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ASSESSMENT OF THE GROWTH OF *LISTERIA MONOCYTOGENES* IN BELGIAN ARTISANAL CHEESES AND INVESTIGATIONS ON FACTORS GOVERNING IT

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Abstract

Providing safe food to consumers remains a key challenge nowadays, especially considering the trend that favours natural products and food free of additives. Among foodborne diseases, listeriosis is the fifth most occurring in EU, with more than 2,500 cases identified in 2018. *Listeria monocytogenes*, the pathogen responsible for this disease, can be carried by various RTE foods, including dairy products. As potential vectors of *L. monocytogenes*, cheeses have to comply with food safety criteria defined by Regulation (EC) No 2073/2005. By default, cheeses are considered as RTE foods allowing the growth of the pathogen during their shelf-life. Therefore, producers have to guarantee that *L. monocytogenes* is not detected in cheeses placed on the market. Nevertheless, various foreign studies have identified cheese varieties not allowing this growth, and even allowing a decrease in the levels of contamination during storage.

Belgian cheeses, especially artisanal products, are relatively unknown, although this country possesses a rich diversity of cheese varieties and producers. Consequently, not many data are available regarding the behaviour of *L. monocytogenes* in these products. Belgian cheese varieties are thus considered as allowing the growth of *L. monocytogenes* during refrigerated shelf-life. Although they are necessary to guarantee consumers' safety, food safety criteria represent a permanent sword of Damoclès for producers. The detection of *L. monocytogenes* can indeed result in huge economic losses and important moral consequences.

The main goal of this thesis was thus to assess the growth of *L. monocytogenes* in diverse Belgian artisanal cheeses, and to understand factors affecting it.

First, a phone survey was performed among 142 Belgian artisanal cheese producers, providing general knowledge on producers, manufacturing processes and varieties. Globally, 16 major types of cheese were identified. One third of varieties were unripened acid-curd cheeses. Another third corresponded to uncooked pressed cheeses, including *Saint-Paulin*-type and *Gouda*-type cheeses, mainly found in Wallonia and Flanders, respectively. Soft cheeses corresponded to 18% of observed varieties. Minor varieties were also identified, including half-cooked and cooked pressed cheeses, blue-veined cheeses, *Ricotta*, *Mozzarella*, *Halloumi* and *Feta*.

From this data, 65 varieties were selected for deeper characterization. Factories were visited and manufacturing process of these cheeses was monitored. Finally, samples were collected for physico-chemical characterization. From these 65 varieties, only two had physico-chemical characteristics naturally inhibiting the growth of *L. monocytogenes*, *i.e.* pH $\leq$ 4.4, or $a_w \leq 0.92$, or pH $\leq$ 5.0 and $a_w \leq 0.94$. It means that most varieties theoretically allowed its growth, confirming the interest of the present thesis. Collected data did not allow to improve current cheese classification tools.

After that, 32 varieties representative of the diversity of artisanal cheeses were selected in order to assess the growth of *L. monocytogenes*. It was decided to perform challenge studies for this purpose, with artificial contamination of final cheeses with *L. monocytogenes*. Briefly, three batches of each variety were studied,

except if predictive models showed no growth of the pathogen during storage. For each batch, 12 pieces were collected. Six were artificially contaminated, remaining pieces being control samples. *L. monocytogenes* was enumerated the first and the last day of storage at 8±1 °C, allowing to determine the growth potential of the pathogen. It was concluded that unripened acid curd cheeses systematically allowed a decrease in the levels of *L. monocytogenes*. Through a new circular, this type of cheese is now recognized as unrisky for human health by Federal Agency for the Safety of the Food Chain. A level of 100 cfu/g of *L. monocytogenes* in cheese is now tolerated. Results for other cheese types were more controversial. Globally, soft cheeses allowed the growth of the pathogen to levels harmful for health. Nevertheless, three batches of a raw milk *Herve* cheese showed a decrease in the contamination. Regarding semi-hard cheeses, huge variability was observed between varieties, between batches and between samples. Physico-chemical and process-associated parameters did not allow to understand these differences. It was surprising to observe that methodologies provided by official guidelines from European Union Reference Laboratory for *Listeria monocytogenes* did not allow to take this variability into account when determining risk associated to a product. A revision of these guidelines should be planned in order to guarantee consumers' safety.

It was thus decided to focus on cheese microbiota, aiming to identify eventual inhibitive bacterial species or consortia. Using next-generation sequencing technologies, bacterial richness and diversity were determined at the genus level. Richness and diversity were significantly higher in soft cheeses, in comparison with other types. Surprisingly, diversity was poor in semi-hard cheeses, and study of the microbiota did not provide useful explanation concerning the variability in behavior of *L. monocytogenes* for this type of products. Regarding *Herve* cheese, metagenetics revealed the presence of an unknown species of the genus *Fusobacterium*, with a relative abundance around 10%.

