, *E. coli* O157:H7 appeared more severely impacted by lactic acid in the presence of *L. monocytogenes* compared to its mono-culture conditions, both in terms of microcolony formation and final cell density, as both were lower then.

The presence of NaCl favored the apparition of microcolonies of *L. monocytogenes* at 0.25% LMPA (*i.e.* apparition of aggregates of 10^3 µm^3), But not at the other LMPA concentrations tested (Figure 2). Of note, some cell aggregates could be observed at the lowest LMPA concentration (0.25%). For *E. coli* O157:H7, there was no variation in microcolony size, but the population of single cells could not be observed anymore with salt supplementation (Figure 2). NaCl also appeared to promote adherence and biofilm development on the polystyrene at the bottom of the wells for both pathogens at 0.25% LMPA (Figure 2, supplementary material Figure S2), as well as at 0.50% LMPA especially for *E. coli* O157:H7 (Figure 2). However, no significant difference regarding microcolony formation and adherence to the bottom of the wells could be observed at 1.00% LMPA concentration compared to the control conditions. In the presence of

NaCl in co-culture, the microcolonies were significantly smaller (10^2 µm^3 instead of 10^4 µm^3) for *E. coli* O157:H7 at 48 h compared to mono-culture conditions (Figures 2, supplementary material Figure S3). However, the mean biovolumes finally reached an equivalent size at 96 h, between mono- and co-culture conditions, indicating that the expansion of microcolonies was only slowed down and temporarily delayed.

In gel matrices supplemented with both lactic acid and NaCl, microcolonies were no longer observed for both *L. monocytogenes* and *E. coli* O157:H7 at all LMPA concentrations (Figure 2), with mean biovolumes (10^2 µm^3) comparable to what was seen in the lactic acid condition. Adherence and biofilm development at the bottom of the wells was still observed for *E. coli* O157:H7 with 0.25% and 0.50% LMPA, but not for *L. monocytogenes* (Figure 2, supplementary material Figure S2). However, for co-cultures in this condition with lactic acid and NaCl, *L. monocytogenes* had a higher biovolume and formed a biofilm with *E. coli* O157:H7 at the bottom of the well (Figure 2, supplementary material Figure S2).

3.3) Single-cell motility of *L. monocytogenes* and *E. coli* O157:H7 in the gel matrices

With no addition of lactic acid or NaCl, single cell motility was only observed in gel matrices at 0.25% LMPA with a mean speed (Smean) of 0.53 µm/s and 0.68 µm/s for *E. coli* O157:H7 and *L. monocytogenes*, respectively (Figure 3, supplementary material Table S2). At higher LMPA concentrations, bacterial cells were sessile and generally part of microcolonies (Figure 2). At 0.25% LMPA supplemented with lactic acid, motility was observed for both pathogens. With 0.25% LMPA supplemented with NaCl, *L. monocytogenes* was motile, but not *E. coli* O157:H7. The same results were obtained at 0.50% LMPA, but lower speed rates were recorded. At 1.00% LMPA, no motility was observed whatever the strain and supplements (Figure 3).

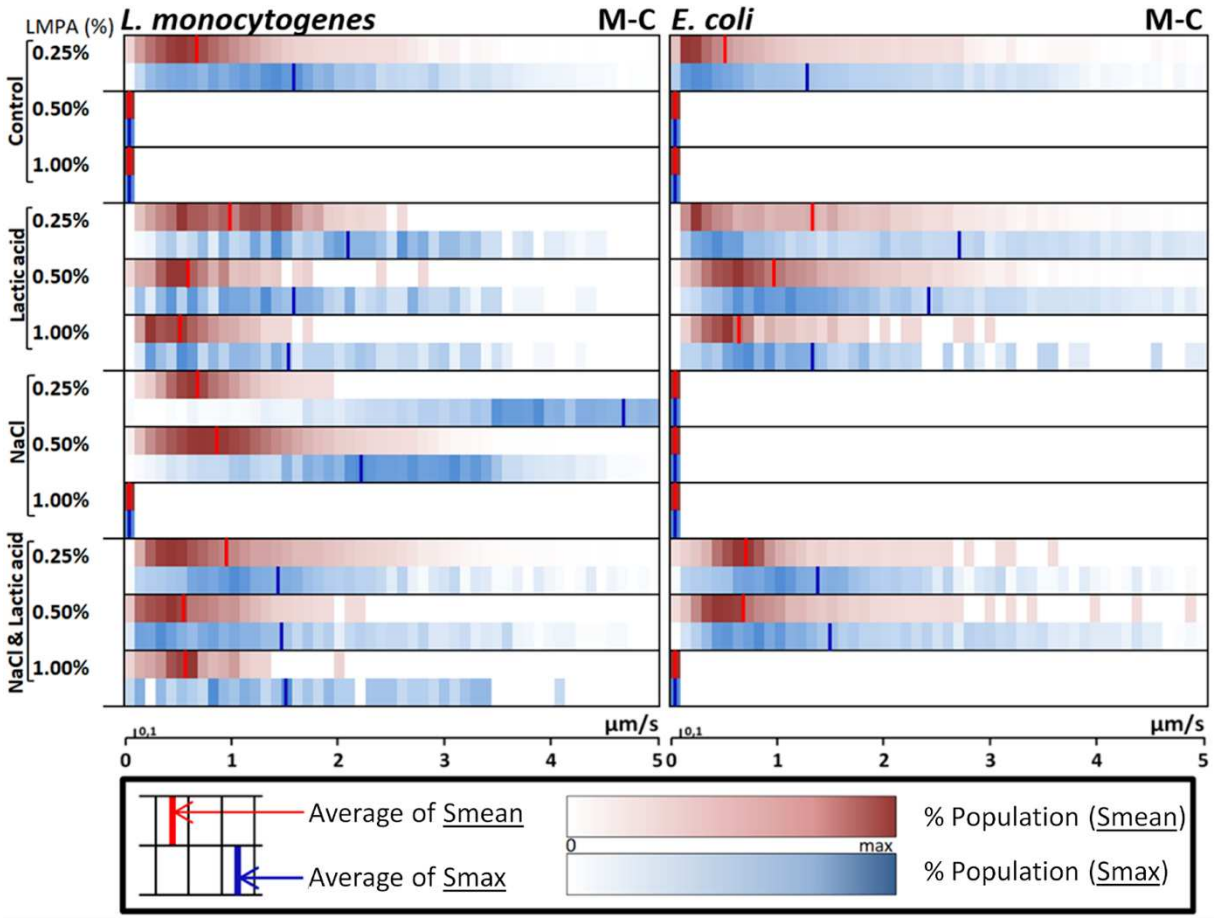


Figure 3: Quantitative analysis of individual cell motility of *L. monocytogenes* and *E. coli* O157:H7 in different gel matrix conditions. The mean speed (Smean in red) and the max speed (Smax in blue) were calculated from the average of 5 to 8 independent samples of bacterial cells observed in time-lapse microscopy as described in the Materials and Methods section. From there, the percentage of population for the different speed values was represented as a density bar. The averages for the Smean and Smax were further calculated and represented as bright colour lines (red and blue, respectively) to highlight shift in motility of the cell population. Gel matrices were obtained at different LMPA concentrations (i.e. 0.25%, 0.50% or 1.00%) *(i)* without the addition of lactic acid or NaCl (control condition), *(ii)* with the addition lactic acid, *(iii)* with supplementation of NaCl, or *(iv)* with both lactic acid and NaCl.

In all tested conditions, the variation of Smean values was less pronounced than for the maximum speed (Smax) of the single cells. In the presence of lactic acid for *E. coli O157*:H7 and in the presence of NaCl for *L. monocytogenes*, there were clear shifts of Smax, with significantly higher values than in other conditions (Figure 3, supplementary material Table S2). For *E. coli* O157:H7 in the presence of lactic acid, Smax was 2.7 and

2.4 µm/s at 0.25% and 0.50% LMPA, respectively (versus 1.3 and 0 µm/s with no supplementation) (supplementary material Table S2). This increase in cell motility upon NaCl or lactic acid supplementation was no longer observed at 1.00% LMPA. These results are in accordance with the microscopic observations and motility tracking (Figure 5). For *L. monocytogenes*, Smax reached 4.8 and 2.2 µm/s with NaCl supplementation at 0.25% and 0.50% LMPA, respectively (versus 1.6 and 0 µm/s with no supplementation) (supplementary material Table S2).

Considering the distribution of the cell population, the two pathogens appeared to behave differently in conditions promoting cell motility (Figure 3); while most cells of *L. monocytogenes* reacted to lactic acid or NaCl supplementation, only a small subpopulation of *E. coli* O157:H7 cells did. With NaCl supplementation in gel matrix, over 80% of *L. monocytogenes* cells had a Smax higher than 2 µm/s, whereas 38% of *E. coli* O157:H7 still had a Smax below 1 µm/s with lactic acid (Figure 3). When both lactic acid and NaCl were added to the gel matrices, the Smean and Smax for *L. monocytogenes* were not significantly different from the results obtained with lactic acid supplementation alone (Figure 3, supplementary material Table S2). For *E. coli* O157:H7, motility for 0.25% and 0.50% LMPA supplemented with lactic acid and NaCl was equivalent to what was observed in the control (Figure 3).

In co-cultures, a slight but significant increase of Smean for *L. monocytogenes* upon NaCl supplementation was observed (supplementary material Figure S4, supplementary material Table S3). In co-culture with lactic acid, the Smean for *L. monocytogenes* was doubled in comparison to mono-culture conditions at 0.25% and 0.50% LMPA. For *E. coli* O157:H7, the opposite was observed, with a general decrease in the Smean values. Co-cultivation of *E. coli* O157:H7 and *L. monocytogenes* in gel matrices thus led to significant change in the motility trends for each strain.

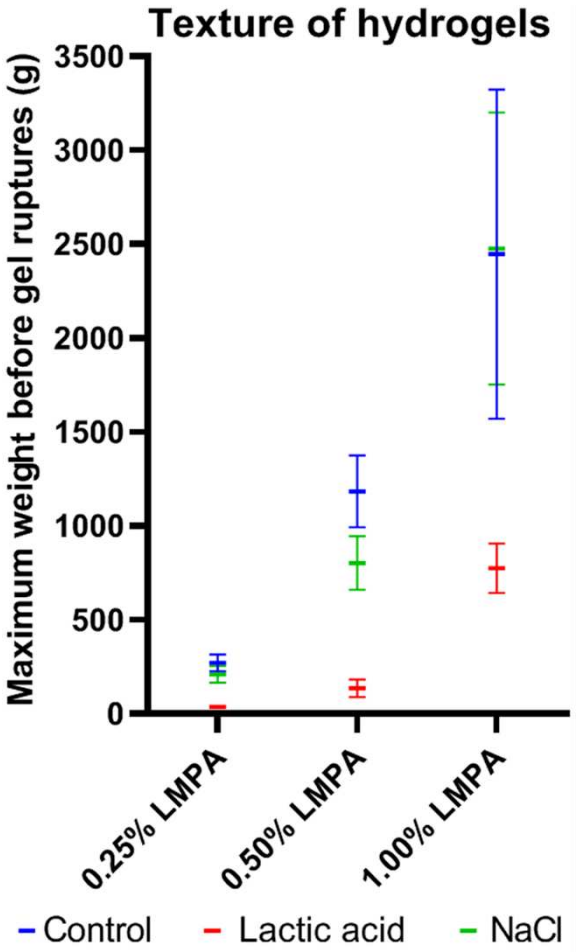


Figure 4: Texture of the gel matrices supplemented with lactic acid or NaCl. Mean of hydrogel hardness (g) at the different LMPA concentrations considered: 1.00%, 0.50% and 0.25%, without supplementation (control condition in black) or supplemented with lactic acid (green) or with NaCl (orange). Error bars indicate the standard deviation (calculated from 10 measurements). Each value presented is the average of 5 to 8 independent replicates.

With the addition of lactic acid or NaCl in 0.50% or 1.00% LMPA gel matrices, cell motility was promoted for both *L. monocytogenes* and *E. coli* O157:H7 (Figure 3). As this could result from variations in the hardness of the gel matrices in these conditions, the texture of the gel matrices was further considered (Figure 4). As expected, a significant increase in the hardness of the gel matrices was confirmed with increasing LMPA concentrations (Figure 4); the gel matrix was 5.5 times harder at 0.50% LMPA and 14.5 times harder at 1.00% LMPA compared to 0.25% LMPA. When lactic acid or NaCl was added, the rheology of the gel matrices was significantly modified as

hydrogels composed of 0.50% LMPA and supplemented with NaCl could support up to 795 g (*i.e.* a 33% decrease in strength) and a gel matrix at 0.50% LMPA with lactic acid only 173 g (*i.e.* 89% decrease in strength) compared to the control condition (gel matrix with no supplementation). The same impact of the supplements is noted for the values 0.25% and 1.00% and overall, the 3 conditions of agarose concentrations, lactic acid and NaCl significantly ($p>0.05$) altered the texture of the gel. This variation in hydrogel hardness could partially explain the increase in cell motility for *L. monocytogenes* in gel matrices with lactic acid. However, for 0.50% LMPA supplemented with NaCl, the Smean and Smax obtained for *L. monocytogenes* were significantly higher than at 0.25% LMPA when the hydrogel hardness of the control condition was in fact lower (Figures 3 and 4, supplementary material Table S2). Similar observations could apply for the Smean and Smax of *E. coli* O157:H7 at 0.50% LMPA with lactic acid versus conditions at 0.25% LMPA, where hydrogel hardness is equivalent but motility is not. In these conditions, variations of the cell motility would thus result from physiological adaptation of bacterial cells rather than from modifications to the texture of the gel matrices.

In order to further characterise the cell motility of *L. monocytogenes* and *E. coli* O157:H7 in the different gel matrix conditions, the effective radius of motility for each bacterial cell was evaluated. At 0.25% LMPA, where *L. monocytogenes* and *E. coli* O157:H7 were less motile, the same effective radius of ~2.5 µm/m was measured (Figure 5, supplementary material Figure S5 video A and D). With the addition of lactic acid, an increase in cellular speed was observed for both pathogens (Figure 3) and tumbling was also observed (Figure 5, supplementary material Figure S5 video B and E); the effective radii in this condition were about 7.5 µm/m and 12.5 µm/m for *L. monocytogenes* and *E. coli* O157:H7, respectively. In gel matrices supplemented with NaCl, no motility could be observed for *E. coli* O157:H7, whereas *L. monocytogenes* had a mean effective radius of 31.8 µm/m (Figure 5, supplementary material Figure S5 video C and F). This increase in effective radius was observed for *L. monocytogenes* with

supplementation of NaCl, with no increase in the Smean (0.68 µm/s) but a significant change in Smax (from 1.57 to 4.77 µm/s) (supplementary material Table S2).

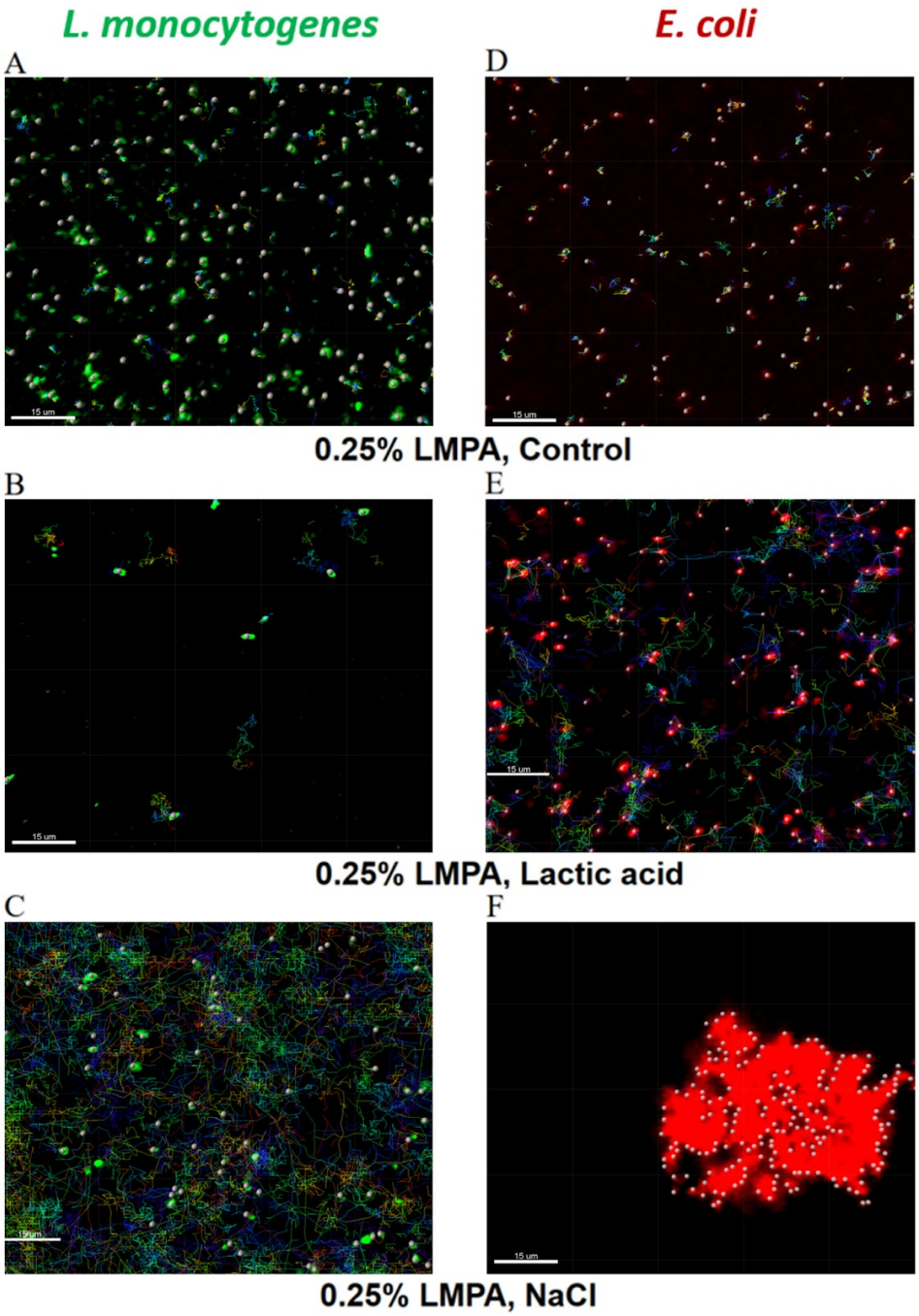


Figure 5: Time-tracking of single-cell motility of *L. monocytogenes* (A, B, C) and *E. coli* O157:H7 (D, E, F) in gel media. Data acquisition was performed at 0.25% LMPA; without the addition of lactic acid or NaCl (control condition; A, D); with the addition of lactic acid (B, E), or with supplementation of NaCl (C, F). Tracks were acquired and represented over the full duration of the time lapse (120 s). All images are 101 µm by 82 µm and correspond to close-ups of images found in the 6 supplementary material movies V7A to V7F.

4) Discussion

Food matrices are rich media harbouring hundreds of microbial species (Chaillou et al., 2015; Yu et al., 2019). Understanding the behaviour of foodborne pathogens in this environment is one of the key prerequisites to refining risk assessment models and ultimately to improving microbiological food safety (Koutsoumanis et al., 2016; Membré and Guillou, 2016). However, the behaviours of bacterial foodborne pathogens are generally assessed in liquid growth media that poorly mimic solid food matrices. At the same time, food matrices are often opaque, preventing direct photonic microscopic observation of these microorganisms to evaluate their physiology and behaviour. To alleviate this limitation, genetically engineered fluorescent *L. monocytogenes* and *E. coli* O157:H7 were grown in transparent nutritive gel matrices for *in situ* observations by CSLM (Bridier et al., 2015; Verheyen et al., 2019). Besides the visualisation of bacterial cells in the gel matrix, the use of LMPA presented the advantages of a low solidification point (~25 °C) limiting bacterial thermal stress, combined with a low melting point (≤65 °C) facilitating experimental handling compared to other jellifying agents such as agar, guar gum or gelatine (Jaeger et al., 2015). Furthermore, LMPA allowed coverage of a wide range of textures relevant to various food matrices, *e.g.* 0.50% LMPA presented a hardness similar to fried beef patties (Uriyapongson, 2007) and 1.00% LMPA was comparable to the texture of mature cheddar (Pollard et al., 2003).