A hypothesis was that the presence of this species could explain the surprising behavior of *L. monocytogenes* in *Herve* cheese. Nevertheless, we did not succeed at isolating the bacterium. Metagenomics on cheese deoxyribonucleic acid sample allowed to assemble and to annotate the theoeretical genome of this bacterium. Nucleotide identity and phylogenomic tree suggested that it belong to a novel species of the *Fusobacterium* genus. Proteome comparison identified potentially unshared proteins families, metabolic pathways and subsystems unshared with other *Fusobacterium* spp. Nevertheless, without isolation of the bacterium, it was impossible to describe the novel species, as well as to assess its potential role in the inhibition of *L. monocytogenes* in *Herve* cheese.

Globally, although markers were identified for unripened acid curd cheeses, it was not possible to determine individual factors affecting the growth or the absence of growth of *L. monocytogenes* in semi-hard cheeses. It is likely that its behavior is more affected by a complex interaction between factors, intrinsic to each cheese variety, and providing sufficient hurdles. This thesis contributed to the global knowledge on Belgian artisanal cheeses, in association with *L. monocytogenes*, but a

lot of work still must be performed during the next years, concering fundamental research, but also concerning the development of universal guidelines and standards.

Résumé

Fournir des aliments sûrs aux consommateurs reste un défi clé de nos jours, avec la volonté de favoriser les produits naturels et les denrées alimentaires exemptes d'additifs. Parmi les maladies d'origine alimentaire, la listériose est la cinquième plus importante dans l'Union Européenne en termes d'occurrence, avec plus de 2.500 cas rapportés en 2018. *Listeria monocytogenes*, le pathogène responsable de cette maladie, peut être transmis par diverses denrées alimentaires prêtes à être consommées, notamment les produits laitiers. En tant que vecteurs potentiels de *L. monocytogenes*, les fromages doivent satisfaire aux critères microbiologiques définis dans le Règlement (CE) N° 2073/2005. Par défaut, les fromages sont considérés comme des denrées alimentaires prêtes à être consommées permettant la croissance du pathogène au cours de leur durée de vie. En conséquence, les producteurs doivent garantir la non-détection de *L. monocytogenes* au sein de leurs fromages avant leur mise sur le marché. Cependant, diverses études étrangères ont permis d'identifier des variétés de fromages ne permettant pas cette croissance, et assurant même parfois une baisse des niveaux de contamination durant le stockage.

Les fromages belges, en particulier les produits artisanaux, sont relativement inconnus, bien que ce pays possède une riche diversité de variétés de fromage et de producteurs. Ainsi, peu de données sont disponibles en ce qui concerne le comportement de *L. monocytogenes* dans ces aliments. Les variétés belges de fromage sont donc considérées comme des aliments permettant la croissance de *L. monocytogenes* en cours de stockage réfrigéré. Bien que nécessaires pour garantir la sécurité des consommateurs, les critères d'hygiène des denrées alimentaires constituent une épée de Damoclès permanente au-dessus de la tête des producteurs. La détection de *L. monocytogenes* peut en effet engendrer de graves conséquences économiques et morales.

L'objectif principal de cette thèse de doctorat a donc été d'évaluer la croissance de *L. monocytogenes* dans différentes variétés artisanales de fromages belges, et de comprendre les principaux facteurs l'influençant.

Premièrement, une enquête téléphonique a été réalisée auprès de 142 producteurs belges de fromage artisanal, fournissant des connaissances générales sur les producteurs, les procédés de fabrication et les variétés. Globalement, 16 grands types de fromage ont été identifiés. Un tiers des variétés correspondaient à des pâtes lactiques fraîches. Un autre tiers était constitué par les fromages à pâte pressée non cuite, incluant les types *Saint-Paulin* et *Gouda*, principalement retrouvés en Wallonie et en Flandre, respectivement. Les pâtes molles représentaient près de 18 % des variétés identifiées lors de l'enquête. Enfin, des variétés mineures ont également été répertoriées, incluant les pâtes pressées mi-cuites et cuites, les bleus, la *Ricotta*, la *Mozzarella*, le *Halloumi* et la *Feta*.