In most conditions tested in the gel matrices, bacteria colonised the media as microcolonies adopting a spherical morphology. While similar observations have already been reported in gelatine gels and/or for other bacterial species (Antwi et al., 2006; Mitchell and Wimpenny, 1997), a clear inverted correlation between the density of founder cells and the radius of the microcolonies was highlighted in this investigation. While giant microcolonies could be observed at the lowest rates of initial inoculation (*e.g.* radius of about 110 µm at 10^2 CFU/mL), the distances between microcolonies decreased as the initial inoculation rate increased (*e.g.* from one millimeter to around 80 µm). Modification of metabolite gradient in the microcolonies

can be expected between those conditions (Jeanson et al., 2015). In addition, the sphericity of the microcolonies increased with the initial concentration of the inoculum, whereas more oblate/lenticular shapes were observed at the lowest initial load. Such lenticular microcolony morphology has been previously reported, but as a result of increasing concentrations of gelatine (Antwi et al., 2006) or agarose (Zhang et al., 2021). In biofilms, the thickness plays a role in phenotypic diversity with the regulation of gene expression (Shemesh et al., 2008). As spherical and lenticular morphologies in microcolonies present different thickness at a given point with possibly different nutrient and metabolic waste gradients, it could be of interest to study the phenotypic diversification of embedded bacteria between the two shapes.

The formation of microcolonies itself is also a food safety issue due to emerging properties caused by heterogeneity and diversification of bacterial phenotypes, which appear as colonies grow larger and produce gradients of metabolites (Saint-Ruf et al., 2014). Quorum sensing can also be a source of diversification in such high-density populations (Garmyn et al., 2011; Ji et al., 1995; Kanamaru et al., 2000). Supplementation of gel matrices in lactic acid and/or NaCl had profound effects on the microcolony development of both pathogens. The final cell density of *E. coli* O157:H7 and *L. monocytogenes* as well as microcolony formation were significantly reduced in the presence of lactic acid and most certainly result from cumulative inhibitory effects of pH and diffusion of undissociated weak acid across the cellular membranes (Arcari et al., 2020; Booth, 1985; Lambert and Stratford, 1999; Russell and Cook, 1995). Supplementation with NaCl in tested hydrogels did not significantly impact the final cell density, but a modification of the spatial distribution in the gel matrix was observed, especially development at the bottom of the wells. Addition of NaCl was previously shown to influence the physiology, including bacterial adhesion and/or biofilm formation abilities of *E. coli* (Cerca and Jefferson, 2008; Hou et al., 2014; Olesen and Jespersen, 2010) or *L. monocytogenes* (Lee et al., 2019; Olesen et al., 2009; Silva et al., 2020; Xu et al., 2010) in various growth conditions. Interestingly, the behaviours of *L. monocytogenes* and *E. coli* O157:H7 appeared to be influenced when they were co-

cultured. Differences in bacterial microcolony formation and final cell density could be observed for *E. coli* O157:H7 in co-cultures supplemented in lactic acid and/or NaCl. Filtered supernatants of one pathogen culture had no impact on the growth when added to the culture of the other pathogen (data not shown), suggesting that the observations stem either from nutrient competition or from a specific mechanism of interference that requires further investigation to be identified. While generally considered in a discrete manner in food safety (from quality control to risk modelling) or medical microbiology (in case of human infections), these zoonotic foodborne bacterial pathogens can co-occur and contaminate the same foodstuffs, especially some types of cheese and meat as well as some vegetable products (Fantelli and Stephan, 2001; Kallipolitis et al., 2020; Lin et al., 1996; Luna-Guevara et al., 2019). The possibility of co-contamination should then not be overlooked (Opperman and Bamford, 2018). Of note, the present experiments were performed at 20 °C, which could be encountered for foodstuffs left on a kitchen work surface or during shopping and transport by consumers from store to home. Obviously, further in-depth investigations performed at refrigerating temperature will be needed to explore the phenomenon at the recommended storage temperatures of perishable products in the food distribution system. In addition, other pertinent foodborne pathogens, such as *Salmonella*, *Campylobacter*, or *Staphylococcus*, as well as their interactions with technological (lactic acid bacteria) and spoilage bacteria (e.g. *Pseudomonas*, *Brochothrix* or *Erwinia*), should also be considered.

Cell motility appeared to be dependent on the texture of the gel matrix, but also on the presence of lactic acid and/or NaCl. As expected, the speed of cells swimming in gel matrices was slower than that reported in liquid broth (Swiecicki et al., 2013) and would rather correspond to speed rate measured at the edge of swarming colonies (Damton et al., 2010). Besides the speed rate, lactic acid is known to have an impact on the rotation of the flagella in *E. coli* as it induces inversion of flagellar rotation from counter-clockwise (CCW) to clockwise (CW) (Xu et al., 2019); CCW rotation is generally associated with straight swimming, whereas CW rotation of the flagella results in

tumbling behaviour in *E. coli* (Larsen et al., 1974). *E. coli* generally does not express motility at low pH, but a bacteriophage of *E. coli* O157:H7 has been demonstrated to induce a swarming phenotype in acidic media (Su et al., 2010). In *L. monocytogenes*, the expression of flagella is known to be repressed in the presence of lactic acid (Cortes et al., 2020) as well as by high concentrations of NaCl (Caly et al., 2009). In both *E. coli* and *L. monocytogenes,* "hopping" and "trapping" behaviour could also contribute to cell motility and occur as observed in porous matrices, where short bursts of free movement are responsible for most of the apparent motility (Bhattacharjee and Datta, 2019). In *L. monocytogenes*, the flagella plays a role in first contact with the host cell and effectiveness of invasion (Dons et al., 2004). In general, *E. coli* infections are more likely to be associated with high motility phenotypes (Kao et al., 2014) and for O157:H7 in particular, the O island (OI) 172 regulates the motility of the pathogen when multiple such OI (148, 122, 15, 48...) are known to be vital for virulence (Xu et al., 2013).

Information obtained in hydrogel matrices emphasized consistent behaviours and responses of bacterial cells to different environmental conditions and/or stresses. Such data are of great importance in establishing more suitable and appropriate predictive models for the control and mitigation of foodborne pathogens in solid/semi-solid food matrices. They could also serve as a basis for the formulation of new and innovative strategies to limit or prevent growth of these pathogens in food products. Investigation of bacterial colonisation in a model of food matrices by bacterial foodborne pathogens demonstrated a wide range of colonisation patterns depending on environmental conditions. Different strains of a given species as *E. coli* O157:H7 or *L. monocytogenes* can exhibit different behaviours and phenotypes (Biscola et al., 2011; Borucki et al., 2003; Orsi et al., 2011; Renter et al., 2003), but the diversification of cell types in spatially organised communities can trigger emerging community properties. Further layers of complexity can be added by biocoenoses forming discrete microecosystems where foodborne pathogens interact with other species of food microbiota; as well as various regulatory and molecular mechanisms including phase variation (e.g. sequence inversions, different methylation sites and levels of methylase expression) or bistability

of gene expression (Ackermann, 2015; Veening et al., 2008). While the present investigation highlights differences in the spatial organisation of some key foodborne pathogens in gel matrices, the integration of the different levels of heterogeneity in food matrices is clearly the next frontier in the improvement of risk assessment of microbial food safety and necessitates consideration of foodborne pathogens at a single-cell level besides the population level classically considered in food matrices (Lianou and Koutsoumanis, 2013). Besides description of growth parameters, achievement of this aim will require acquisition of in-depth knowledge of molecular mechanisms at play in phenotypic heterogeneity, in combination with mathematical modelling of the behaviour of foodborne pathogens.

Declaration of competitive interest

None

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Supplementary material

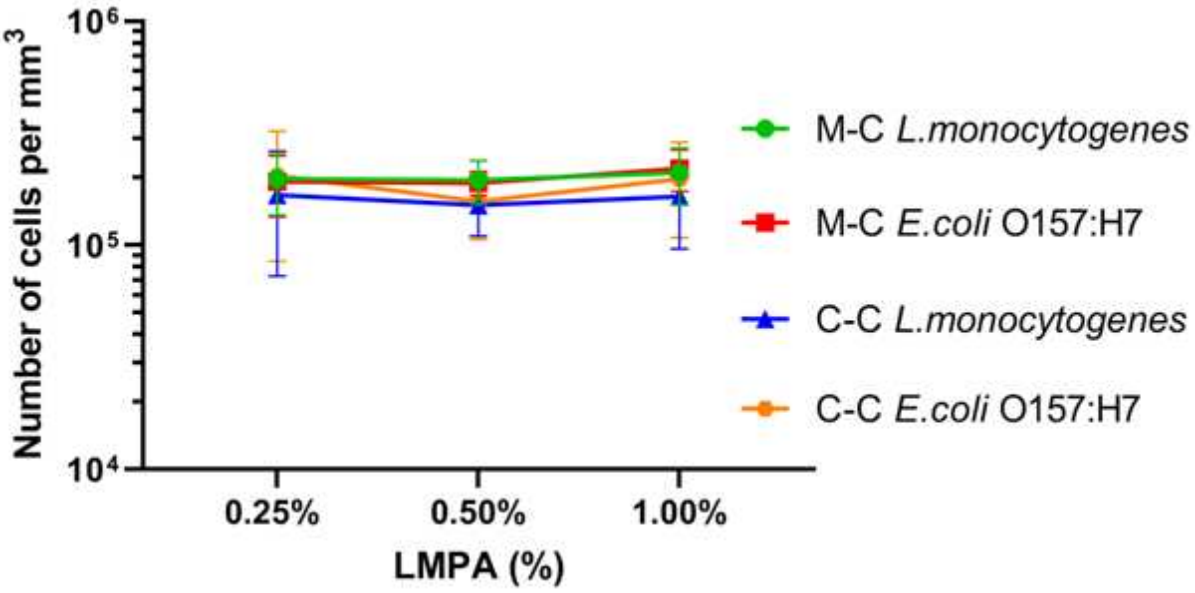


Figure S1: Quantitative analysis of the cell population per image (Materials and Methods) of *L. monocytogenes* and *E. coli* O157:H7 in mono- and co-cultures between three LMPA concentrations. Media were inoculated at 10^2 CFU/mL and cultivated at 20°C for 96 h.

Log(CFU/ml)	**TSB**	**TSB 0.25% LMPA**	**TSB pH5**	**TSB pH5 0.25% LMPA**	**TSB NaCl**	**TSB NaCl 0.25% LMPA**
L. monocytogenes	9.36±0.19	9.48±0.13	9.00±0.43	4.87±0.25	9.39±0.20	8.02±0.31
***E. coli* O157:H7**	9.25±0.11	9.30±0.10	9.00±0.29	7.00±0.24	9.08±0.18	8.62±0.19

Table S1: Table of final CFU/mL achieved in either TSB or 0.50% LMPA in conditions: control, lactic acid, NaCl. Media were inoculated at 10^2 CFU/mL and cultivated at 20°C for 96 h. Supplementation by lactic acid or NaCl is as described in the Materials and Methods.

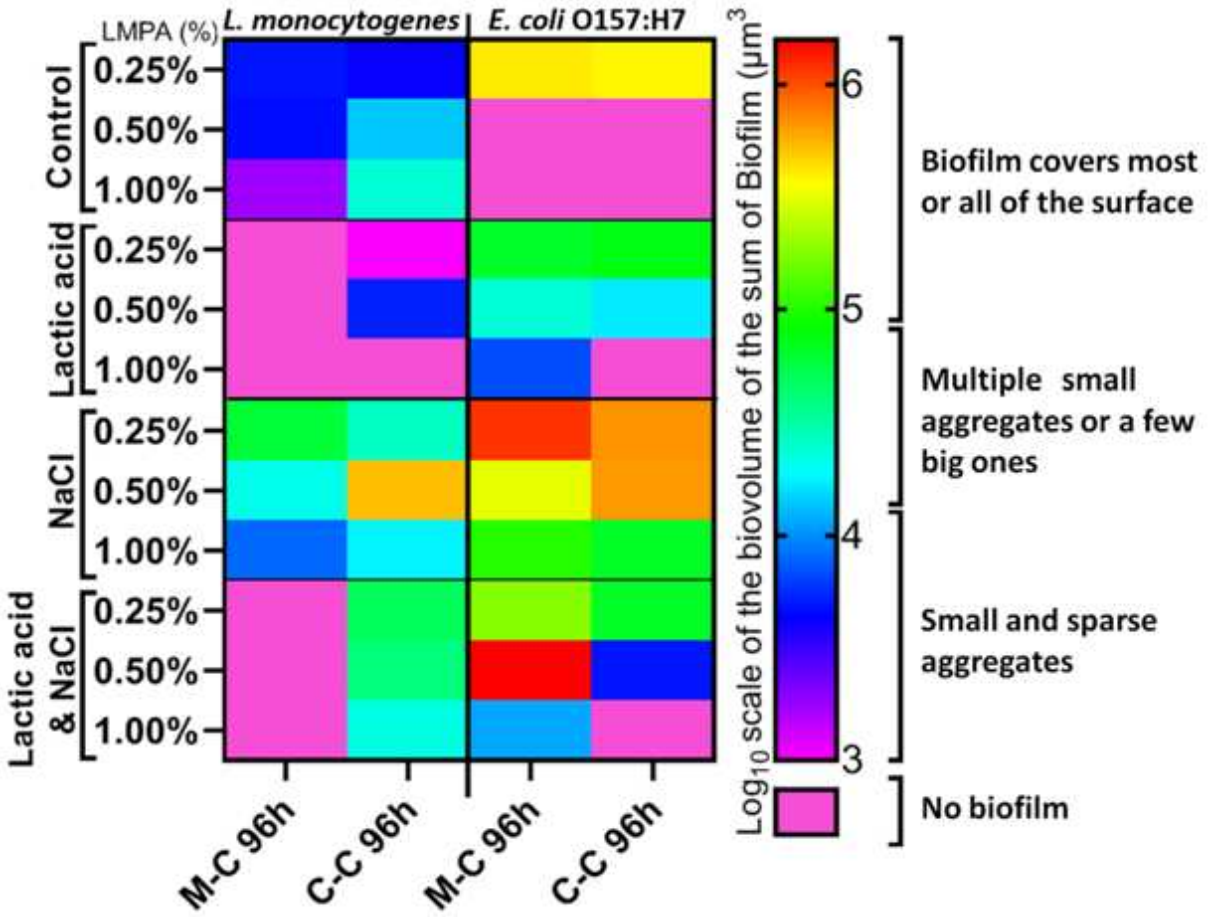


Figure S2: Impact of lactic acid and/or NaCl supplementation on biofilm of *L. monocytogenes* and *E. coli* O157:H7 in gel matrices. Heatmap of the mean biovolume of biofilms in the different gel matrix conditions. The colour scale is expressed in $\log_{10}$ (μm^3) with cooler tones indicating smaller amounts of biofilm and warmer colors indicating bigger amounts of biofilm. Data originate from CLSM observations performed at 96 h from mono-culture (M-C) and co-culture (C-C) conditions.

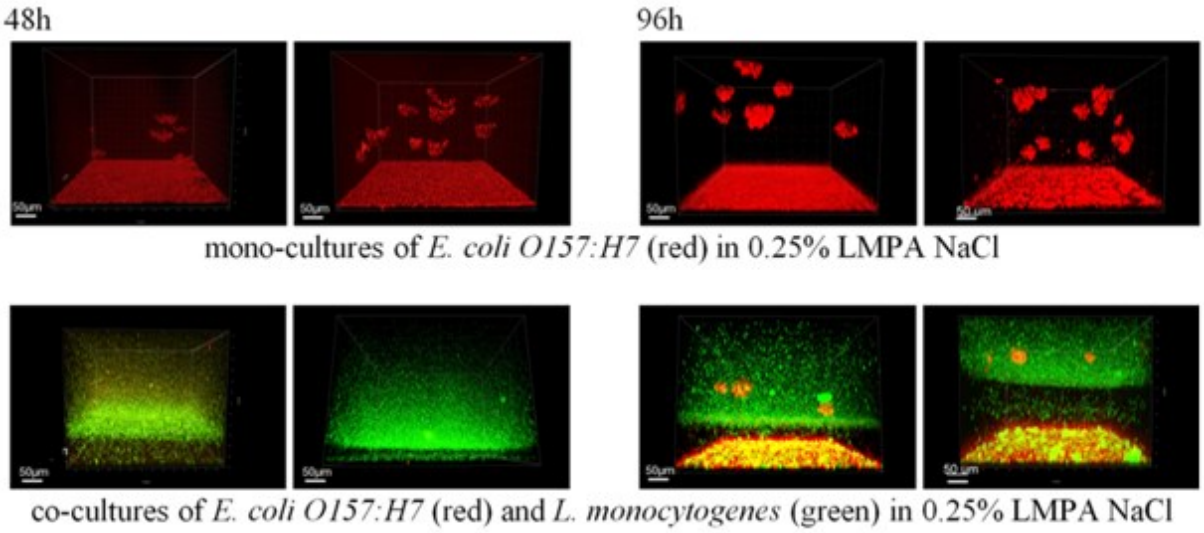


Figure S3: Qualitative observation of the growth delay for microcolonies of *E. coli* O157:H7 caused by the presence of *L. monocytogenes.* Gel matrices were obtained at 0.25% LMPA and supplemented with NaCl. For mono-cultures of *E. coli* O157:H7, four observations are shown, two at 48 h (left side) and two at 96 h (right side). The same was done with co-cultures of *L. monocytogenes* and *E. coli* O157:H7.

Conditions:	Smean µm/s	Standard deviation Smean	Smax µm/s	Standard deviation Smax
***L. monocytogenes* mono-cultures**				
0.25% LMPA control	0.68	0.42	1.57	1.11
0.50% LMPA control	0.00	0.00	0.00	0.00
1.00% LMPA control	0.00	0.00	0.00	0.00
0.25% LMPA lactic acid	0.99	0.47	2.07	1.01
0.50% LMPA lactic acid	0.61	0.38	1.59	0.98
1.00% LMPA lactic acid	0.54	0.30	1.55	1.02
0.25% LMPA NaCl	0.68	0.26	4.77	1.92
0.50% LMPA NaCl	0.95	0.53	2.22	1.16
1.00% LMPA NaCl	0.00	0.00	0.00	0.00
0.25% LMPA lactic acid & NaCl	0.96	0.71	1.39	1.05
0.50% LMPA lactic acid & NaCl	0.57	0.37	1.47	1.10
1.00% LMPA lactic acid & NaCl	0.58	0.30	1.56	1.02
***E. coli* O157:H7 mono-cultures**				
0.25% LMPA control	0.53	0.54	1.27	1.34
0.50% LMPA control	0.00	0.00	0.00	0.00
1.00% LMPA control	0.00	0.00	0.00	0.00
0.25% LMPA lactic acid	1.36	1.04	2.70	1.84
0.50% LMPA lactic acid	0.97	0.64	2.41	1.73
1.00% LMPA lactic acid	0.67	0.48	1.36	1.06
0.25% LMPA NaCl	0.00	0.00	0.00	0.00
0.50% LMPA NaCl	0.00	0.00	0.00	0.00
1.00% LMPA NaCl	0.00	0.00	0.00	0.00
0.25% LMPA lactic acid & NaCl	0.72	0.39	1.39	1.05
0.50% LMPA lactic acid & NaCl	0.70	0.48	1.58	1.16
1.00% LMPA lactic acid & NaCl	0.00	0.00	0.00	0.00

Table S2: Table of average Smean and Smax values and standard deviations for each condition of mono-culture (see Figure 4). Each value was calculated from a few hundred to a thousand measurements.

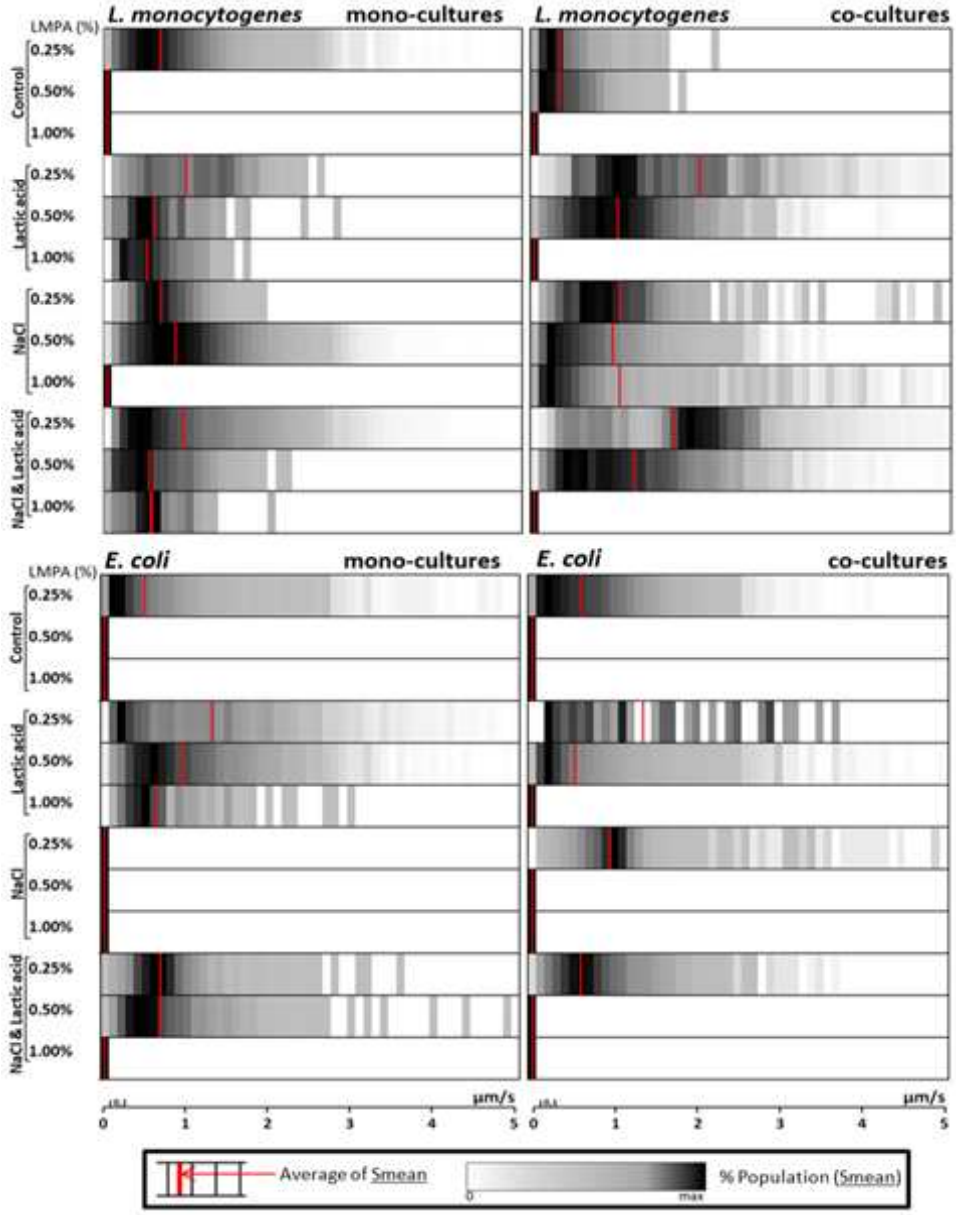
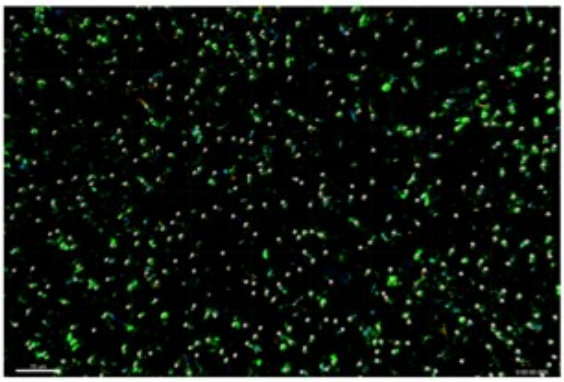


Figure S4: Quantitative analysis of cell motility of *L. monocytogenes* and *E. coli* O157:H7 in different gel matrix conditions for mono- and co-cultures (A). The mean speed (Smean) was calculated from bacterial cell observations in time-lapse microscopy as described in the Materials and Methods section. From there, the percentage of population for the different speed values was represented as a density bar. The averages for Smean were further calculated and represented as bright colour lines (red) to highlight shift in motility of the cell population. Gel matrices were obtained at different LMPA (i.e. 0.25%, 0.50% or 1.00%) concentrations (i) without the addition of lactic acid or NaCl (control condition), (ii) with the addition of lactic acid (pH 5), (iii) with supplementation of NaCl, or (iv) with both lactic acid and NaCl.

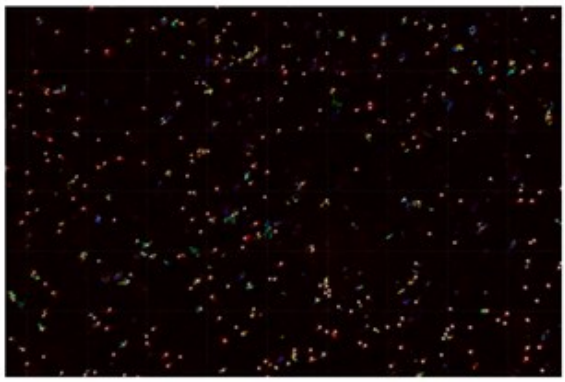
Conditions:	Smean µm/s	Standard deviation Smean
***L. monocytogenes* co-cultures**		
0.25% LMPA control	0.35	0.29
0.50% LMPA control	0.37	0.26
1.00% LMPA control	0.00	0.00
0.25% LMPA lactic acid	2.08	1.33
0.50% LMPA lactic acid	1.07	0.58
1.00% LMPA lactic acid	0.00	0.00
0.25% LMPA NaCl	1.09	1.03
0.50% LMPA NaCl	0.92	0.43
1.00% LMPA NaCl	1.08	1.52
0.25% LMPA lactic acid & NaCl	1.74	0.85
0.50% LMPA lactic acid & NaCl	1.28	0.84
1.00% LMPA lactic acid & NaCl	0.00	0.00
***E. coli* O157:H7 co-cultures**		
0.25% LMPA control	0.66	0.49
0.50% LMPA control	0.00	0.00
1.00% LMPA control	0.00	0.00
0.25% LMPA lactic acid	1.38	1.01
0.50% LMPA lactic acid	0.57	0.54
1.00% LMPA lactic acid	0.00	0.00
0.25% LMPA NaCl	1.00	0.56
0.50% LMPA NaCl	0.00	0.00
1.00% LMPA NaCl	0.00	0.00
0.25% LMPA lactic acid & NaCl	0.79	0.46
0.50% LMPA lactic acid & NaCl	0.00	0.00
1.00% LMPA lactic acid & NaCl	0.00	0.00

Table S3: table recapitulating the average Smean values and standard deviations for each condition of mono- and co-cultures. Each value was calculated from a few hundred to a thousand measurements.

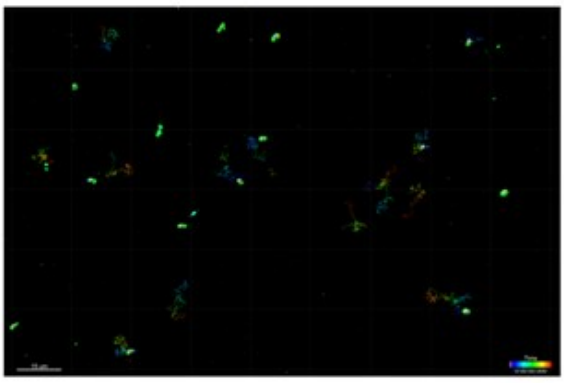
Video5A: *L. monocytogenes* in 0.25% LMPA control



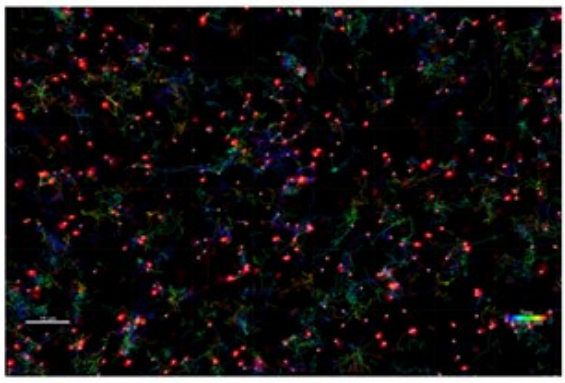
Video5D: *E. coli* O157:H7 in 0.25% LMPA control



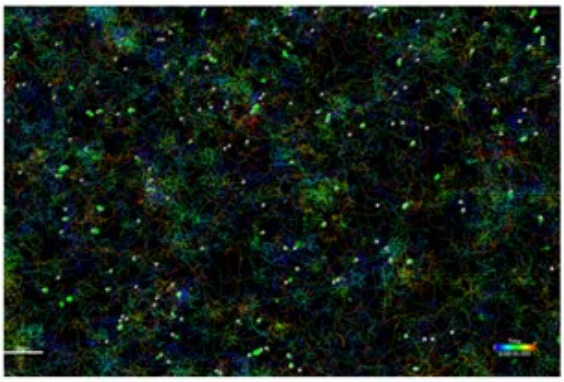
Video5B: *L. monocytogenes* in 0.25% LMPA acid



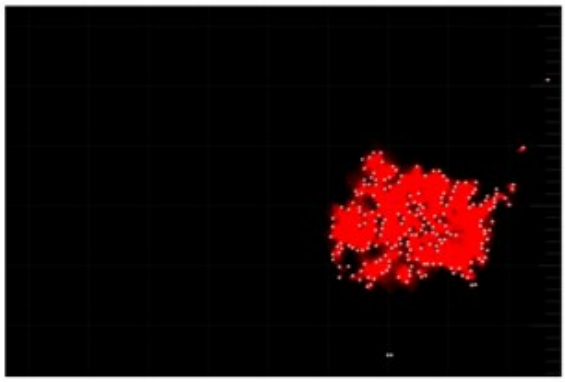
Video5E: *E. coli* O157:H7 in 0.25% LMPA acid



Video5C: *L. monocytogenes* in 0.25% LMPA NaCl



Video5F: *E. coli* O157:H7 in 0.25% LMPA NaCl



Supplementary movies S5: All time lapse sequences were recorded for cultures after 96 h at 20°C. Time lapses have a 2-minute length with a frequency of 0.44s for a total of 275 images. Video were taken with imaris software at 24 frames/s and are 183 µm by 122 µm in size. Dragon-tails show 5 seconds of tracking. An image with the full tracking of the video is presented below. A close-up of the same image is used in figure 5 of the article.

2) Spatialisation de l'expression génique dans les microcolonies quand celles-ci sont dans un hydrogel

La présence de conditions acides favorise l'expression périphérique de *gadB* dans les microcolonies.

Il a été démontré que, au sein de populations bactériennes isogéniques et organisées dans l'espace, une diversité fonctionnelle peut émerger dans la communauté bactérienne, telles que la tolérance au stress. En tirant parti de la microscopie confocale à balayage laser (CLSM) combinée à une fusion transcriptionnelle fluorescente rapportant à l'échelle de la cellule unique l'expression de la glutamate décarboxylase *gadB* dans *E. coli* O157:H7, il a été possible de visualiser pour la première fois les schémas spatiaux de l'expression des gènes bactériens en microcolonies au sein d'une matrice gélifiée. Le gène *gadB* est impliqué dans la tolérance d'*E. coli* aux conditions acides et sa forte surexpression a été observée localement à la périphérie des microcolonies cultivées dans des hydrogels acides. Cette spatialisation de l'expression de *gadB* n'était pas corrélée avec les populations vivantes/mortes qui semblaient distribuées de manière aléatoire dans les microcolonies. Alors que la population planctonique des pathogènes est inactivée par une exposition à HCl pH=2 pendant 4h mimant un stress acide gastrique, les bactéries cultivées dans les hydrogels ont été peu affectées par ce traitement, en particulier dans des conditions où *gadB* était spatialement surexprimé. Les conséquences de ces résultats pour la sécurité alimentaire sont ensuite discutées.

Stratégie de la construction génique employée

Pour suivre l'expression de gènes d'intérêt, plusieurs stratégies génétiques ont été déployées dans les deux modèles bactériens. Les constructions basées sur *L. monocytogenes* sont présentées en annexe, tandis que le matériel et méthode de la stratégie indirecte du système rapporteur fluorescent de *E. coli* utilisée dans cette étude est présentée en détail dans l'article 2.

La stratégie de construction pour *E. coli* O157:H7 prévoyait initialement l'utilisation de deux plasmides, un plasmide exprimant de manière constitutive une RFP (fluorescence rouge) pour marquer les cellules et un autre plasmide avec un système de gène rapporteur basé sur la GFP (fluorescence verte) pour le suivi de l'expression d'un gène. Afin d'obtenir une normalisation optimale du niveau de fluorescence du rapporteur dans chaque cellule, il a été décidé de développer un plasmide unique contenant les deux systèmes de fluorescence.

Article original "Spatially localized expression of *Escherichia coli* O157:H7 glutamate decarboxylase *gadB* in gelled matrix microcolonies" :

(soumission prévue dans "*NPJ Science of Food*")

Spatially localized expression of glutamate decarboxylase *gadB* in *Escherichia coli* O157:H7 microcolonies in hydrogel matrix

Cédric Saint Martin[1,2], Nelly Caccia[2], Maud Darsonval[1], Marina Grégoire[1], Grégory Jubelin[2], Florence Dubois-Brissonnet[1], Sabine Leroy[2], Romain Briandet[1]* & Mickaël Desvaux[2]*

[1] Université Paris-Saclay, INRAE, AgroParisTech, MICALIS Institute, 78350 Jouy-en-Josas France,

[2] Université Clermont Auvergne, INRAE, UMR454 MEDIS, 63000 Clermont-Ferrand, France,

*Corresponding authors: M. Desvaux (mickael.desvaux@inrae.fr) & R. Briandet (romain.briandet@inrae.fr)

Abstract

Functional diversity within isogenic spatially organised bacterial populations has been shown to trigger emergent community properties such as stress tolerance. Taking advantage of Confocal Laser Scanning Microscopy (CLSM) combined with a transcriptional fluorescent fusion reporting at single cell scale the expression of the glutamic acid decarboxylase *gadB* in *E. coli* O157:H7, it was possible to visualise for the first time spatial patterns of bacterial gene expression in microcolonies grown in a gelled matrix. The *gadB* gene is involved in *E. coli* tolerance to acidic conditions and its strong over-expression was observed locally on the periphery of embedded microcolonies grown in acidic hydrogels. This spatialisation of *gadB* expression did not correlate with live/dead populations that appeared randomly distributed in the colonies. While the planktonic population of the pathogens was eradicated by an

exposition to HCl pH=2 for 4h mimicking a stomachal acidic stress, bacteria grown in gel-microcolonies were poorly affected by this treatment, in particular in conditions where *gadB* was spatially overexpressed. The consequences of these results for food safety are further discussed.

Keyword

E. coli O157:H7, food matrix, microcolonies, spatial expression, *gadB*, fluorescent transcriptional fusions for Confocal Laser Scanning Microscopy (CLSM), Food safety

Abbreviations

CLSM : stands for "Confocal Laser Scanning Microscopy"

LMPA : stands for "Low Melting Point Agarose"

1) Introduction

Escherichia coli is a commensal bacteria found in the gut of mammals that plays an integral part in the digestive process. However, some strains of *E. coli* are pathogenic and represent a public health issue when they reach the production lines of food industries. Besides obvious evisceration accidents tainting the meat at the slaughter state, food contamination of animal products (meat and milk products), vegetable or water usually occur through direct or indirect (e.g. transfer) fecal contamination [1–3]. Further handling and storage temperature are then major contributors to their growth and survival in food products [4]. Shigatoxin producing *E. coli* (STEC) are the third most common foodborne zoonosis in Europe [5] and amongst them, the serotype O157:H7 is a commonly identified agent in patients. *E. coli* O157:H7 are enterohaemorragic *E. coli* (EHEC), causing bloody diarrhoea by breaking the intestinal lining. A possible outcome of the shigatoxin (Stx) passing in the bloodstream is damage to the kidneys that can lead to a hemolytic uremic syndrome (HUS), with fatal outcomes in 5% of cases [6]. Children are especially at risk and *E. coli* O157:H7 is still the main cause of pediatric

HUS [7]. While the international regulations usually require the absence of this pathogen in 25g of product (ISO/TS 13136:2012), it is detected above 100 CFU/g in more than 1% of all red meats, the main vector of infection for this pathogen [5,8].

Structured food matrices are heterogeneous assemblages of local microenvironments harbouring multiple micro-gradients that can evolve with time and microbial activity [9]. Bacteria cells thus face different biotopes in which their growth and behaviour can diverge from observations in liquid laboratory media. These environmental conditions can prompt high phenotypic diversity in microbial populations as the cells adapt to local microenvironments [10,11]. In comparison with their planktonic counterparts, phenotypic diversity in structured communities can influence bacterial fitness and behaviour, such as increase expression of virulence genes [12], higher tolerance to antimicrobials and thermal stress [13,14], or better cell motility in the food matrix [15]. While several studies reported emergent properties of bacterial community in food matrices at the population level [13,16–19], no experimental evidence is yet reported on the spatial heterogeneity of gene expression at single cell scale in those biotopes.

Amongst the challenges that food microbiota face, the stomachal phase after food ingestion is credited for the highest population reduction as it involves survival to extreme acidic pH conditions for several hours. High tolerance to acidic conditions is therefore an asset for foodborne pathogens. The Glutamic Acid Decarboxylase (GAD) system is present in many bacteria that can survive these extreme acidic conditions [20–24]. In *E. coli*, the GAD system is composed of three components: two glutamate decarboxylases, *i.e.* GadA and GadB, which remove an H^+ by converting glutamate into γ-aminobutyrate (GABA), and the glutamate/GABA antiporter GadC. Cytoplasmic GadB migrates near the membrane when pH is below 5.6 [25]. Contrary to *gadA*, *gadB* and *gadC* are co-transcribed and the expression of both *gadA* and *gadBC* is transcriptionally regulated by RpoS and two AraC-like regulators, *i.e.* GadX and GadW, as well as effectors with two inhibitors, i.e. the cyclic AMP receptor protein and H-NS. H-NS and

RpoS in particular determine the temporal expression, the former inhibiting *gadB* expression, whereas transcription of *gadB* is promoted by RpoS once the stationary phase is reached [26].

In order to decipher and model fitness and behavior of food-borne pathogens, synthetic microbial ecology approaches were used in structured food matrices where the complexity of the communities and the factors of influence are reduced to their minimum, but increased in their controllability [27]. Such approaches have been used, for example, to describe how matrix parameters affect bacterial growth and morphodynamics of microcolonies [16,28]. In a recent contribution, we have shown that the volume, distribution and sphericity of microcolonies of *E. coli* O157:H7 in hydrogel is dependent of the size of the founder inoculum, but also on the concentration of acids and salts that are frequent in food products [29].

In this study, we took advantage of a hydrogel matrix to visualise the expression of *gadB* in *E. coli* O157:H7 cells in microcolonies using confocal laser scanning microscopy (CLSM). Bacterial strains with a dual fluorescent reporter system were engineered to monitor the spatially patterned expression of *gadB* at the single cell level. The survival of planktonic cells to an extreme acid stress mimicking stomachal passage was further assessed versus cells in microcolonies to relate observed phenotypic heterogeneity in microcolonies with community function.

2) Material and methods

Bacterial strains and culture conditions

From cryotubes stored at -80°C, bacterial strains were plated on Petri dishes with TS-A (Tryptone Soya Agar, Oxoid, USA) and incubated overnight at 37°C. One bacterial colony was picked up and inoculated in TS-B (Tryptone Soya Broth, Oxoid, England) before overnight incubation at 37°C under orbital shaking. When required, growth

media were supplemented with chloramphenicol (Cm 25 µg.mL^{-1}; EUROMEDEX, reference 3886-B, China).