Sur base des ces données, 65 variétés ont été sélectionnées pour une caractérisation plus approfondie. Ainsi, les fromageries concernées ont été visitées et les procédés de fabrication ont été suivis. Enfin, des échantillons de produits finis ont été prélevés pour être caractérisés physico-chimiquement. Parmi ces 65 variétés

fromagères, seules deux présentaient des caractéristiques physico-chimiques permettant naturellement une inhibition de la croissance de *L. monocytogenes*, *i.e.* un pH ≤ 4,4, ou une a_w ≤ 0,92, ou enfin un pH ≤ 5,0 et une a_w ≤ 0,94. Cela signifie donc que la majorité des variétés permettent théoriquement la croissance du pathogène, confirmant l'intérêt de la présente thèse. Les données collectées n'ont pas permis d'améliorer les outils de classification des fromages actuellement disponibles.

Après cela, 32 variétés représentatives de la diversité des fromages artisanaux belges ont été sélectionnées en vue d'évaluer la croissance de *L. monocytogenes* en leur sein. Il a été décidé de réaliser des tests de provocation à cette fin, impliquant une contamination artificielle des fromages par *L. monocytogenes*. Brièvement, trois lots de chaque variété ont été étudiés, à l'exception des variétés pour lesquelles les outils informatiques de prédiction de croissance avaient au préalable démontré l'impossibilité pour le pathogène de s'y développer. Pour chaque lot, 12 pièces ont été prélevées. Six ont été artificiellement inoculées, les autres servant de témoins. *L. monocytogenes* a été dénombrée les premier et derniers jours de stockage à 8 ± 1 °C, permettant de déterminer le potentiel de croissance du pathogène pour chaque lot. Il a été conclu que les pâtes lactiques fraîches permettaient systématiquement une décroissance des niveaux de contamination par *L. monocytogenes*. Par le biais d'une nouvelle circulaire, ce type de fromage est mainteant reconnu comme sûr pour la santé humaine par l'Agence Fédérale pour la Sécurité de la Chaîne Alimentaire. Un niveau maximal de 100 ufc/g de *L. monocytogenes* est maintenant toléré pour ces produits. Les résultats relatifs aux autres types de fromages ont été plus controversés. Globalement, les fromages à pâte molle ont permis la croissance du pathogène jusqu'à des niveaux dangereux. Néanmoins, trois lots d'un *Herve* au lait cru ont présenté une décroissance des niveaux de contamination. Concernant les fromages à pâte mi-dure, une grande variabilité a été observée entre variétés, entre lots et entre pièces. Les paramètres physico-chimiques et les données associées aux procédés de fabrication n'ont pas permis de comprendre ces différences. Il a été choquant de constater que les méthodologies actuelement détaillées par le Laboratoire de Référence de l'Union européenne pour *L. monocytognes* ne permettaient pas de tenir compte de cette variabilité en déterminant les risques liés à une denrée. Une révision de ces méthodes devrait être à l'ordre du jour afin de garantir de façon efficace la sécurité des consommateurs.

Il a été décidé de s'intéresser au microbiote des fromages, en vue d'identifier des espèces bactériennes ou des consortia pouvant potentiellement inhiber *L. monocytogenes*. Au moyen des nouvelles technologies de séquençage, la richesse et la diversité bactérienne ont été déterminées au niveau du genre. Ces deux paramètres étaient significativement plus élevés au sein des fromages à pâte molle en comparaison aux autres types. De façon surprenante, la diversité bactérienne était très faible dans les fromages à pâte mi-dure, et l'étude du microbiote n'a pas permis de formuler des hypothèses intéressantes expliquant la variabilité observée pour ces produits. En ce qui concerne le *Herve*, la métagénétique a révélé la présence d'une

bactérie inconnue appartenant au genre *Fusobacterium*, avec une abondance relative de l'ordre de 10 %.

Une hypothèse a été de se dire que la présence de cette bactérie pourrait expliquer les observations surprenantes réalisées précédemment, concernant la décroissance des niveaux de *L. monocytogenes* lors du stockage de ces produits. Néanmoins, nous ne sommes pas parvenus à isoler la bactérie concernée. La métagénomique appliquée sur les extraits d'ADN obtenus à partir du fromage a permis d'assembler et d'annoter le génome théorique de cette bactérie. L'identité nucléotidique et l'arbre phylogénomique ont suggéré qu'elle pourrait appartenir à une nouvelle espèce du genre *Fusobacterium*. La comparaison de protéomes a permis d'identifier des familles protéiques, voies métaboliques et sous-systèmes potentiellement non partagés avec d'autres espèces de *Fusobacterium*. Néanmoins, sans être parvenu à isoler la bactérie, il demeure impossible de décrire la nouvelle espèce, ainsi que d'évaluer son rôle potentiel dans l'inhibition observée de *L. monocytogenes* au sein de ce fromage de *Herve*.