Genetic construction

The *E. coli* O157:H7 CM454 [30,31] served as the wild type strain in this study. We used an T7 polymerase (T7pol) amplification technique inspired from previous reports [32,33], where the cassette *T7pol::Cm^R* is inserted after the genetic sequence of the gene *gadB* using the Datsenko Wanner [34] recombination technique (supplementary material Figure S1). Regions of identity were added at the ends of the cassette by the forward primer (5'CCGAAACTGCAGGGTATTGCCCAACAGAACAGCTTTAAACATACCTGATAACAGGAGG TAAATAATGCACACGATTAACATCGC3') and reverse primer (5'AAATTGTCCCGAAACGGGTTCGTTTCGGACACCGTTACCGTTAAACATGGAGTTCTGAG GTCATTACTG3'). The correct insertion of *T7pol::Cm^R* in the construct was verified by PCR using forward primer (5'CTCCCTGAAATATCTCAGTG3') and reverse primer (5'TTGCTAATGCCAGTGAACAG3'), and then sent for sequencing (Eurofins Genomics). Based on the sequence of the pHL40 plasmid [32], a new plasmid was synthetised (GeneArt, ThermoFisher Scientific) bearing the P$_{T7pol}$*::GFPmut3::*T$_{p7pol}$ as a GFP reporter but modified by insertion of P$_{BBa_J23119}$*::mCherry2::*T$_{BBa_B0062}$ (iGEM parts) for constitutive expression of a red fluorescent protein (RFP). This new plasmid, called pHL60 was transformed into competent *gadB::T7pol::Cm^R* bacterial cells. This system is an indirect reporter of *gadB* transcription as the transcriptional fusion of *gadB::T7 polymerase* allows an amplified production of GFP (GFPmut3) from pHL60 and normalisation of the level of expression respective to the constitutive expression of the RFP (mCherry2) from the same plasmid, to minimise variations of the fluorescence associated with variations in the number of plasmids from one cell to another. To validate the genetic construction, the reporting planktonic expression of *gadB* was tested on six pH values from 4.5 to 7.0 using a microplate reader (Synergy H1, Biotek) (Supplementary material figure S2).

Transparent hydrogel matrices for fluorescent imaging

As previously described [29], the hydrogel matrices were obtained by mixing TS-B with 0.50 % low melting point agarose (LMPA; UltraPure Agarose, Invitrogen, reference 16500-500, USA). Overnight pre-cultures were adjusted to 10^5 CFU/ml. After boiling to ensure complete melting, the TS-LMPA pH=7 was cooled down at 40°C to prevent thermal stress before adding the bacterial inoculum to obtain 10^4 CFU/ml final. When necessary, the medium was adjusted to pH=5 with HCl (10^{-5} M; VWR reference 30018.298). After homogenizing and gentle stirring to avoid bubble formation, the inoculated gel matrix was immediately distributed in each well of a 96-well microtiter plate of microscopic grade (Microclear, Greiner Bio-One, France). The microtiter plates were then incubated at 20°C and observed under CLSM after 96 hours of incubation.

All microscopic observations were performed with a Leica HCS-SP8 confocal laser scanning microscope (CLSM) at the INRAE MIMA2 imaging platform (www6.jouy.inrae.fr/mima2). The GFP (GFPmut3; $\lambda_{ex}500$; $\lambda_{em}513$) and RFP (mCherry2; $\lambda_{ex}589$; $\lambda_{em}610$) were excited respectively with laser bands 488 nm and 561 nm. For Live/dead exploration, SYTO9 ($\lambda_{ex}485$; $\lambda_{em}501$) and IP ($\lambda_{ex}535$; $\lambda_{em}617$) were excited respectively with laser bands 488 nm and 561 nm. Observations were carried out with a 63x objective lens (numerical aperture = 1.20) for 184µm x 184µm fields. Bidirectional acquisition speed of 600 Hz allows a frame rate of 2.3 images per second in both cases. For 3D stack analysis, a 1 µm step between z levels was used. For each condition, a minimum of 60 stacks were acquired in over a dozen independent wells. Microscopic pictures were treated on IMARIS v9.64 (Bitplane, Switzerland) to generate sections and projections. Kymograms reporting the spatial analysis of *gadB* expression in microcolonies were performed using BiofilmQ v0.2.2 [35]. BiofilmQ image segmentation was performed with a threshold value set at 0.1 with cubes of 1.8 µm (vox=10). The absence of fluorescence gradients in *E. coli* O157:H7 microcolonies was ascertained with a strain constitutively expressing GFP (supplementary material figure S3).

Acidic digestion challenge

To test the ability of *E. coli* O157:H7 population to survive the strong acidic stress during the stomachal passage, 3 ml of planktonic or gel-colonies cultures (72h, 20°C, pH=7 or pH=5) were transferred in 27 ml of NaCl 9g/L saline solution (control groups) or 27 ml of a saline solution adjusted with HCl (5 M) to pH=2. The cups were then incubated at 37°C for 4 hours under a 90 rpm shaking to simulate matrix digestion. All media were then homogenised to disperse bacteria (IKA Ultra-Turrax T25; Janke Kunkel) and the resulting suspensions were immediately plated on agar for enumeration and determination of the log reduction in CFU/ml before and after acidic treatment.

Statistics

Graphics and ANOVA variance analysis were performed with Prism 9 (Graphpad; CA, USA). Differences were considered significant when $P<0.05$ with P being the critical probability associated with the Fisher test.

3) Results

A) Spatial patterns of *gadB* expression in gel-microcolonies

The morphodynamic of microcolonies formed in acidic hydrogel differs from those obtained in neutral hydrogels: they appear more spherical than the ovoid found in the control, and where microcolonies at neutral pH have a clearly defined boundary, those in acidic hydrogels possess edge structures that resemble streamer populations from the core colony and form a crown around it (Figure 1). Despite differences in spatial arrangements, no significant differences in mean radius were observed between neutral (28 µm) or acidic conditions (27 µm) (Figure 1, obtained from measurement on around 40 colonies, $P>0.05$).

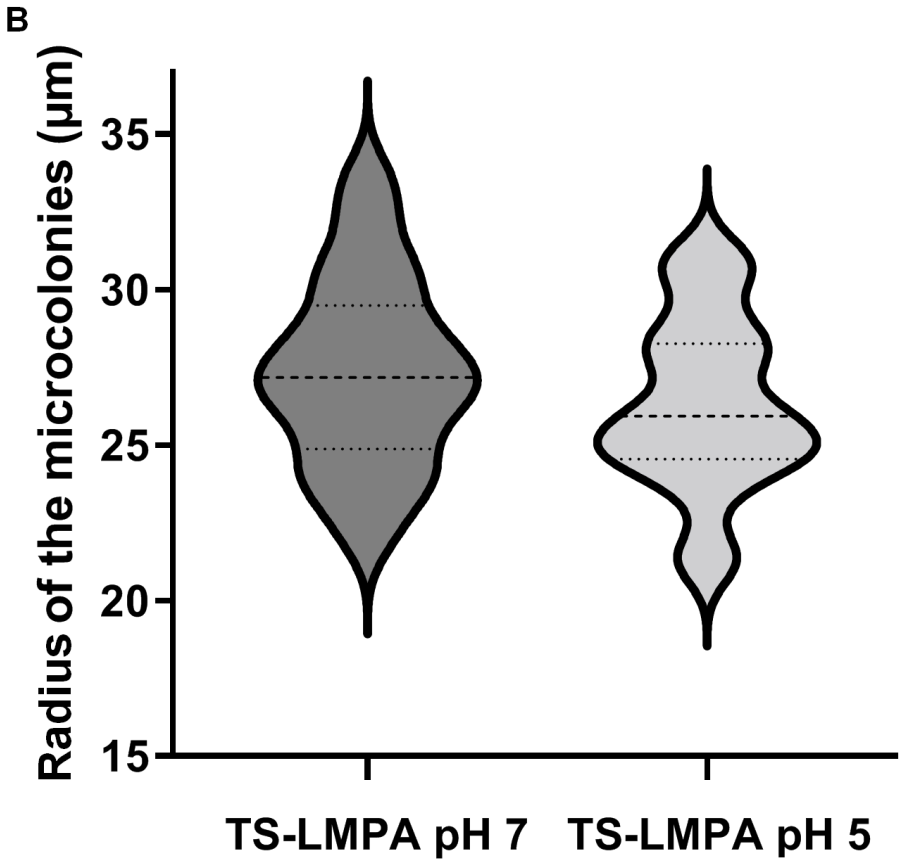


Figure 1 : Radius of the microcolonies. Representation of the radius of the microcolonies in µm with a larger width indicating a concentration of the number of values. For each case, the slashed line is the mean value of radius, and dotted lines define the 75 % probability interval. Calculated from 40 independent microcolonies.

Microscopic observations of the fluorescence reporting the expression of *gadB* in cells inside microcolonies (Figure 2) shows radically different situations between the two hydrogels. In neutral conditions, this gene is expressed at a low level throughout the whole microcolony with no specific spatial arrangement (Figure 2A, supplementary material Figure S4). By contrast, the expression of *gadB* is strongly overexpressed in the periphery of the colonies formed in acidic hydrogel (Figure 2B, supplementary material Figure S4). Those qualitative observations were reinforced by a quantitative exploration of the axial distribution of *gadB* expression (Figure 3). For both hydrogels, a kymograph integrating 40 independent microcolonies represents *gadB* transcription from the center of the microcolony (d_{CM}=0 µm) to the edge of the colonies (d_{CM} = 20-35 µm) and beyond, where the gene expression is monitored by the green fluorescent intensity normalised with red fluorescent intensity. In neutral hydrogel, *gadB* expression is low and almost constant over the radius of the microcolonies (Figure 3A). For acidic hydrogels a sharp band of *gadB* over-expression is observed at a distance between 25 and 30µm from the center of the microcolonies (Figure 3B). Since the radius of the microcolonies is 27-28 µm (±5 µm) in those experimental conditions, it concords with the observed spatial expression at the edge of colonies. Interestingly, when microcolonies are in near contact, the two sides facing each other do not present an over-expression of *gadB* or the shedding of single cells visible in other areas of the periphery (supplementary material Figure S5). Similarly, if two microcolonies merge at contact as they grow, they will behave like a single colony in regard to *gadB* spatial expression.

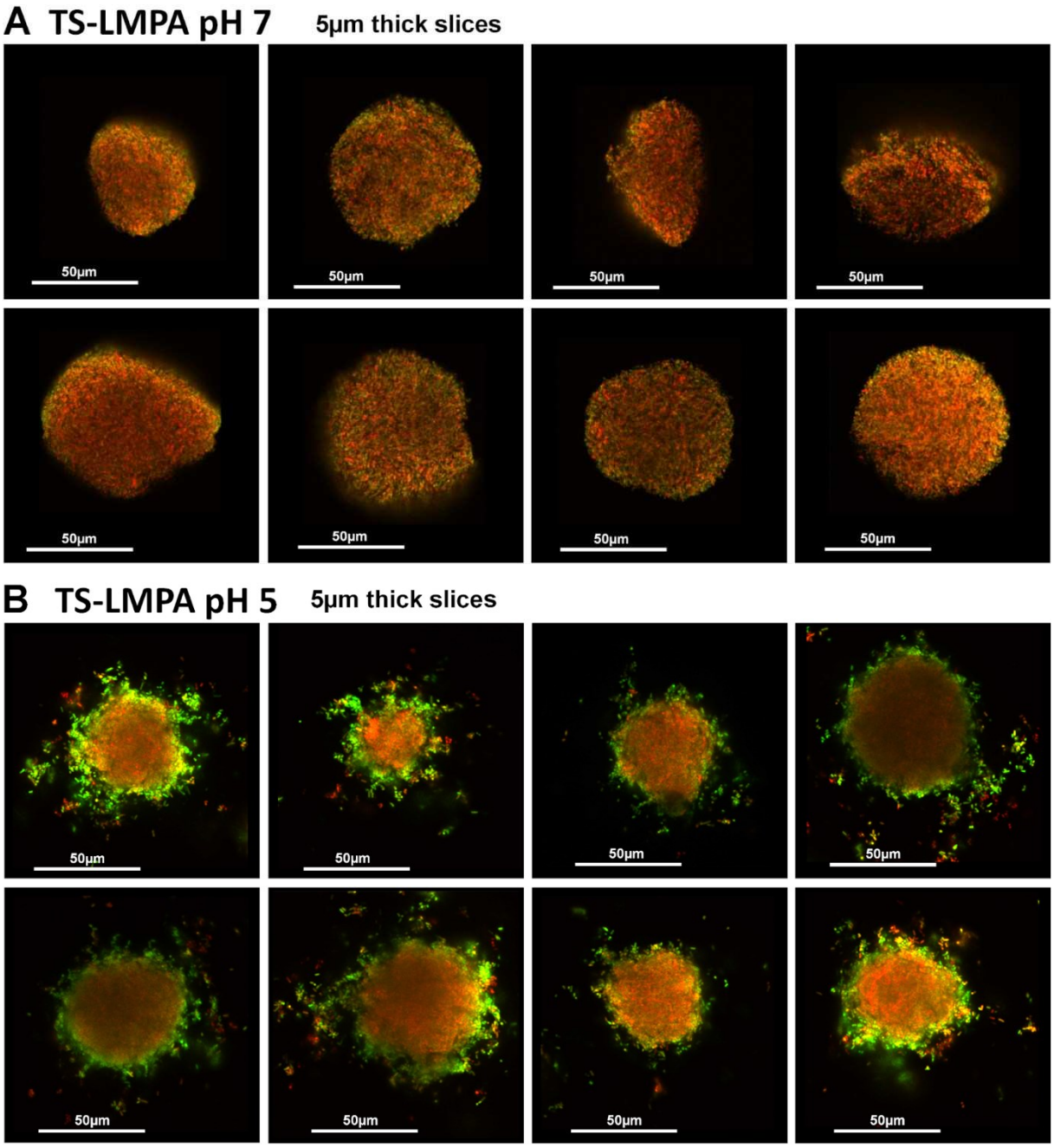


Figure 2: Representative images of *gadB* spatial patterns of expression for *E. coli* O157:H7 cultivated in neutral (pH=7) or acidic (pH=5) hydrogels. A series of 5µm slices of microcolonies is presented in control TS-LMPA pH=7 (A) and the acid matrix TS-LMPA pH=5 (B). The red fluorescence is constitutive and the green fluorescence is expressed as a function of *gadB* transcription. (The supplementary material figure S3 presents the same representation for the constitutive GFP in both conditions).

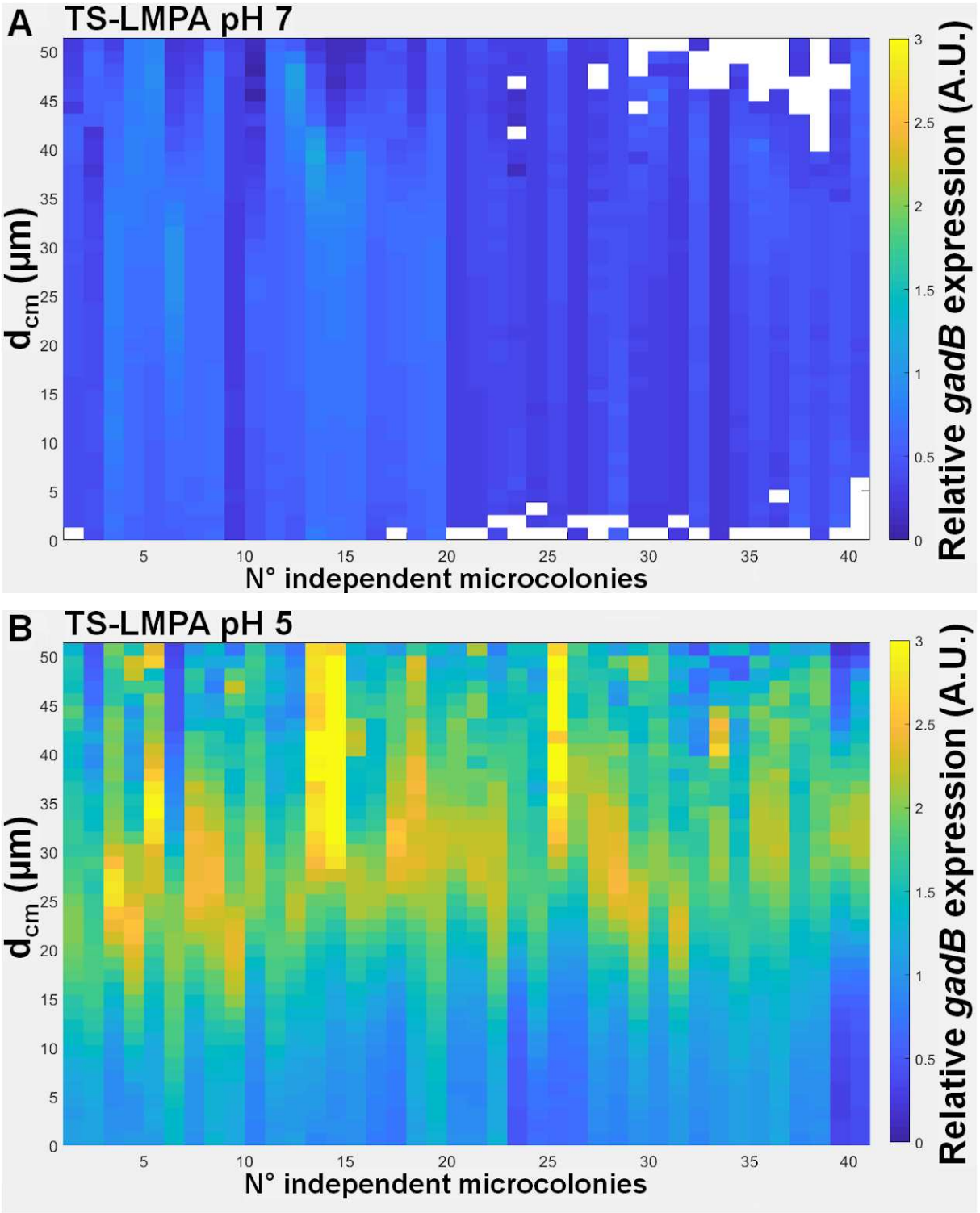


Figure 3 : Relative expression of *gadB* in function of the distance from the center of microcolony. (A) In this representation, the relative *gadB* expression is expressed as the ratio of green fluorescence intensity over red fluorescent intensity (GFP reporter/RFP constitutive) in function of the distance from the center of the microcolony (d_{CM}) for forty microcolonies grown in neutral (A) or acidic hydrogels (B).

Experiments performed with a constitutive expression of the green fluorescence protein did not show the spatialisation of the expression as presented above (supplementary material S3).

A time course microscopic analysis allows the observation of *gadB* expression spatialisation in acidic hydrogels as early as microcolonies become visible under the microscope, ~48h after inoculation (data not shown).

Finally, the spatial repartition of dead cells in gel-microcolonies as shown by live/dead fluorescent staining indicated a random distribution of the red dead cells, with no preferential localisation in the microcolonies associated with *gadB* expression (supplementary material Figure S6).

B) Increased survival to acidic stomachal stress of *E. coli* grown in gel-microcolonies

Considering that *gadB* is linked to acid tolerance, survival to stomachal acidic stress of *E. coli* O157:H7 populations grown planktonically or in hydrogel matrices was evaluated by enumeration on agar. Cultures of *E. coli* O157:H7 adjusted to 4 logCFU/ml were incubated at pH=7 or pH=5 in either TS-B or TS-LMPA. In TS-LMPA after 96h of incubation at 20°C, the populations reached 8.6 logCFU/ml at pH=5 and 9.5 logCFU/ml at pH=7; in TS-B, it reached 9.4 logCFU/ml at pH=7 and 9.6 logCFU/ml at pH=5. All populations were significatively affected by the exposure to the stomachal acid stress (4 hours at pH=2). Bacteria grown in planktonic conditions were highly sensitive to this treatment with a total loss of the culturable population in every replicate of the manipulation (Figure 4A and 4B). On the contrary, cells in gel-microcolonies presented a high tolerance to this extreme acidic stress. The best tolerance was observed for the microcolonies grown in acidic hydrogel with a log reduction as low as 0.29 log CFU/ml, significantly lower than the reduction observed for microcolonies grown in neutral hydrogel with a log reduction of 0.78 CFU/ml, ($P<0.05$).

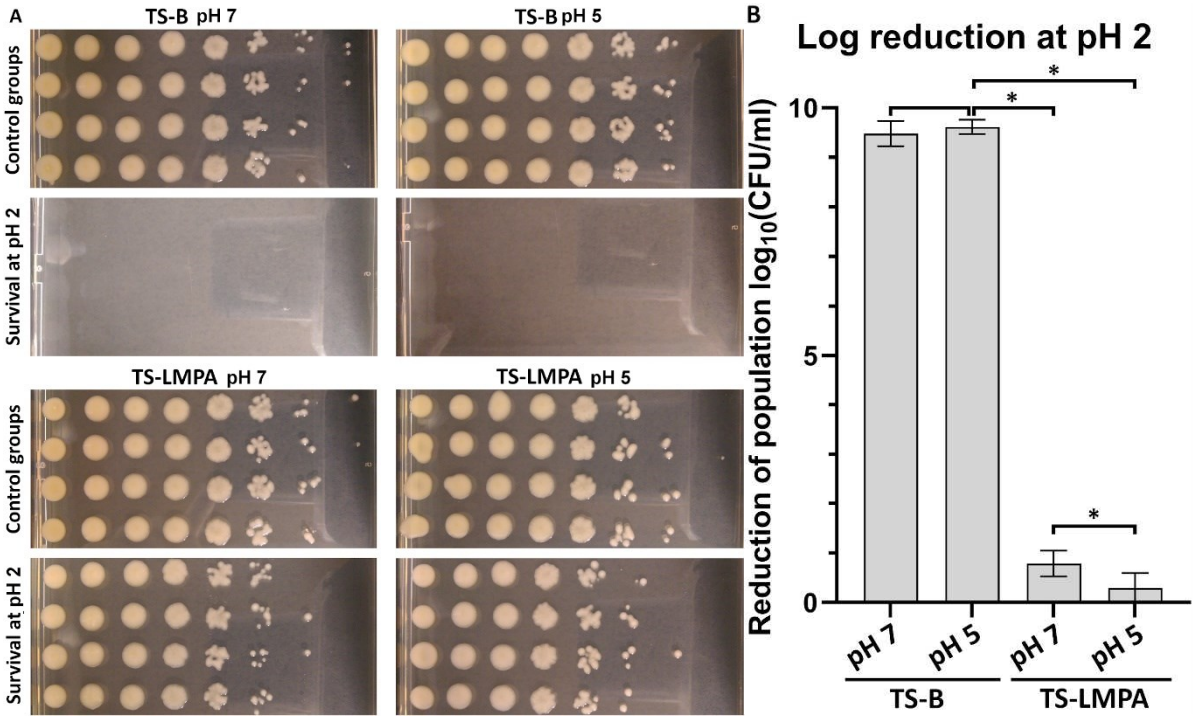


Figure 4 : Survival of planktonic cultures and hydrogel microcolonies of *E. coli* O157:H7 upon acidic exposure. Bacterial cells were exposed for 4 hours at HCl pH=2. (A) Images represent serial dilution plates that compare the bacterial survival before and after the acidic stress exposition. The results of planktonic cells are present at the top and those of microcolonies in hydrogels at the bottom. The left column shows bacteria grown at pH=7 before the survival test and on the right column those that were grown at pH=5. The control groups were exposed to a saline solution at pH=7, and for survival at pH=2, cells were exposed to the saline solution at pH=2. Each plate has eight lines and eight columns, columns from left to right are successive dilutions (dilution 10 leftmost; dilution 108 rightmost) and the four lines are repetitions of one biological replicate. (B) Representation of the mean reduction of population log between the control group and the one exposed to the saline solution pH=2. Liquid media are on the left, hydrogels on the right and a star indicate significant difference between values ($P<0.05$). Data resulted from at least six biological replicates**.**

4) Discussion

Microbial behaviour in laboratory liquid growth media can strongly deviate from what is observed in solid food matrices [18,36–40]. Environmental heterogeneity of structured media is one of the four main causes of cellular variation, alongside genetic variation, aging and stochasticity of gene expression [41]. Environmental heterogeneity of structured media can trigger a large diversity of phenotypic cell expression in the same biotope, promoting the cohabitation of cells with different spectra of behaviours, such as stress response or virulence [42,43]. However, the opacity of numerous food matrices prevent the use of most live imaging and miscroscopic approaches. To overcome these limitations, several studies took advantage of synthetic gelled systems to simplify and control the parameters of growth of embedded bacteria. Here, low melting point agarose was used as a gelling agent, as it forms hydrogels in which cells can be dispersed without thermal stress and with tunable textures and compositions allowing to mimic various food environments such as grounded meat or cheese [29]. The experiments presented in this work show that the morphology of microcolonies in the media complemented with HCl are different from the control with in particular bacteria shedding from the periphery of the microcolonies. This effect can be explained by a combined action of relaxed gel structures due to low pH and the higher motility of *E. coli* O157:H7 when acidic conditions are encountered [29,44].

Over the course of this study, we observed a clear overexpression of the gene *gadB* for a subpopulation of cells localised on the periphery of microcolonies formed in acidic hydrogels. When *gadB* is overexpressed it is at levels 2-3 times higher than in neutral conditions, which is in accordance with the ratio that was obtained in planktonic cultures (Supplementary material Figure S2). We confirmed that this spatialisation was neither associated with dead cells (supplementary material Figure S6), nor a limitation of oxygen for GFP maturation in the center of the microcolony (supplementary material Figure S3). The use of a single plasmid bearing both the genes for the constitutive and induced fluorescence means that, at the image analysis step, we prevented bias due to

different plasmid copy numbers or difference in coloration from a mix of dye/genetic reporters [45]. The use of two lasers with different properties of matrix penetration can lead to a bias in the Z axis with a fluorescence ratio dependent on the height, but 3D analysis of all the cells in a microcolony reduces the bias as any offset at the bottom of the agglomerate is compensated by an opposite offset at the top.

Spatial patterns of genetic expression were previously reported for other genes in other bacterial species using different structured biosystems, such as localised expression of *E. coli* sigma factors and type 1 pili, as well as *Pseudomonas aeruginosa* β-lactamase in biofilms [46–48]. To our knowledge, such patterns of gene expression were never reported in food or hydrogel matrices. In further studies, the use of an acid naturally found in meat or cheese such as lactic acid would bring us closer to conditions of a real-life food matrix.

There seems to be a correlation between a subpopulation overexpressing *gadB* and a population significantly more tolerant to an exposition of 4h at pH=2. Furthermore, regardless of the initial pH, populations incubated in a semi-solid media have a better tolerance to acid stress than those grown in liquid broths, where no surviving cells were detected. This underlines possible bias in building predictive models for food matrices from data obtained in liquid conditions, as previously shown in other studies[18,36,37,49]. It has been suggested by studies that the components of the matrix could have a buffer effect that protects embedded cells by preventing the drop in pH. Another study in a gelified dairy matrix, where bacteria were not allowed growing in the gel before the acidic stress, reported no protective effect compared to liquid media [50]. Here, in order to limit a buffer effect, the volume of saline solution at pH=2 was higher than in the hydrogel; pH in hydrogels at the end of the experiments was tested and appeared to be at 2. Another explanation for the heightened survival in gels would be the spatial organisation of the population. The microcolonies were not dispersed as the hydrogels survived the 4 hours of incubation at pH=2, albeit with evidence of deliquescence such

as unraveling of filaments and loss of stiffness. The ability of spatially organized populations of bacteria to better survive acid stress has been already described for pathogenic bacteria but also for auxiliary microbiota and probiotics, such as *Lactobacillus* strains [51,52]. The bacteria may also secrete extracellular polymeric substances (EPS) when grown in communities as described in biofilms [53,54]. For *E. coli* O157:H7, tolerance to low pH of bacteria could also involve the DNA binding Protein Dps which is known to enhance survivability when local nutrients are exhausted [55,56].

From this study in a hydrogel matrix, *gadB* appeared to be more expressed at the periphery of the *E. coli* O157:H7 microcolonies in acidic conditions. This correlated with an increased tolerance to the type of acid stress that can be encountered by bacterial cells after ingestion of food. This finding could have consequences on the infectious dose and bacterial virulence, as well as risk assessment based on bacterial behaviour in planktonic conditions, and at population level without considering the heterogeneity of cells in microcolonies. Modelisation of pathogens growth and survival should take in account the gelled environnements where the spatialisation of genetic expression and its resulting populational effects could deeply affect pathogens behaviour and virulence after ingestion.

Declaration of competitive interest

None

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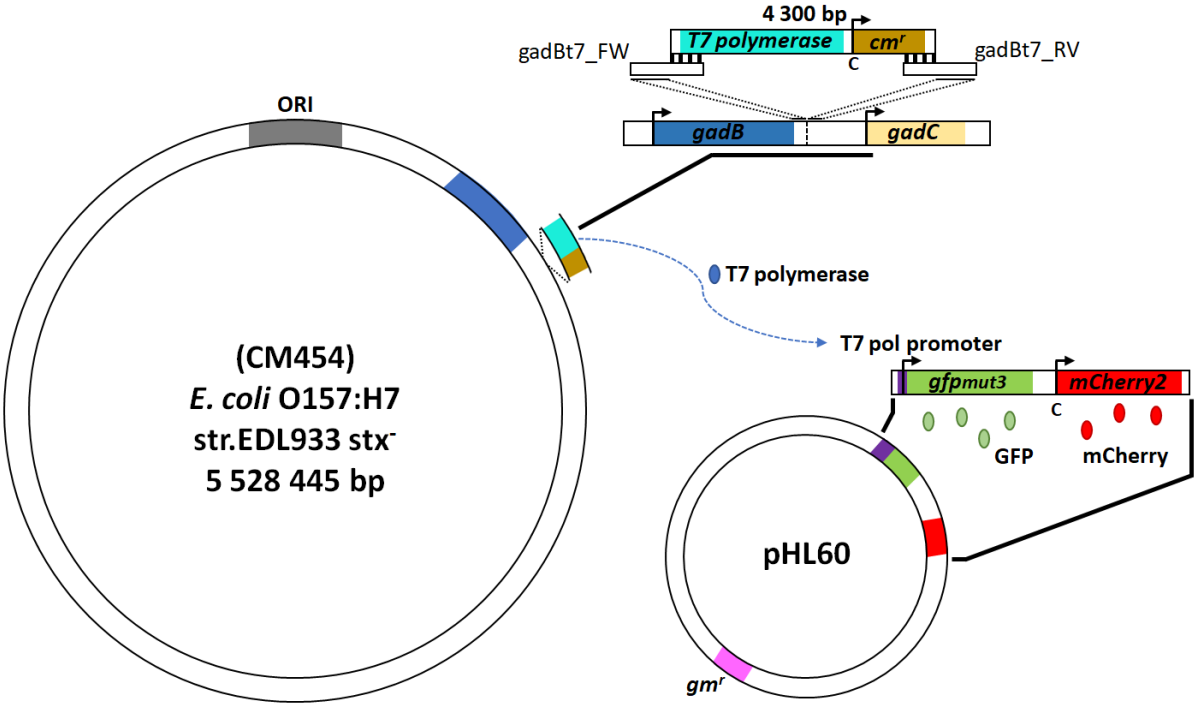
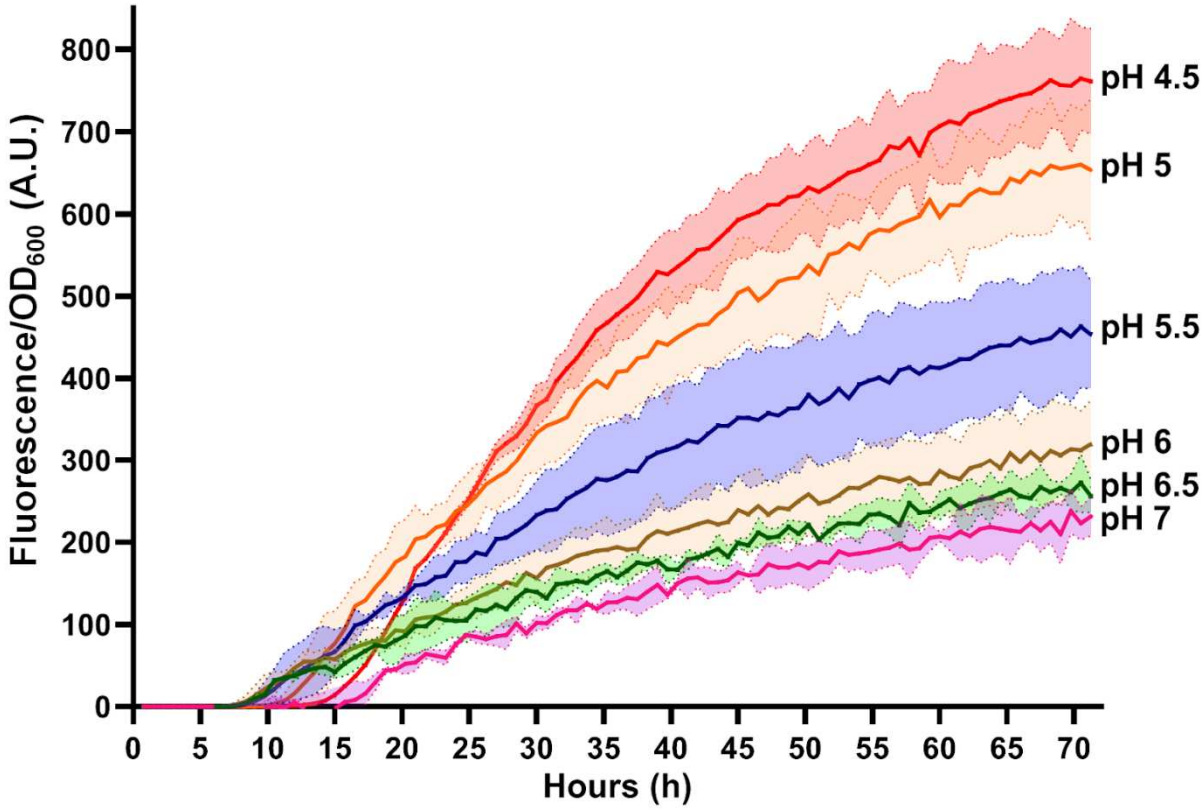
Supplementary material

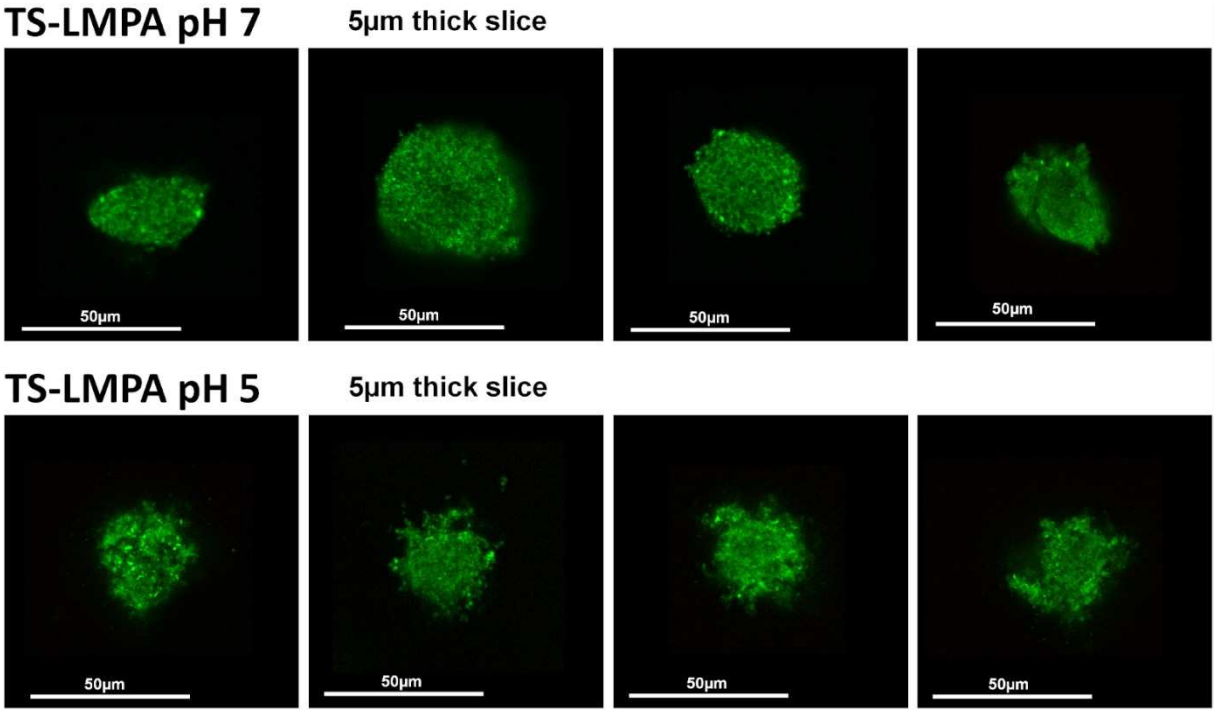


Supplementary material figure S1 : Strategy of the genetic construction. (A) chromosome of *E. coli* O157:H7 with a zoom on the region of the insert and how the different CDS are present in this situation. The cassette bearing the *T7 polymerase* (turquoise) and resistance gene *cm*^{r} (in brown) is elongated by PCR with two primers (gadBt7_FW and gadBt7_RV) so that an homology sequence exists with insertion site. This site is located between the gene interest *gadB* (in blue) and *gadC* (in yellow). On the right the low copy plasmid pHL60 bearing the fluorophore is represented. It contains the constitutively expressed *mCherry2* (in red) sequence and *gfpmut3* (in green) under the control of the *T7 pol promoter* (in purple), as well as a gentamicin resistance gene *gm*^{r} (in pink).

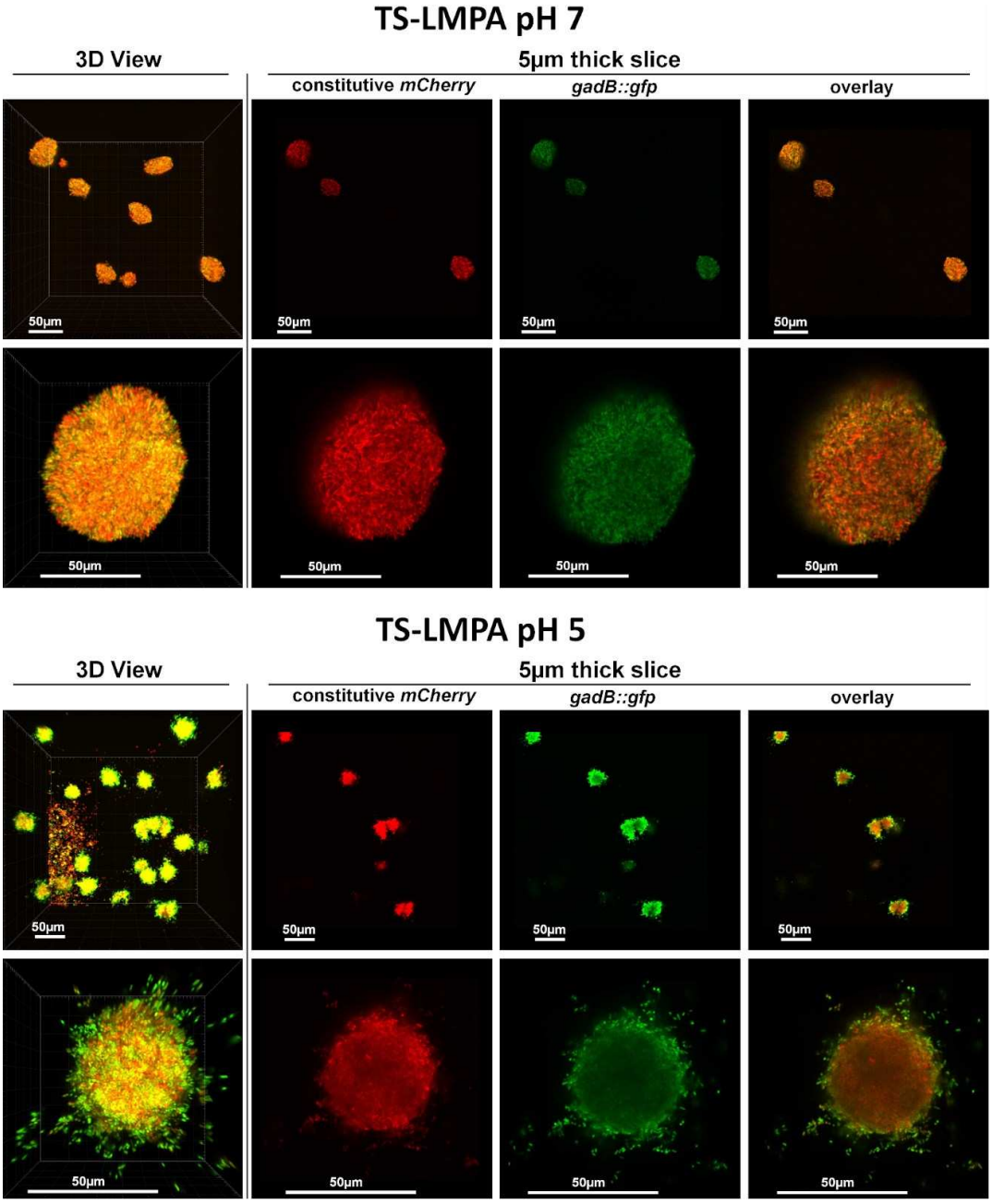


Supplementary material figure S2 : Relative expression of *gadB* in a population in function of time and under different acidity levels. The fluorescence of populations of *E. coli* O157:H7 *gadB::GFP* are represented in function of the OD_{600}. For each curve representing the mean signal over time, the standard deviation of values is shown as an area of lighter color.