Globalement, bien que des marqueurs aient pu être identifiés pour les pâtes lactiques fraîches, il n'a pas été possible de déterminer des facteurs individuels pouvant affecter la croissance ou l'absence de croissance de *L. monocytogenes* au sein des fromages à pâte mi-dure. Il est possible que son comportement soit plutôt affecté par l'interaction complexe entre différents facteurs intrinsèques à chaque variété de fromage et permettant ainsi de fournir une barrière suffisante à la croissance du pathogène. Cette thèse a contribué à la connaissance globale relative aux fromages artisanaux belges, en lien avec *L. monocytogenes*, mais pas mal de pistes peuvent encore être explorées durant les prochaines années, concernant la recherche fondamentale mais aussi le développement de lignes directrices et normes universelles plus appropriées.

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List of acronyms

ALOA: Agar *Listeria* according to Ottaviani and Agosti

AMOVA: analysis of molecular variance

ANSES: Agence nationale de Sécurité sanitaire de l'Alimentation, de l'Environnement et du Travail (France)

APAQ-W: Agence wallonne pour la Promotion d'une Agriculture de Qualité (Wallonie)

ATP : adenosine triphosphate

a_w: water activity

BLAST: Basic Local Alignment Search Tool

BLASTP: Protein BLAST

BHI: brain-heard infusion

BPW: buffered peptone water

CAC: Codex Alimentarius Commission

CBL: Confédération belge de l'Industrie laitière

CDS: coding sequence(s)

cfu/g: colony-forming unit per gram

Ct: cycle treshold

DG SANCO: Directorate-General of Health and Consumers

DNA: deoxyribonucleic acid

ddNTP: dideoxyribonucleotide triphosphates

dNTP: deoxyribonucleotides triphosphates

EC: European Commission

ECDC: European Center for Diseaese Prevention and Control

EFSA: European Food Safety Authority

EC: European Commission

EU: European Union

FAO: Food and Agriculture Organization

FASFC: Federal Agency for the Safety of the Food Chain

FDR: false discovery rate

FNRS: Fonds national pour la Recherche scientifique

FRIA: Fonds pour la Formation à la Recherche dans l'Industrie et dans l'Agriculture

GC content: guanin and cytosin content

GLM: generalized linear model

GSHC: *Gouda*-type semi-hard cheese(s)

HOMOVA: homogeneity of molecular variance

ILVO: Vlaams Instituut voor Landbouw- en Visserijonderzoek

ISO: International Organization for Standardization

KEGG: Kyoto Encyclopedia of Genes and Genomes

HACCP: hazard analysis and critical control points

LAB: lactic acid bacteria

MEGA: Molecular Evolutionary Genetics Analysis

MFFB: moisture on a fat-free basis

MPa: megapascal

MRS: De Man, Rogosa and Sharpe

MRSC: mold-ripened soft cheese(s)

NCBI: National Center for Biotechnology Information

NGS: next generation sequencing

NMDS: non-metric multidimensional scaling

NRC: National Reference Center

NSLAB: non-starter lactic acid bacteria

NVWA: Nederlandse Voedsel- en Warenautoriteit

PATRIC: Pathosystems Resource Integration Center

PCA: plate count agar

PCR: polymerase chain reaction

PDO: Protected Designation of origin

PGfam: cross-genus protein families

qPCR: quantitative polymerase chain reaction

PDO: Protected Designation of Origin

RAST: Rapid Annotation using Subsystem Technology

rRNA: ribosomic ribonucleic acid

RSC: ripened soft cheese(s)

RTE foods: ready-to-eat foods

SHC: semi-hard cheese(s)

SH/HC: semi-hard and hard cheese(s)

SPSHC: *Saint-Paulin* semi-hard cheese(s)

SRSC: smear-ripened soft cheese(s)

STEC: *Shiga*-toxin-producing *Escherichia coli*

TBX: tryptone bile X-glucuronide

UACC: unripened acid-curd cheese

USDA-FSIS: United States Department of Agriculture – Food Safety and Inspection Service

VBNC: viable but non-culturable

YGC: yeast extract glucose chloramphenicol

δ: growth potential

δ_{th}: theoretical growth potential

Chapter 1

Introduction

Global context and definitions

In the Middle East and Central Europe, 8,000 years ago, people started to produce cheese in order to extend milk conservation (Salque *et al*., 2012; Gillis and Ayerbe, 2018; Gobbetti *et al.*, 2018). In 2017, world cheese production was more than 20,900,000 tons (Irlinger and Spinnler, 2020). More than 1,200 cheese varieties could be found worldwide (Barthelemy and Sperat-Czar, 2001). All of these fulfil the definition provided by Codex STAN 283-1978:

"Cheese is the ripened or unripened soft, semi-hard, hard, or extra-hard product, which may be coated, and in which the whey protein/casein ratio does not exceed that of milk, obtained by:

- *(a) coagulating wholly or partly the protein of milk, skimmed milk, partly skimmed milk, cream, whey cream or buttermilk, or any combination of these materials, through the action of rennet or other suitable coagulating agents, and by partially draining the whey resulting from the coagulation, while respecting the principle that cheese-making results in a concentration of milk protein (in particular, the casein portion), and that consequently, the protein content of the cheese will be distinctly higher than the protein level of the blend of the above milk materials from which the cheese was made; and/or*
- *(b) processing techniques involving coagulation of the protein of milk and/or products obtained from milk which give an end-product with similar physical, chemical and organoleptic characteristics as the product defined under (a)."*

In Belgium, around 109,000 tons of cheese were produced in 2018. The same year, Belgian people ate 14.4 kg of cheese per capita, while European Union (EU) average consumption is 17.0 kg/capita (Confédération belge de l'Industrie laitière (CBL), 2019).

Cheese is a particularly interesting matrix, as a lot of factors can influence its final characteristics, including milk animal origin, milk heat treatment, milk skimming, curdling method, lactic starters used, lactose removal, pressing or not and ripening duration. All these factors have an impact on cheese texture, aromas and flavors.

Cheese spoilage, listeriosis and food safety criteria

Milk and production environment can have a negative influence on cheese, resulting in a threat for food safety. Various pathogenic bacteria are susceptible to be carried by cheese, including *Salmonella* spp., *Staphylococcus aureus*, *Escherichia coli* O157:H7 and *Listeria monocytogenes*. The latter one is responsible for listeriosis, a foodborne disease for which an increasing trend was observed in EU during the last decade. As an example, EU member states reported 2,549 listeriosis cases in 2018. The same year, listeriosis was the fifth most prevalent foodborne disease, after campylobacteriosis (246,571 cases), salmonellosis (91,857), *Shiga*-toxin producing *E. coli* (STEC) infections (8,161) and yersiniosis (6,699). Case fatality was 15.6% for patients affected by listeriosis (status known for 57.6% of the cases; **Figure** 1-1; European Food Safety Authority- European Center for Diseaese Prevention and Control (EFSA-ECDC), 2019b). Only considering people at risk,

especially neonates, pregnant women and old or immunocompromised people, case fatality can reach 30%. In Belgium, National Reference Center (NRC) for *Listeria monocytogenes* reported 73 and 74 listeriosis cases in 2017 and 2018, respectively (Sciensano, 2019).
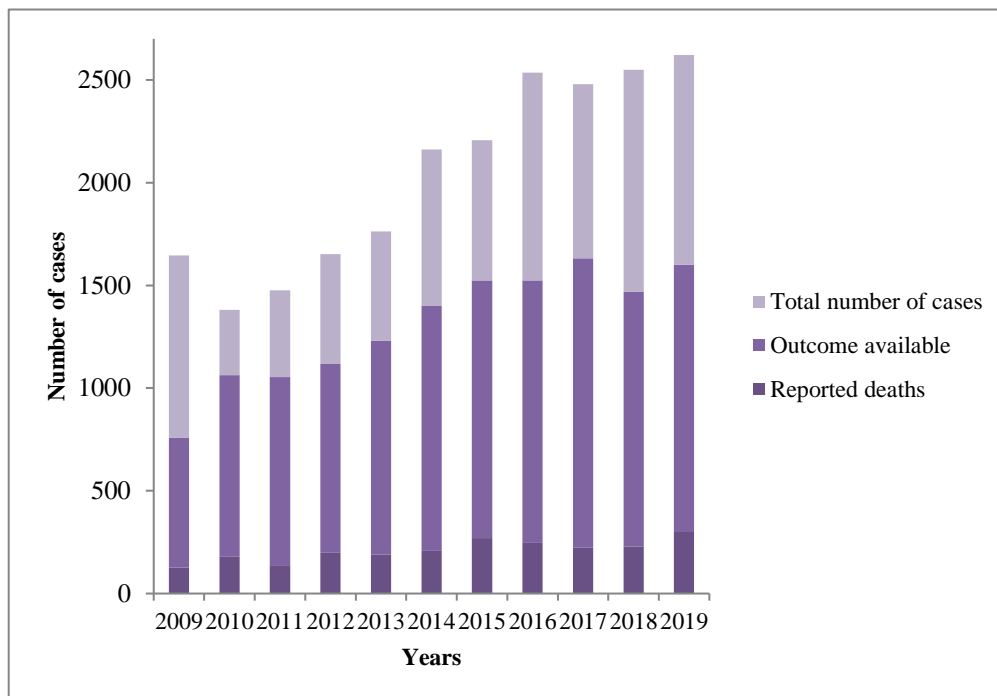
Figure 1-1. Evolution of the number of listeriosis cases per year during the last decade. Proportion of cases for which the disease outcome was known, proportion of deaths are also displayed (data gathered from EFSA-ECDC reports published since 2009).