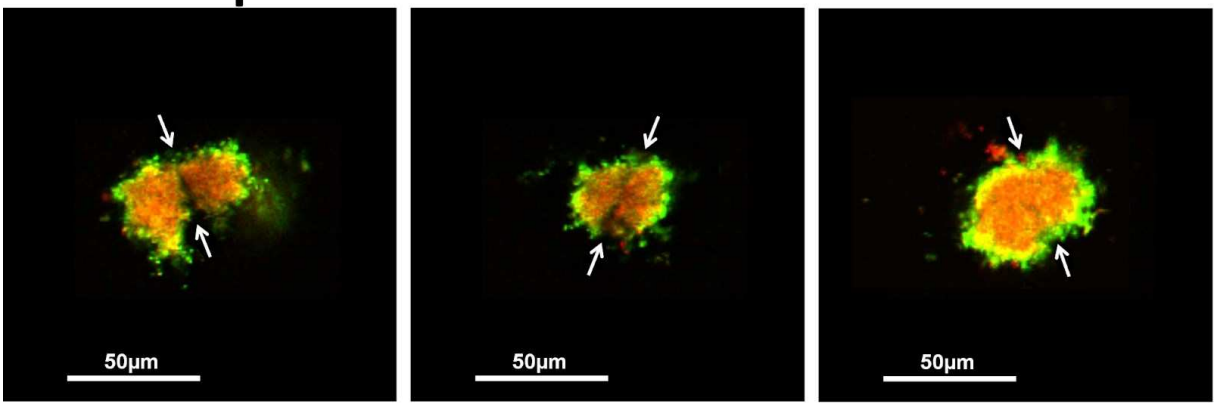
In accordance with tests in acidic gels, it shows that detected fluorescence becomes incrementally stronger each time the pH is lowered . This rise in expressed fluorescence is not linear with greater leaps of intensity once the condition is below pH=6.0. Points over time show that fluorescence intensity at pH 4.5 can be 3-4 times higher than reported values for the pH=7.0 control. The lag time before the green fluorescence is detected is high at pH 7.0 (15 hours) compared to pH= 5.5 where it starts after eight hours. In media pH=5.0 and pH=4.5, the lag time becomes progressively longer (12 then 14 hours).



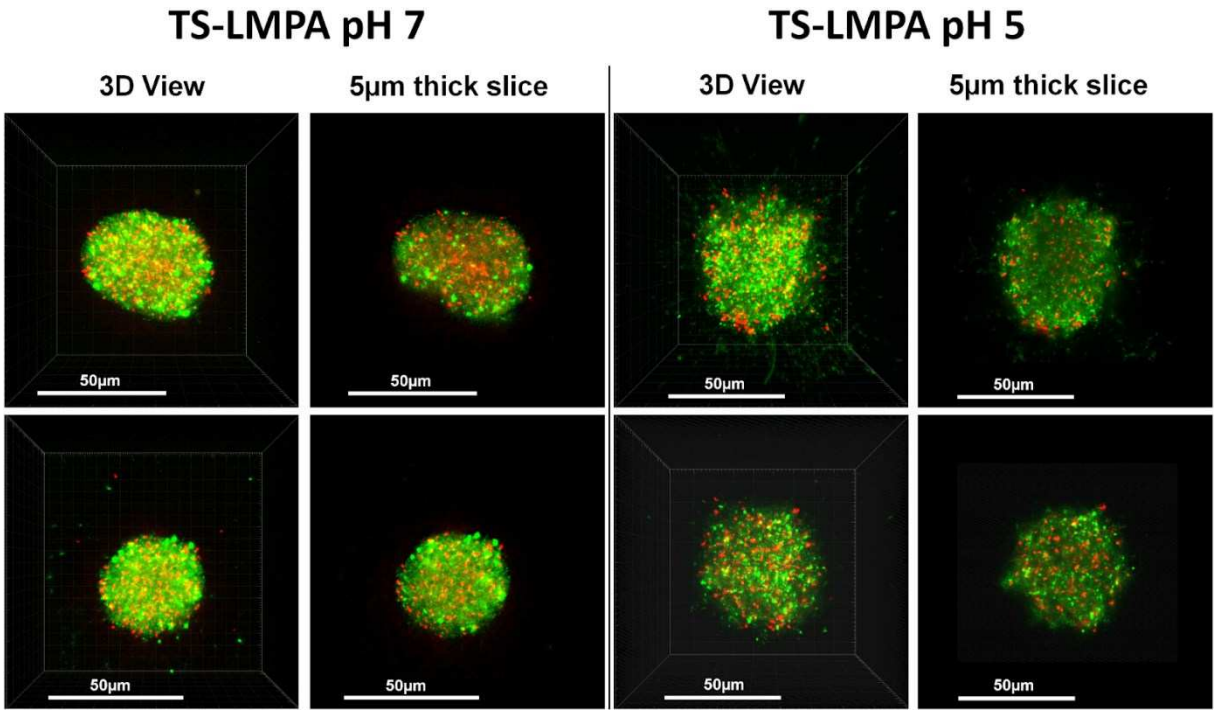
Supplementary material Figure S3 : Qualitative presentation of the constitutive GFP spatial expression in microcolonies at pH=7 or pH=5 A control group with a constitutive GFP to verify that at pH=7 (up) and/or pH=5 (down) the green fluorescence is not already spatialised. Each image is a 5µm slice of a stack.



Supplementary material Figure S4 : Qualitative presentation of *gadB* spatial expression in microcolonies at pH=7 or pH=5 Microscopic observations of the expression of *gadB* in the control TS-LMPA pH=7 (top) and in the acid matrix TS-LMPA pH=5 (bottom). For both groups, the 2 successive rows use a 40X then a 63X objective to present a collection of microcolonies and a close observation of a single example. Each image is shown as a 3-dimensional representation (leftmost column) and in each case a series of 5µm slices show both fluorescent channels (middles columns) and the overlay (Rightmost column). The first row of each condition was taken with a 40x air objective (numerical aperture = 0.85) to explore a 290 µm x 290 µm fields.



Supplementary material Figure S5 : The case of joint microcolonies. Examples where two microcolonies of *E. coli* O157:H7 are touching or merging in TS-LMPA pH=5. White arrows indicate the separation between each microcolonies.



Supplementary material Figure S6: Live/Dead staining in the gel microcolonies. Bacterial cells in microcolonies grown in neutral (left side) or acidic (right side) were labeled with the cell impermeant propidium iodine (red, dead cells) and the cell permeant SYTO 9 (Green, all cells).

Partie III : Discussion générale et perspectives

Il existe quatre causes majeures de la diversité phénotypique de populations bactériennes (Bury-Moné and Sclavi, 2017), mais si la variation génétique, l'expression stochastique et la composante temporelle ont été largement décrites, l'influence de l'hétérogénéité de l'environnement est moins caractérisée dans le contexte de la sécurité microbiologique des aliments. Les matrices alimentaires sont constituées de multiples micro-environnements physico-chimiques et nutritionnels. Ces conditions favorisent une stratégie de "*bet-hedging*" avec l'apparition de sous-populations qui sont responsables de l'apparition de propriétés émergentes, en particulier dans des conditions hostiles à la croissance bactérienne.

Au cours de ce travail de thèse, je me suis particulièrement intéressé à l'observation du comportement de deux pathogènes alimentaires *E. coli* O157:H7 et *L. monocytogenes* dans des matrices structurées. Pour ce faire, un milieu synthétique modèle simplifié a d'abord été mis au point et la croissance des cellules a été caractérisée en termes de distribution spatiale, de motilité et de morphologie des microcolonies selon les conditions du milieu. L'expression du gène *gadB* permettant de réguler le pH intracellulaire a été suivi à l'aide d'une fusion fluorescente transcriptionnelle, et les images ont été analysées par des segmentations sémantiques et d'instances pour quantifier l'expression au niveau de la cellule unique dans les microcolonies.

Croissance de micro-organismes en milieux structurés

L'une des premières étapes de la thèse a consisté à utiliser des hydrogels comme modèles synthétiques simplifiés de matrices alimentaires. Ceux-ci ont permis de caractériser l'impact de la structuration du média sur les paramètres de croissance et la morphodynamique des microcolonies. En particulier, ils ont permis de montrer l'effet de l'inoculum initial sur le volume et la sphéricité des microcolonies et le changement de répartition de la population dans l'espace. Nous avons également observé une forte stimulation de la motilité des cellules par ajout de suppléments acides ou NaCl dans

l'hydrogel. Ces milieux gélifiés sont éloignés des conditions réelles auxquelles ils ne sauraient se substituer. Il s'agit d'un système simple et contrôlé posant les fondations pour une complexification par palier du milieu synthétique. En particulier, pour avoir un modèle équilibré entre représentation du milieu réel et limites techniques, il faudrait améliorer quatre paramètres. 1) La composition du milieu devrait être plus diverse, 2) l'insertion des bactéries dans le média gagnerait à être mieux contrôlée spatialement, 3) des macrostructures pourraient être reproduites par évolution des techniques de formation du gel, et 4) finalement il reste l'ajout de communautés bactériennes.

L'impression 3D permettrait d'aborder les trois premiers points. Celle-ci permet l'instauration de macrostructures simples par addition de couches successives de milieu qui impactera d'autres paramètres comme la vitesse de séchage ou l'exposition à l'environnement extérieur. Les machines sont également capables d'alterner entre plusieurs sources de pâte pour la création de l'objet, modulant ainsi la composition locale. Enfin, l'instauration de motifs dans la contamination du milieu est également rendue possible par l'utilisation de buses supplémentaires qui dispensent des microgouttelettes de cultures sur les filaments lors de leur dépôt. L'utilisation de l'impression 3D constituerait alors une (r)évolution des techniques de conception pour l'étude en matrices structurées (Kyle, 2018). Le dernier point, portant sur l'ajout de communautés bactériennes, est plus difficile à gérer car il fait appel à un enchevêtrement d'interactions inter-espèces impactant la croissance et la survie par des phénomènes de compétition, inhibition et symbiose. Dans ces conditions, la distribution des inocula de différentes espèces peut en elle-même faire la différence entre l'élimination d'une souche et sa survie (Krishna Kumar et al., 2021). Des tests pourraient être réalisés sur des flores sauvages ou des flores technologiques contenant des bioconservateurs comme *Lactobacillus plantarum* et *lactobacillus reuteri*, ainsi que des espèces endogènes de la viande et des fromages comme *Carnobacterium divergens*, *Leuconostoc carnosum* et *Lactococcus lactis*, *Streptococcus thermophilus*.

Hétérogénéité d'expression génique dans les microcolonies

La possibilité que l'hétérogénéité d'expression soit spatialement organisée dans les microcolonies était notre hypothèse de départ en raison de la proximité de cette organisation spatiale avec celle des biofilms ou ces phénomènes sont largement décrits. Il a été ici démontré que le gène de tolérance à l'acide *gadB* est surexprimé en périphérie des microcolonies en milieu acide. Ce phénomène se corrèle également avec l'apparition d'une plus grande tolérance au stress acide digestif au niveau populationnel. Ce résultat est prometteur pour l'étude de l'hétérogénéité d'expression d'autre gènes impliqués dans la tolérance aux conditions de stress comme par exemple *oxyR* (senseur du peroxyde d'hydrogène), *fnr* (senseur de l'hypoxie), ou plus généralement *rpoS* et σ^B (tous les deux facteurs de transcription alternatif pour la réponse au stress); ainsi que ceux associés à des fonctions de colonisation e.g. *cah* (auto-agrégation), *secA2* (sécrétion hors de la membrane), *degU* (facteur de transcription orphelin, accrochage aux parois), ou encore de la maintenance de la cellule e.g. *dam* (méthylation de l'ADN), *recA* (réparation des coupures de l'ADN). La mise au point des constructions permettant le suivi n'est cependant pas aisée, à la fois du côté de la biologie moléculaire, mais surtout en raison de l'impact de la construction sur l'étape de l'analyse d'image. Avec notre système, pourtant conçu pour amplifier l'expression génétique, l'hétérogénéité d'expression ne devient visible et quantifiable que par rapport à un marqueur cellulaire constitutivement exprimé. Un double marquage qui peut mettre en exergue les modifications du taux de transcription de la fusion transcriptionnelle est le système le plus efficace que nous ayons trouvé pour suivre cette hétérogénéité d'expression cellulaire. L'utilisation de marqueurs chimiques aurait engendré des questions sur l'homogénéité de la coloration, et en particulier des tests au SYTO 9 et SYTO 61 dans les hydrogels ont montré des variations fortes d'intensité de marquage entre cellules d'une même microcolonie.

L'étude de l'hétérogénéité pourrait aussi s'étendre à des techniques suivant directement la transcription et l'analyse du protéome. L'exploration des cinétiques de transcription au niveau de la cellule unique est possible grâce aux progrès matériels

actuels permettant d'atteindre la détection robuste de modification de la transcription entre différents génomes à ce niveau (Sandberg, 2014). La FISH en particulier a été adaptée pour une étude en matrice et permet le suivi de dizaines de milliers de cellules (Aldridge and Teichmann, 2020), ce qui la rend très avantageuse par rapport aux autres méthodes qui requièrent la destruction de l'organisation spatiale de la communauté. Des problèmes en termes de quantification de l'ADN par cellule sont cependant attendus (Stegle et al., 2015). Du côté de la protéomique, 90% du protéome de *E. coli* est modifié de manière post-traductionnelle (Soufi et al., 2015) e.g. phosphorylation, glycosylation, oxydo-réduction, hydroxylation (Fortuin et al., 2015; Spät et al., 2015). Ces modifications sont facilement réversibles et participent à la régulation du phénotype (Grangeasse et al., 2015), comme par exemple dans la virulence des pathogènes (Michard and Doublet, 2015). Des techniques basées sur l'interaction protéine/enzyme (FRET, splitFAST) ou la conformation de la protéine permettraient de suivi en hydrogel cette activation de phénotypes qui ne n'est pas liée à l'expression génétique.

Microbiologie prévisionnelle et applications concrètes

Quand un modèle prédictif de la croissance est considéré, il faut prendre en compte "l'intervalle" des paramètres où il est effectif et la "pertinence" de ces conditions comparé au milieu alimentaire (Ross and McMeekin, 2003). De manière générale, plus l'écosystème est complexe, moins les modèles sont précis, mais d'un autre côté, plus un écosystème est simple plus on crée des erreurs dû au manque d'exhaustivité des facteurs pris en compte (Baranyi et al., 1996; McMeekin and Ross, 2002). L'emploi de matrices structurées pour l'étude de la dynamique de croissance et de survie des bactéries en présence de microstructure est de plus en plus commun (Gomand et al., 2019; Kamanina et al., 2021; Verheyen et al., 2018; Wessman et al., 2011). L'écosystème que l'on souhaite modéliser est un mix de facteurs abiotiques e.g. température, pH, Aw, qui modifient les conditions de la matrice, ainsi que de facteurs biotiques (e.g. métabolisme, inoculum, expression stochastique) qui influencent le comportement

phénotypique de la cellule (figure 21 ci-dessous). Ces deux types de facteurs, biotique et abiotique, sont également en interaction. Il est par conséquent très difficile de modéliser la croissance des populations avec toutes ces sources de variations durant la latence, la probabilité de croissance, la cinétique de duplication… Le "*Quantitative Microbial Risk Assessment*" (QMRA) est d'autant plus difficile que l'établissement de la dose infectieuse est modifié par l'apparition de sous-populations hyper- ou hypo-virulentes. Si le risque d'un pathogène est mal estimé, alors même si la croissance est correctement déterminée, le risque persiste pour le consommateur d'ingérer une dose suffisante pour déclencher une infection alimentaire. La sévérité de la maladie est également dépendante de cette variation avec des souches plus tolérantes à certaines conditions ou qui échappe à la détection par le système immunitaire. Les données descriptives acquises lors de cette étude permettront de mieux définir les fonctions primaires (e.g. cinétiques de croissance, survie cellulaire, dose-réponse) et secondaires (état physiologique, nature du stress…)(González et al., 2019) des modèles mathématiques, ce qui permettra de renforcer les capacités de prédiction de la croissance des cellules et l'estimation du risque sanitaire.

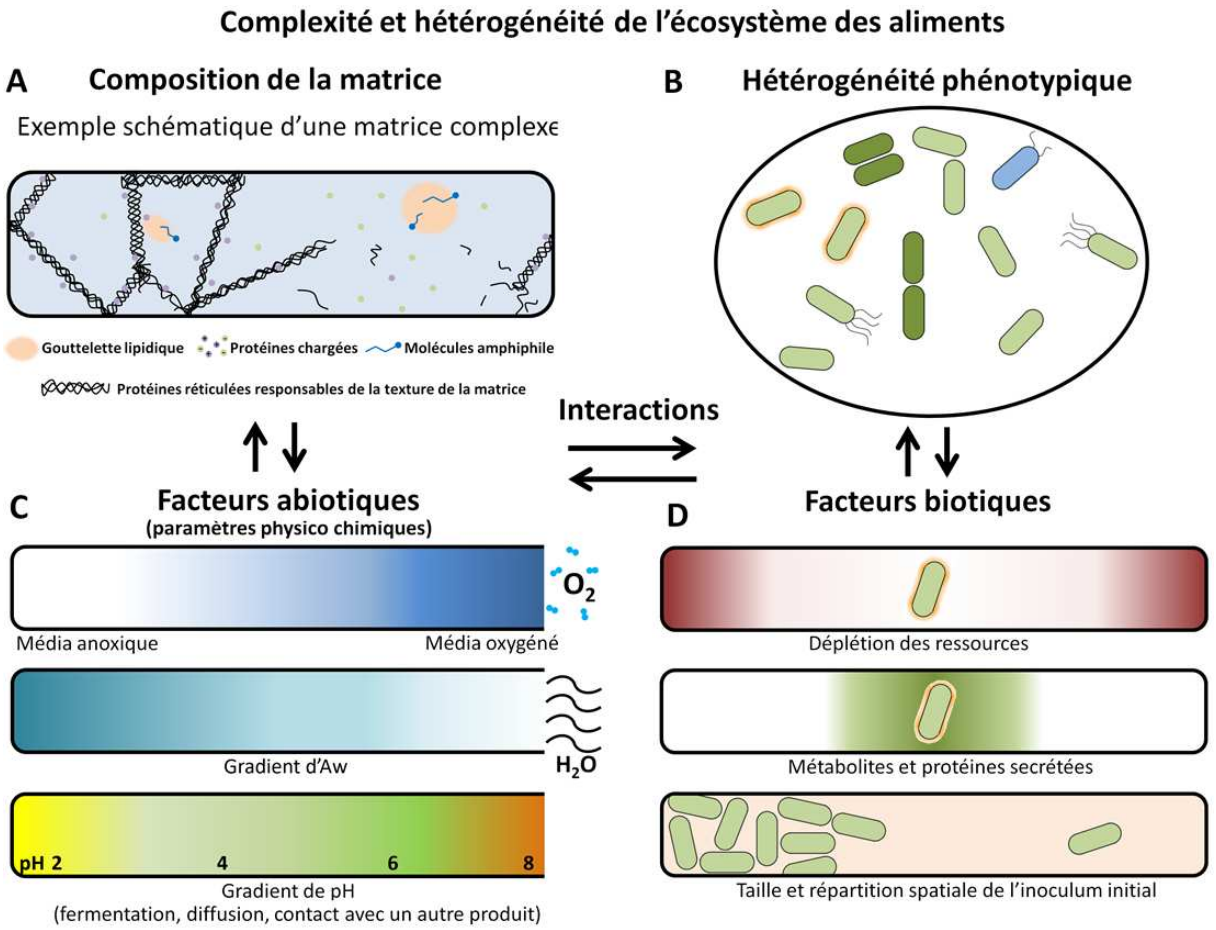


Figure 21 : Récapitulatif des quatre types de facteurs responsables de la difficulté de modéliser la croissance cellulaire en matrice alimentaire. La complexité du système découle de l'interaction de plusieurs facteurs. A) La composition de la matrice qui n'est pas uniforme et crée des conditions locales qui divergent de l'observation macroscopique. B) L'hétérogénéité propre aux populations bactériennes par adaptation physiologique ou l'activation stochastique de gènes. C) Les facteurs abiotiques venant de l'extérieur et modifiant les conditions physico-chimiques de la matrice. En particulier on retrouve la perméation du média par des gaz ou la perte d'eau qui modifie l'Aw, l'évolution du pH et le contrôle de la température. D) Les facteurs biotiques découlant de la croissance des bactéries et leur répartition spatiale. Ce sont les conséquences du métabolisme cellulaire qui consomme et excrète différents composés pour maintenir la survie et/ou la croissance cellulaire, ainsi que les perturbations sur ce mécanisme dû à la présence de l'activité d'autres cellules à proximité.