L. monocytogenes is a ubiquitous Gram-positive bacillus. Its main reservoirs are soil, silage and ground or surface water (Freitag *et al*., 2009). The bacterium is psychrotrophic and able to grow at temperature below the freezing point, *i.e.* -2 °C (Agence nationale de Sécurité sanitaire de l'Alimentation, de l'Environnement et du Travail (ANSES), 2011). Therefore, various foods have been identified as potential vectors of *L. monocytogenes*, including dairy products, meat, delicatessen, smoked salmon, cantaloupe, salads, fruits, celery and ice cream (McCollum *et al.*, 2013; Buchanan *et al.*, 2017; EFSA-ECDC, 2019a, Self *et al.*, 2019; Smith *et al.*, 2019). Due to hazards associated with transmission of *L. monocytogenes* by food, safety criteria regarding the presence of the pathogen in RTE foods are strict. These are defined by Regulation (EC) No 2073/2005 (European Commision (EC), 2005). Annex I of this Regulation is divided into three chapters:
-    Chapter 1: Food safety criteria;
-    Chapter 2: Process hygiene criteria;
-    Chapter 3: Rules for sampling and preparation of test samples.

Food safety criteria regarding the presence of *L. monocytogenes* in ready-to-eat (RTE) foods are summarized in Annex 1 Chapter 1 (**Table** 1-1). Three food categories are considered, namely RTE foods intended for neonates and for medical purposes (category 1.1), RTE foods able to support the growth of *L. monocytogenes* (category 1.2) and RTE foods unable to support the growth of the pathogen (category 1.3). *L. monoctogenes* should not be detected during shelf-life of RTE foods belonging to category 1.1. A level of 100 cfu/g is tolerated during shelf-life for categories 1.2 and 1.3. However, an extra criterion is required before RTE foods from category 1.2 are placed on the market: *L. monocytogenes* cannot be detected in 25 g of food. Regulation (CE) No 2073/2005 also considers that RTE foods are unable to support the growth of *L. monocytogenes* when:
- pH $\leq$ 4.4;
- $a_w \leq$ 0.92;
- pH $\leq$ 5.0 and $a_w \leq$ 0.94;
- Shelf-life $\leq$ 4 days.

In the United Sates, a zero-tolerance is applied before food is placed on the market as well as during shelf-life (Lakicevic and Lastasijevic, 2017).

Table 1-1. Food safety criteria regarding the presence of *L. monocytogenes* in RTE foods (adapted from Chapter 1 of Annex I of Regulation (CE) No 2073/2005).

Food category	Criterion	Stage of application
1.1 RTE foods intended for neonates and for special medical purposes	No detection in 25 g	Whole shelf-life
1.2 RTE foods able to support the growth of *L. monocytogenes*	100 cfu/g	Whole shelf-life
	No detection in 25 g	Before product is placed on the market
1.3 RTE foods unable to support the growth of *L. monocytogenes*	100 cfu/g	Whole shelf-life

When *L. monocytogenes* is detected in RTE food from category 1.2 before it is placed on the market, producer cannot sell the product anymore until he is able to manufacture three consecutive batches in which the pathogen is not detected. In addition to food safety hazard, *L. monocytogenes* is also a threat for RTE foods producers for which economic and moral consequences can be huge.

Most dairy products, including cheeses, have to be considered as part of category 1.2. For the period 2010-2017, 6.3% of listeriosis outbreaks were associated with contaminated cheese consumption (EFSA-ECDC, 2019b). However, as already explained, multiple types of cheese can be found on the market. It is known that in addition to pH and a_w, other factors can influence the fate of *L. monocytogenes* during cheese manufacture and storage, including cheese resident microbiota or undissociated lactic acid concentration (Wemmenhove *et al*., 2018). In depth

investigations on the growth of *L. monocytogenes* in various types of cheese are thus interesting.

The debate on the use of raw milk for cheese manufacture is a hot topic in Belgium, since an artisanal producer of raw milk *Herve* cheese decided to stop its activities because *L. monocytogenes* was detected in his products (Bodeux, 2015). In Belgium, an increasing number of cheese factories is observed, at smaller or larger scale. Cheese production landscape is complex in this country, with different trends and habits in Flanders or Wallonia. Furthermore, Belgian cheese producers are not grouped under common specifications, as it is for instance the case in France, for major protected designation of origin (PDO) cheeses, like *Comté*, *Reblochon* or *Camembert*. *Herve* is for instance the only Belgian PDO cheese (Gillis and Ayerbe, 2018). Finally, Belgian cheeses are not well described by the available scientific literature.

Given the circumstances and the gaps in the knowledge of Belgian cheeses, it was necessary to perform a large-scale study in order to collect data on these products, to assess the risk associated with the presence of *L. monocytogenes*, and to understand factors determining its growth/no growth.

Cheese manufacture and classification

The goal of this section is to provide an overview of cheese manufacture, presenting main steps required to obtain cheese, and potential variations allowing the obtention of specific cheese types. The distinction between unripened acid-curd cheeses (UACC) and ripened cheeses is also detailed. After that, various approaches allowing cheese classification are described, as well as major types, illustrated with examples.

1. General outline for cheese manufacture

Figure 1-2. General outline for manufacture of UACC. Red box corresponds to a facultative step.

and **Figure** 1-3 provide a caption of the main steps required during UACC or ripened cheese manufacture at an artisanal scale, respectively. Not all steps listed hereafter are compulsory for all varieties. Temperature and duration of each step also vary depending on the targeted type of cheese.
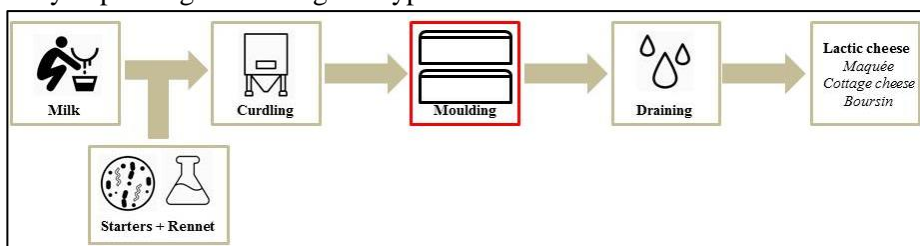
Figure 1-2. General outline for manufacture of UACC. Red box corresponds to a facultative step.
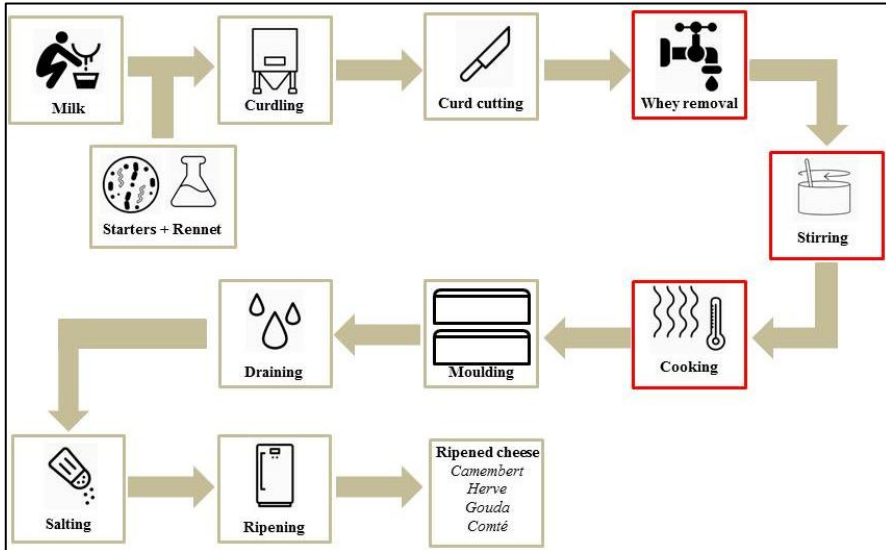
Figure 1-3. General outline for manufacture of ripened cheeses. Red boxes correspond to facultative steps.

Optimal milk temperature to start cheese production is at least 20 °C (approximately room temperature), depending on cheese varieties. Thermization or pasteurization can be applied to eliminate pathogenic bacteria, but cheeses made from pasteurized milk have fewer flavors than raw milk cheeses (Goudédranche *et al.*, 2011a; Gobbetti *et al.*, 2018). Skimmed milk can also be used to produce low-fat cheeses. The following points describe the general production process of UACC and ripened cheeses, using four major references, namely Goudédranche *et al.* (2001a), Fox *et al.* (2017), Gillis and Ayerbe (2018) and Gobbetti *et al.* (2018).