Concrètement, un résultat de cette étude intéressant pour la recherche appliquée est l'hyper-tolérance à des pH acides extrêmes des populations formant des microcolonies. Ces structures sont associées à une meilleure survie de bactéries pathogènes face à un stress mimant la digestion gastrique, et il serait donc avantageux de moduler la formulation des aliments pour limiter ce type de structures (Saint Martin et al., 2022). A l'inverse, il serait souhaitable que les flores technologiques ou positives soient sélectionnées pour former des microcolonies afin d'améliorer leur survie et d'enrichir le microbiote intestinal. Par exemple, une souche de *Lactobacillus* formant de plus grandes colonies serait préférable à une autre souche de la même espèce formant plusieurs petites microcolonies. Il serait également possible d'introduire certains ferments directement sous la forme de micro-biofilms ou microcolonies plutôt que sous forme planctoniques pour favoriser la formation de larges microcolonies dans la matrice. Une autre problématique soulevée par les travaux est l'intégration de la notion de motilité des bactéries dans les milieux semi-gélosées. Celle-ci semble possible dans des matrices alimentaires pour les bactéries motiles. Comment ce processus participe-t-il à la colonisation de l'aliment ? Est-il source de diversité phénotypique chez les pathogènes ou au contraire est-il bénéfique en limitant la formation des microcolonies ? Enfin une autre perspective à explorer concerne l'influence de cette diversification phénotypique lors de la croissance de pathogènes dans les matrices alimentaires sur la virulence de la souche. De nombreux facteurs de virulence possèdent une activité liée à l'état physiologique de la cellule. Serait-possible de maintenir des bactéries pathogènes dans une état d'hypo-virulence par une formulation adaptée des matrices alimentaires ?

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Valorisation du travail

Publications scientifiques

(1) **C. Saint Martin**, M. Darsonval, M. Grégoire, N. Caccia, L. Midoux, S. Berland, S. Leroy, F. Dubois-Brissonnet, M. Desvaux, R. Briandet, "Spatial organisation of *Listeria monocytogenes* and *Escherichia coli* O157:H7 cultivated in gel matrices", Food Microbiology, Volume 103, 2022, 103965, ISSN 0740-0020, https://doi.org/10.1016/j.fm.2021.103965.

(2) **C. Saint Martin**, N. Caccia, M. Darsonval, M. Grégoire, S. Leroy, F. Dubois-Brissonnet, R. Briandet, M. Desvaux, "Spatially localized expression of *Escherichia coli* O157:H7 glutamate decarboxylase *gadB* in gelled matrix microcolonies" (en préparation).

(3) **C. Saint Martin,** G. Jubelin, M. Darsonval, S. Leroy, C. Leneveu-Jenvrin, V. Stalh, L. Guillier, R. Briandet, M. Desvaux*, F. Dubois-Brissonnet*, "Physiological diversity and cellular heterogeneity of bacterial pathogens in food matrices: consequences on food safety" (en préparation).

Communications orales

(4) R. Briandet, Y. Dergham, **C. Saint Martin**, V. Gueneau, J. Deschamps, J.C Piard. « Biofilms dans l'industrie : comment lutter contre ou les utiliser à bon escient ?», Clubster NSL (Nutrition Santé Longevité), Webinar, Avril 2021.

(5) **C. Saint Martin,** R. Briandet, M. Desvaux. "Spatial organization of food pathogens *Listeria monocytogenes* and *Escherichia coli* in jellified media", Rencontres des microbiologistes du pôle clermontois, 10 Avril 2021, Clermont Ferrand.

(6) R. Briandet, Y. Dergham, D. Le Coq J. Deschamps, V. Gueneau, **C. Saint Martin**, P. Sanchez-Vizuete. "Actualité et perspectives sur les biofilms dans l'industrie pharmaceutique», Symposium Pharmaceutique BioMérieux 2021. 17 Juin 2021, Marcy L'Etoile.

(7) **C. Saint Martin,** R. Briandet, M. Desvaux. "Spatial organization of food pathogens *Listeria monocytogenes* and *Escherichia coli* in jellified media", ISEKI Food Association, 6th International Conference ISEKI-FOOD "Sustainable development goals in food systems: challenges and opportunities for the future" 23-25 Juin 2021, Visioconférence.

(8) **C. Saint Martin,** R. Briandet, M. Desvaux. "Spatial organization of food pathogens *Listeria monocytogenes* and *Escherichia coli* in jellified media" 16ème Congrès National de la Société Française de Microbiologie (SFM) 22-24 Septembre 2021, Nantes.

(9) **C. Saint Martin,** R. Briandet, M. Desvaux. "Spatial organization of food pathogens *Listeria monocytogenes* and *Escherichia coli* in jellified media" Journée de restitution du Réseau Mixte Technologique (RMT) Actia Florepro et projet ANR Redloss "Prédiction des altérations microbiologiques et place de la biopréservation dans la conservation des aliments" 12 Octobre 2021, Paris.

Annexes

1) Autres gènes d'intérêt pour une étude de la spatialisation de l'expression en microcolonies

Comme présenté dans les perspectives, d'autres gènes présentent le potentiel pour une expression spatialisée dans les microcolonies. Il s'agit de facteurs impliqués dans diverses fonctions telles que:

1.1) La colonisation des milieux

La formation du biofilm est un procédé complexe nécessitant l'intervention de plusieurs voies métaboliques qui sont tour à tour activées et réprimées suivant le stade de la construction de l'agglomérat. Trois gènes ont été sélectionnés dans ce but, *cah* de *E. coli* O157:H7 ainsi que *degU* et *secA2* de *L. monocytogenes*.

Chez *E. coli* O157:H7, le gène *flu* découvert en 1980 by Diderischen et depuis rebaptiser *cah* puis *ag43*, l'antigène 43 est un auto-transporteur appartenant au système de sécrétion de type V (sous type a, T5aSS) qui joue un rôle dans l'auto-agrégation bactérienne par un mécanisme de reconnaissance d'un autre Ag43 sur la membrane d'une cellule voisine (van der Woude and Henderson, 2008). Ag43 joue un rôle important dans la première phase de l'apparition des biofilms en milieu minimum comme le M9 mais est moins indispensable dans les milieux riches comme le LB (*Lysogeny Broth*) (Danese et al., 2000).

La régulation de l'expression de *cah* (antigène 43) est complexe (Figure 22) et implique un mécanisme de variation de phase par compétition entre DAM et OxyR pour l'accès à trois sites GATC dans la région promotrice de *cah* (Diderichsen, 1980; Henderson and Owen, 1999). A chaque duplication du génome, si la méthylase DAM méthyle l'adénine du site GATC, l'inhibiteur de transcription OxyR ne peut plus se fixer sur ce brin d'ADN

(Owen et al., 1996). Un double brin hémiméthylé réduit la capacité de fixation de OxyR mais la présence de OxyR sur un double brin empêche toute méthylation de GATC (Haagmans and Van Der Woude, 2000), le système est ainsi biaisé vers le phénotype off du gène. Le ratio de forme oxydé/réduite de OxyR n'a pas d'impact supplémentaire sur la transcription de *agn43* (Wallecha et al., 2003).

Une dernière forme de régulation existe sous la forme d'une inhibition de fonction par la présence de Fimbriae de type 1, une autre protéine membranaire autoaggrégative également soumise à la variation de phase. La taille supérieure de Fim1 empêche les interactions Agn43/Agn43 de se produire (Hasman et al., 2000).

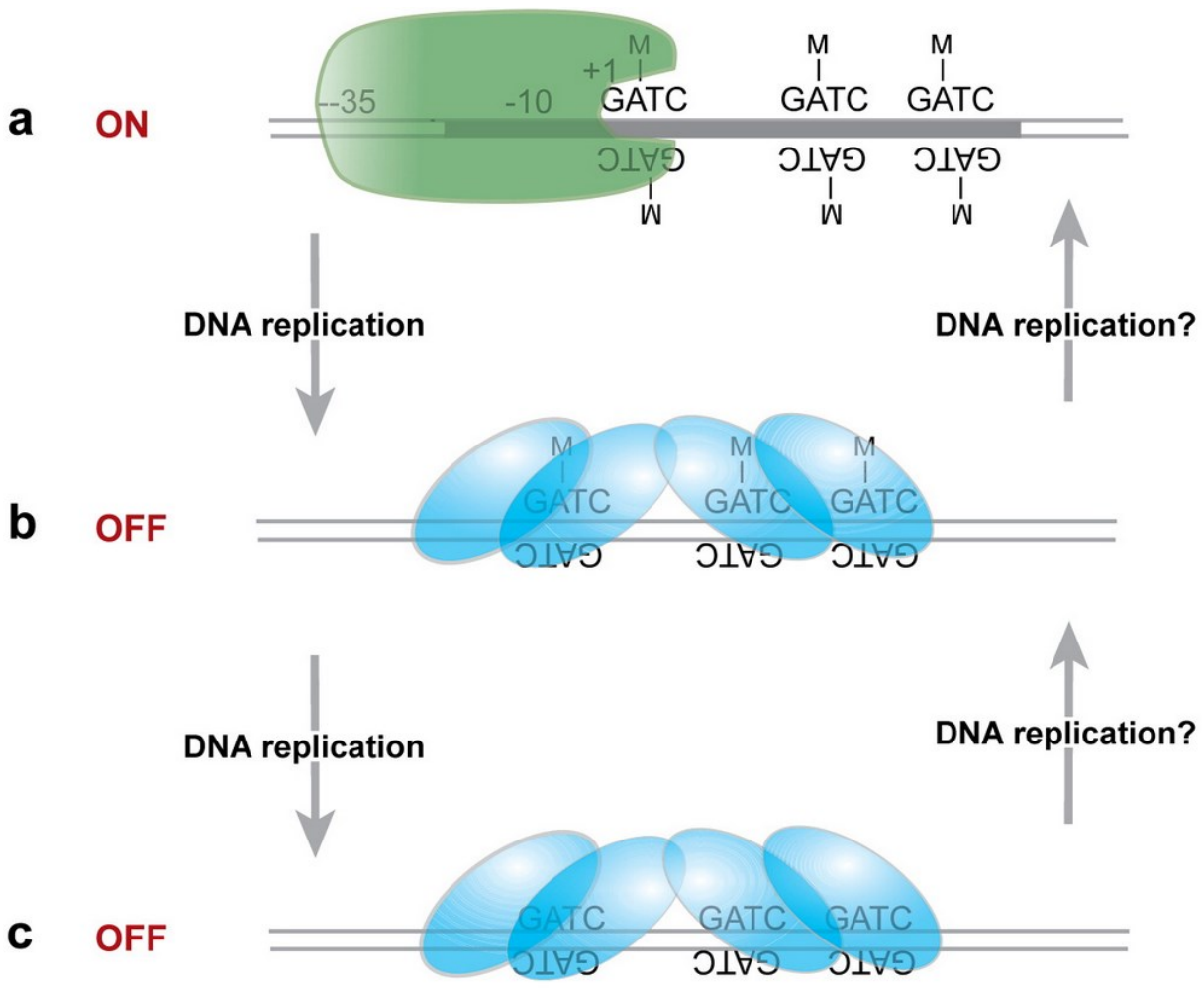

Figure 22 : Représentation du système de contrôle par variation de phase responsable de l'expression du gène *cah*. Les sites GATC, présents sur l'ADN après les promoteurs -35/ -10 et le codon start, servent de cible à la fois pour la méthyltransférase Dam qui rajoute un groupe méthyl (M) à

l'adénine de la séquence, et un tétramère d'OxyR qui a une grande affinité pour l'ADN non méthylé, une plus faible capacité de liaison avec l'ADN hémiméthylé et n'interagit pas avec l'ADN complètement méthylé. La transcription est possible uniquement si aucune protéine régulatrice OxyR n'est fixée à l'ADN (phase on en A), autrement l'expression est impossible (phase off en B et C).
D'après (van der Woude and Henderson, 2008).

Pour *L. monocytogenes*, les deux gènes identifiés n'ont pas une action directe sur la colonisation mais sont nécessaires pour sa mise en place. Le premier code pour le régulateur de transcription DegU. DegU est un régulateur de la transcription orphelin chez *L. monocytogenes*, c'est-à-dire que la bactérie ne possède pas le senseur kinase DegS nécessaire pour former le système à deux composants DegSU tel qu'il a été décrit dans *B. subtilis* (Kunst et al., 1988). Dans *B. subtilis*, DegSU est principalement lié à la détection du choc osmotique (Dartois et al., 1998) et un stress NaCl conduit à la phosphorylation de DegU par DegS. Chez *L. monocytogenes*, DegU orphelin garde un rôle pléiotropique dans la régulation des phénotypes (Figure 23), ce malgré l'absence de *degS* sur le chromosome et donc le manque d'une forme phosphorylée. DegU a été démontré comme jouant un rôle dans la formation du flagelle (Williams et al., 2005), la chemotaxis, la croissance à haute température, la formation du biofilm (Gueriri et al., 2008) et la virulence (Knudsen et al., 2004). En particulier l'impact de DegU sur la motilité, la virulence et l'adhésion cellulaire serait en réponse à la présence de NaCl (Olesen et al., 2009). L'acidité du milieu est un promoteur de l'expression de *degU* chez *L. monocytogenes* (Neuhaus et al., 2013), mais curieusement ce n'est pas le cas avec le NaCl (Olesen et al., 2009).

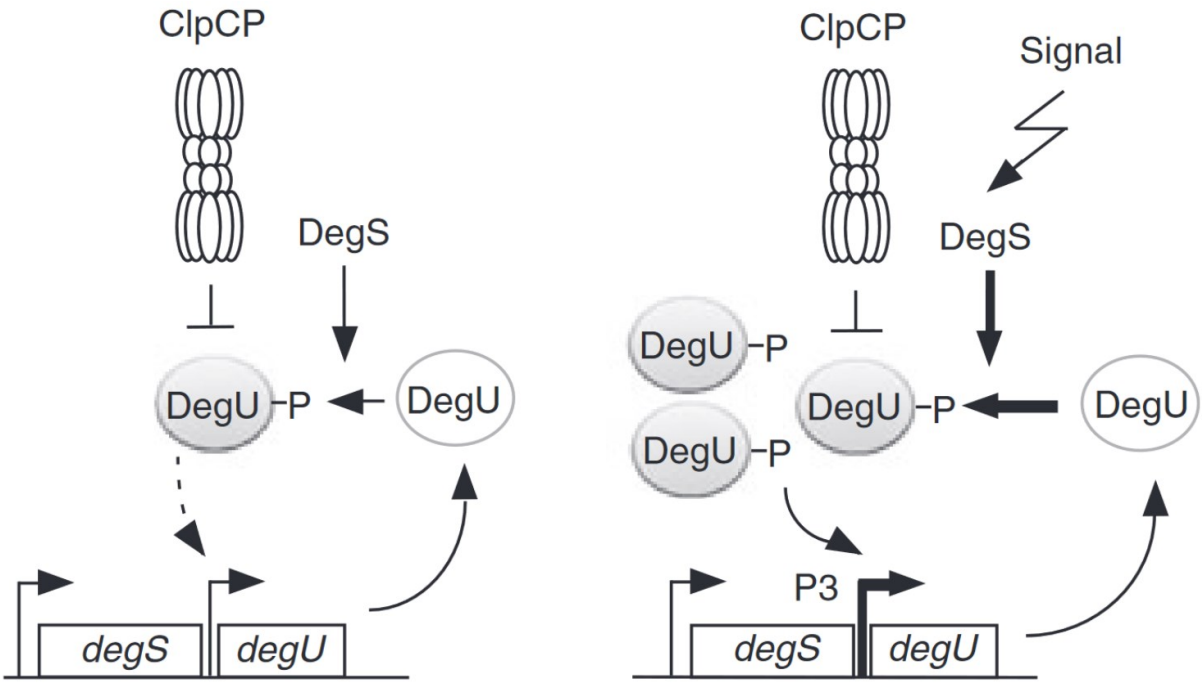

Figure 23 : Cycle d'activation de DegU chez *B. subtilis*. En condition de repos, la phosphorylation de DegU est faible et Degu-P est maintenu à un niveau basal par l'activité de protéolyse spécifique de ClpCP. L'équilibre est rompu par un signal externe qui en modifiant la membrane active l'histidine kinase DegS qui phosphoryle rapidement DegU. DegU-P existe alors en quantité suffisante pour promouvoir sa propre transcription et renforcer la boucle d'activation.
D'après (Ogura and Tsukahara, 2010).

La seconde protéine étudiée dans le cadre de la colonisation chez *L. monocytogenes* est SecA2. L'énergie nécessaire à la sécrétion est généralement due à une seule ATPase SecA1, mais chez certaines Mycobactéries et bactéries à Gram positif, il est possible d'observer une seconde protéine homoloque, SecA2 (Feltcher and Braunstein, 2012). Dans ce cas de figure, SecA1 régie la sécrétion de la majorité des protéines de manière canonique tandis que SecA2 est associé à des fonctions plus spécifiques tel que la formation de la membrane, la parthénogénèse (Renier et al., 2014, 2013) et la virulence (Lenz et al., 2003). Sans SecA2, les cellules de *L. monocytogenes* prennent la forme de chainettes qui sédimentes (figure 24).

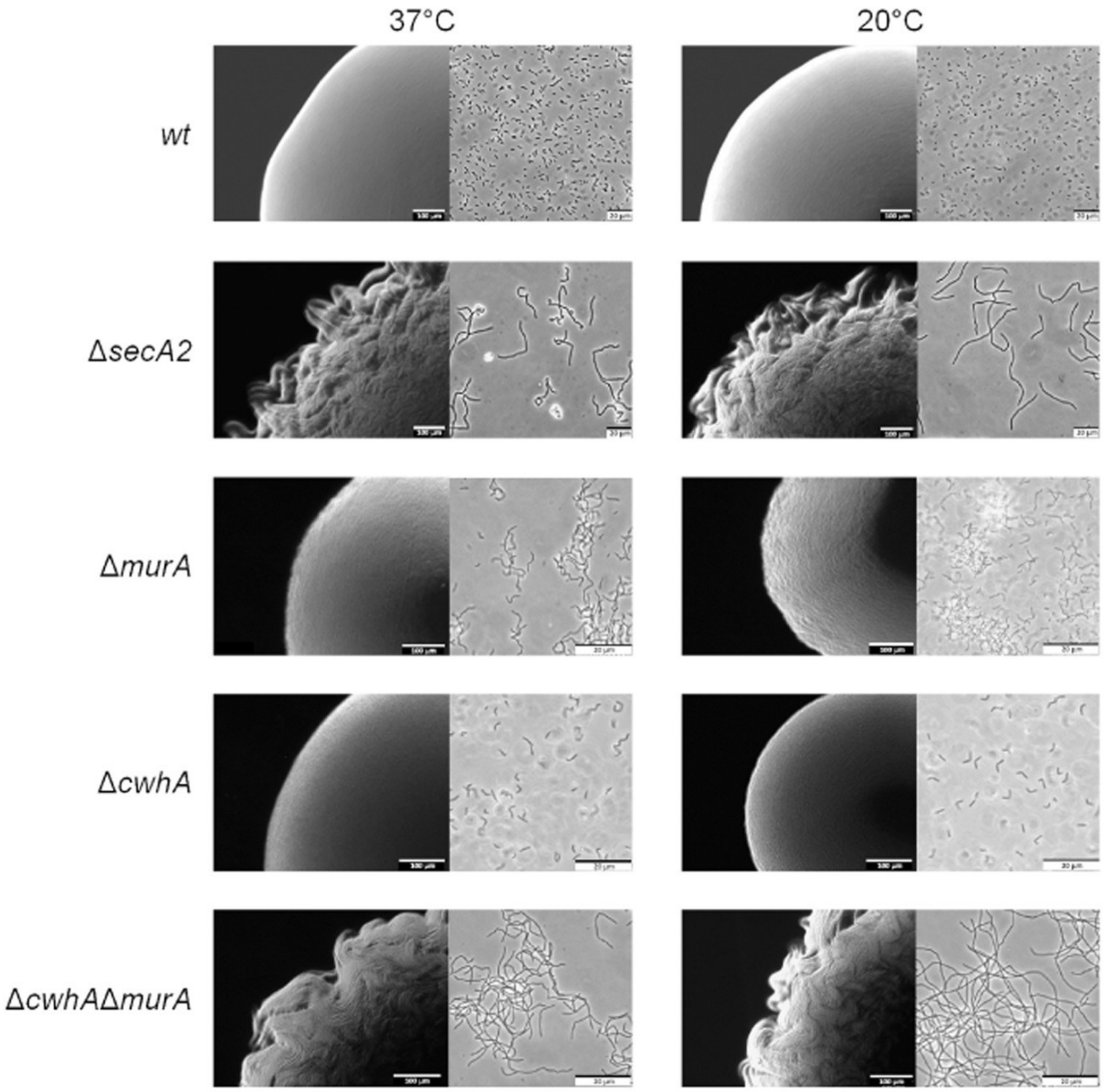

Figure 24 : Morphologie de colonies de *L. monocytogenes* EGD-e sauvage ou de mutant isogénique pour les gènes *secA2*, *murA* et *cwhA*. Après une croissance en BHI à 37°C (à gauche) ou 20°C (à droite), des observations microscopiques en contraste de phase montrent un phénotype «*smooth*» pour les colonies sauvages et Δ*cwhA* aux deux températures. Dans le mutant Δ*murA* des vagues sont visibles en surface de la colonie à 37°C et 20°C. Enfin, pour le double mutant Δ*murA*Δ*cwhA* ainsi que pour Δ*secA2* un phénotype «*rough*» est observé avec un entremêlement de chaînettes de bactéries, également pour les deux de température.

D'après (Renier et al., 2014).

1.2) La tolérance aux conditions de stress avec la réponse générale et spécifique de l'oxygène

La régulation générale du métabolisme face à des stress

Les organismes unicellulaires existent dans des milieux qui peuvent évoluer rapidement entre des conditions bénignes et des situations hostiles couplées à une pauvreté de ressources. Pour survivre et être compétitives, les bactéries possédant des voies métaboliques adaptées à ces différents environnements et surtout un moyen de coordonner la réponse pour rapidement passer d'un mode de croissance à celui de survie. Cela sous-entend un contrôle global de l'expression génétique qui implique RpoS/σ^B chez *E. coli* et *L. monocytogenes*.

Le régulateur RpoS a une activité auto-activatrice sur l'opéron *rpoS*, et pour les Gammaproteobacteria comme *E. coli,* cette autorégulation est réalisée en conjonction avec Crl (Cavaliere et al., 2015; Santos-Zavaleta et al., 2019). Crl augmente les effets activateurs ou inhibiteurs des régulateurs de transcription de l'opéron et a plus d'effet si il y a un faible taux de Rpos dans la cellule (Typas et al., 2007). Les différentes étapes de transcription, traduction et protéolyse de RpoS sont impactées par les conditions du milieu (Hengge-Aronis, 2002).

Les ilôts de pathénogicités de type O sont souvent dépendant de Rpos pour leur activation, ce qui lie intimement stress et virulence pour des souches comme O157:H7 dont la séquence génomique contient de nombreux exemples de ces ilôts (Dong and Schellhorn, 2009).

Chez *L. monocytogenes*, σ^B, est aussi impliqué dans la réponse générale au stress (Becker et al., 1998; Begley et al., 2006; Ferreira et al., 2001; Kazmierczak et al., 2003; O'Donoghue et al., 2016; Wiedmann et al., 1998), avec environ 55 gènes qui sont différemment exprimés par l'activation de la cascade σ^B. Celle-ci est déclenché par la dissolution du complexe séquestrant σ^B (Chen et al., 2004, 2003; Yang et al., 1996) (figure 25). Le régulateur est également impliqué dans l'expression de gènes de

virulence (Camejo et al., 2009; Kazmierczak et al., 2006; Mraheil et al., 2011), la formation du biofilm et colonisation (Lemon et al., 2010; Tiensuu et al., 2019; Todhanakasem and Young, 2008) ainsi que la motilité (Toledo-Arana et al., 2009).

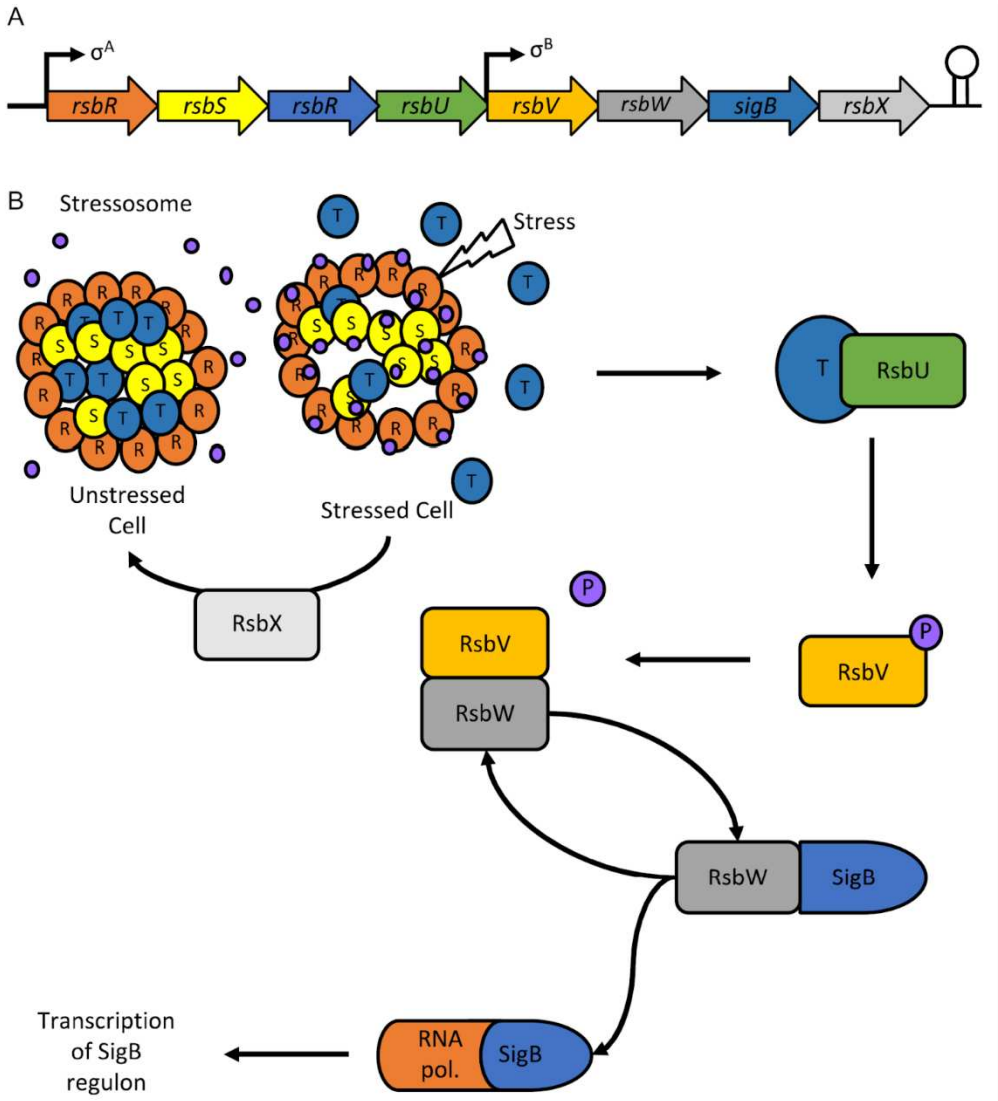

Figure 25 : Opéron *sigB* et la régulation de l'activité de σ^B. A) Opéron *sigB* de *L. monocytogenes,* la transcription peut commencer à deux positions et se finit sur la séquence du terminateur formant une structure en tige-boucle. B) En condition optimale RsbR, RsbT et RsbS forment un stressosome stable. Sous l'action d'un stress RsbT phosphoryle RsbR et RbsS ce qui permet à RbsT de se dissocier du complexe. RbsT libre interagit avec RbsU qui devient actif et déphosphoryle RsbV-P. Rsbw est un facteur anti-sigma qui séquestre σ^B en temps normal mais il a plus d'affinité pour RsbV. σ^B est désormais libre de se lier à une polymérase à ARN pour initier la transcription du régulon à partir du second site de transcription.

D'après (Dorey et al., 2019).

La réponse au stress oxydant ou à l'hypoxie

La présence d'oxygène dans le milieu crée inévitablement des conditions où la formation d'espèces réactives de l'oxygène (ROS) est possible. Les bactéries disposent de senseurs comme OxyR et FNR pour organiser une réponse à la présence d'O_2 dans le milieu.

Membre des facteurs de transcription type LisR (Christman et al., 1989), OxyR est sensible en particulier au peroxyde d'hydrogène par l'intermédiaire de deux cystéines (Lee et al., 2004; Tao et al., 1989; Zheng et al., 1998). En condition normale OxyR est trouvé sous forme d'un tétramère réduit, et une fois oxydé celui-ci peut lier l'ADN (Figure 26). Le glutathion réduit le pont disulfure et OxyR contrôle l'expression de la glutathione réductase (GSH) qui régénère le glutathion par une boucle de rétrocontrôle. OxyR promeut l'expression de nombreux gènes en lien avec les réparations de l'ADN, l'utilisation des peroxydes et l'activité de réduction (Zheng et al., 2001). La présence de H2O2 n'a pas d'impact sur la transcription de *oxyR* (Storz et al., 1990), il n'y a donc pas d'auto-activation par OxyR réduit sur *oxyR*. Par contre, OxyR sous ses deux formes peut réprimer sa propre transcription (Toledano et al., 1994). OxyR dispose d'autres attributs comme la régulation de l'adhésion et de la formation du biofilm et la virulence des souches urinaires (Johnson et al., 2013). Ces capacités de régulation de la transcription pour d'autres gènes sont indépendantes de l'état d'oxydo/redox de OxyR. Par l'intermédiaire du petit ARN non codant OxyS, OxyR impacte aussi l'expression de *flhA* et *rpoS* (Altuvia et al., 1998; Zhang et al., 1998).

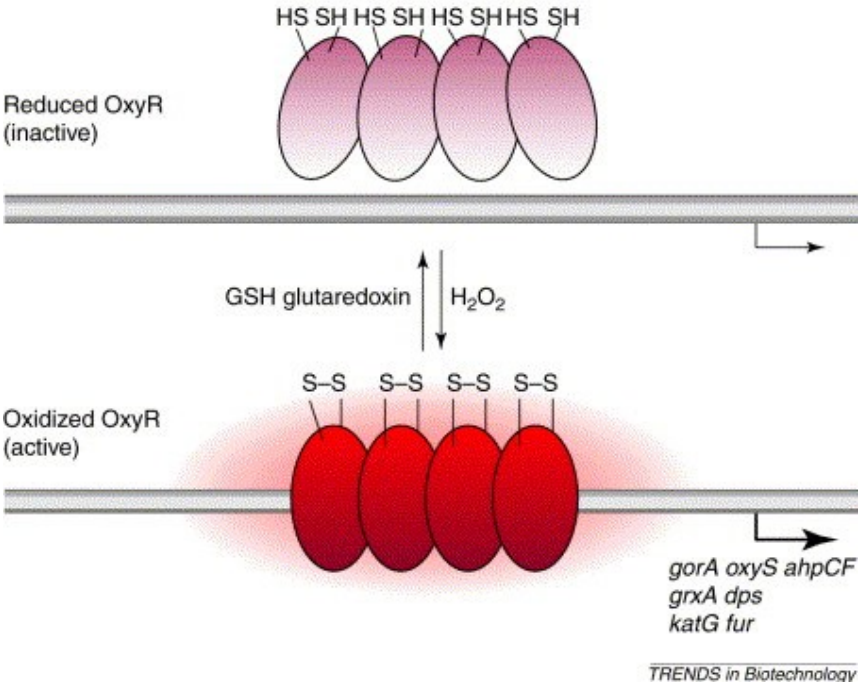

Figure 26 : Régulation de transcription par OxyR. Sous forme réduite, OxyR forme des tétramères mais ne peut interagir avec l'ADN. Seule la forme oxydée du tétramère lie la séquence cible sur le promoteur.

D'après (Pomposiello and Demple, 2001).

Les anaérobes facultatives comme *E. coli* sont régulièrement exposées à des milieux anoxiques (intestins) ou oxygénés et FNR régule le métabolisme bactérien en fonction de son statut mono- ou di-mérique. Ce statut oligomérique est dépendant de la concentration d'O_2 car chaque FNR peut fixer un cofacteur $[2Fe\text{-}2S]^{2+}$, et en condition anoxique deux cofacteurs forment $[4Fe\text{-}4S]^{2+}$ permettant la dimérisation (Bueno et al., 2012). FNR est un régulateur de transcription pour de nombreux gènes et a un fort impact sur le métabolisme (Barnard et al., 2004; Kang et al., 2005), en particulier l'activité de régulation de plus de 63 gènes est modifiée chez *E. coli* (Green et al., 1996; Lazazzera et al., 1996). O_2 réagit avec le soufre de Fe-S, ce qui sépare le cofacteur et entraîne le retour à la forme monomérique inactive de FNR.

1.3) La régulation et la maintenance de l'ADN

Coupure double brin de l'ADN, maintenance chez *E. coli* et *L. monocytogenes* par RecA

La réparation des doubles cassures de l'ADN nécessite l'activité de recombinaison homologue possédée par les recombinases, un groupe très conservé avec 60-70% d'homologie dans l'ensemble des génomes bactériens étudiés (Haldenby et al., 2009; Hofstatter et al., 2016; Rocha et al., 2005). Parmi ces recombinases, RecA est la plus connu de par sa découverte plus ancienne et son exploration dans *E. coli* (Clark and Margulies, 1965).

La protéine est composé d'un domaine N-terminal suivi par un cœur, le «*central core ATPase domain*» (CAD), constitué dans l'ordre, d'un site de liaison de l'ATP, un site de fixation de Mg^{2+},un site liaison au ssDNA dit site 1, un site de liaison au dsDNA homologue dit site 2 et finalement le domaine C-terminal contenant un autre site interagissant avec dsDNA ainsi que d'un second site de captation des ions Mg^{2+} (Mazin and Kowalczykowski, 1998).

Le mode d'action de RecA est complexe (Cox, 2007a; Lee et al., 2016; Rosselli and Stasiak, 1991; Yu et al., 1995) et fait appel à de nombreuses protéines accessoires (Cox, 2007b; Lavery and Kowalczykowski, 1992). Il est présenté en détail dans la figure 27.

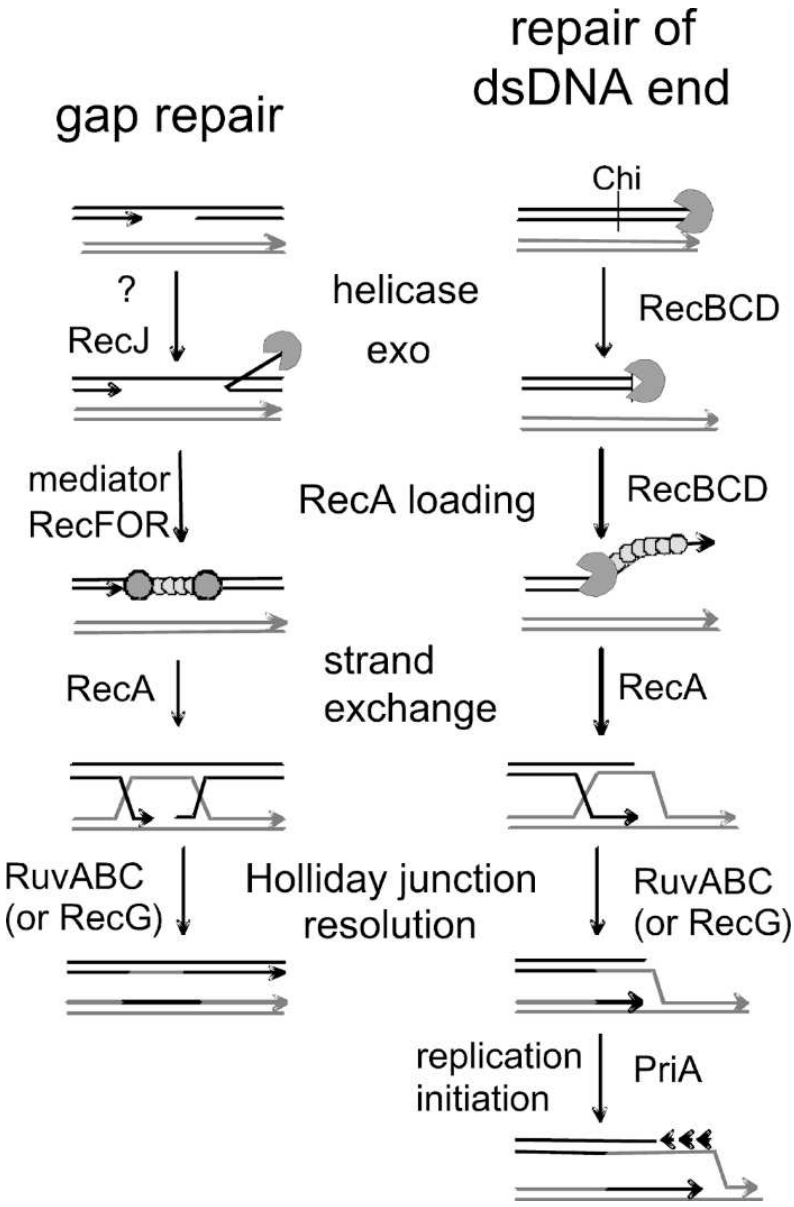

Figure 27 : Système de recombinaison homologue de *E. coli* pour la gestion des trous dans la séquence ou les cassures double brin. A) Pour un trou dans la séquence, la 5' ssDNA exonucléase élargie la section simple brin pour recruter RecA sur le ssDNA. B) Pour une cassure double brin, le recrutement d'un complexe hélicase-nucléase va digérer l'ADN jusqu'au site χ le plus proche et RecA est chargé sur l'ADN. C) Dans les deux situations, RecA recherche une homologie de séquence sur un autre dsDNA et une fois la cible trouvée forme une jonction de Holliday par échange de brin. En étape postsynaptique RecA maintient la jonction tandis qu'elle migre sur le long de l'ADN. Une dernière étape est nécessaire dans le cas de la double cassure avec la synthèse du brin antisens.

D'après (Rocha et al., 2005).

L'adénine methyl-transférase (DAM) est un régulateur majeur de l'épigénétique des gènes de *E. coli*.

Dam est une méthylase de l'adénine de la séquence GATC (Bergerat et al., 1989; Herman and Modrich, 1982), cette dernière est retrouvé en double exemplaire dans la région promotrice de 50% des gènes chez *E. coli* (Herman and Modrich, 1982; Oshima et al., 2002) et est en concurrence avec des régulateurs de la transcription (Aloui et al., 2013; Wallecha et al., 2002). Dam possède donc un impact fort sur le phénotype de la bactérie, mais avec seulement 100-130 Dam par cellule (Boye et al., 1992), des variations stochastiques de l'expression sont attendues. La sur-abondance de Dam en particulier augmente les chances de mutations (Løbner-Olesen et al., 2003; Marinus et al., 1984).