1. Unripened acid-curd cheeses

a) Lactic curdling

The step during which milk becomes a solid tridimensional protein gel is called curdling. During UACC manufacture, curdling majoritary occurs under the action of lactic acid bacteria (LAB), transforming lactose into lactic acid, resulting in a decreasing pH (pH < 5 at the end of curdling). Acidification results in a solubilization of colloidal phosphate and calcium, and in a modification of electrostatic charges at the surface of caseine micelles, through neutralization of acid functions and protonation of amine functions. Milk electrostatic equilibriums are thus modified and casein micelles aggregate. Lactic curdling is slow (16 to 24 h at room temperature). Ancestrally, acidification was performed by resident raw milk microbiota, but the process was difficult to standardize and to predict. Nowadays, microbiota is controlled by the addition of mesophilic and/or thermophilic bacterial starter cultures, including *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus*

delbrueckii subsp. *lactis*, *Lactobacillus helveticus*, *Lactococcus lactis* or *Streptococcus thermophilus*.

b) Draining

After curdling, curd is drained in cheesecloths or in shapes, during 6 to 24 h. UACC are then ready to be packaged and sold. Salting can sometimes be included.

2. Ripened cheeses

a) Curdling

Ripened cheeses can be obtained from lactic or enzymatic curds, or more often from a combination of both techniques. Most ripened cheese varieties are produced with a predominance of rennet coagulation. In this case, rate of acidification is less important than during UACC manufacture, curd pH often remaining > 6. Rennet was originally collected from the abomasum of calves, lambs or goats (Garcia-Gomez et al., 2020). It is mainly composed of two proteases: chymosin and pepsin. κ-casein, the key element stabilizing the emulsion of casein micelles, is cleaved by chymosine. Nowadays, vegetal, fungal and bacterial alternatives to rennet are available on the market (Jaros and Rohm, 2017).

Type and concentration of coagulating agent have an influence on the final organoleptic properties of cheeses (Jaros and Rohm, 2017; Garcia-Gomez *et al*., 2020). Recommended dose of rennet is at least 15 mL for 100 L of milk, but concentration can reach 40 mL/100 L for some hard cheeses.

b) Curd cutting

When expected texture is reached, curd is cut, increasing exchange surface between curd and whey. A smaller grain size favorizes syneresis, *i.e.* expulsion of whey. The size of curd grains depends on cheese type. Coarse cutting is preferred to produce soft cheese, while semi-hard and hard cheeses expect a rice-grain size.

c) Whey removal

During the production of some ripened cheeses, up to 50% of whey is removed after curdling and replaced by a given amount of hot water. Main purposes are: (a) decreasing lactose content, allowing the control of pH during draining, pressing and ripening and (b) favorizing syneresis with the increase in curd temperature.

d) Stirring and cooking

Stirring is applied in order to increase syneresis and prevent grains agglomeration. Cooking up to 55°C favorizes contraction of curd proteic network. At the end of the process, resulting cooked cheese, including *Emmental* and *Gruyère*, are harder.

e) Shaping

Each cheese variety requires specific shapes, especially PDO cheeses, for which size and dimensions are well defined. The method to fill shapes also depends on cheese variety.

f) Draining/pressing and demolding

Shaped curd is only drained by gravity during soft cheese production. Pressing allows the extraction of more whey during semi-hard/hard cheeses (SH/HC) manufacture. During draining and/or pressing, LAB continue to produce acid, and pH often drops around 5.2. When enough whey has been evacuated, shapes are removed.

g) Salting

Salting is generally performed after shapes removal and occurs using several methods: (a) spreading of dry salt on cheese surfaces, (b) brining and (c) direct addition of salt in vat after curdling. According to the literature, cheese NaCl content is often lower than 2%. Salt has various functions in cheese, including a preservative role (decrease in a_w) and a contribution to flavor.

h) Ripening

Ripening is a critical step in cheese manufacture, allowing the development of cheese typicity, with the production of new aromas and flavors. Texture is also modified during ripening. These changes are associated to metabolic activity of ripening microbiota. This consortium is a complex assemblage of bacteria, yeasts and molds, which act on all cheese major constituents, *i.e.* lactose, triacylglycerols and proteins. Individuals forming this consortium come from serveral origins: (a) milk resident microbiota, (b) starters and (c) production environment. Non-starter lactic acid bacteria (NSLAB) originating from milk are numerous, including *Lactobacillus*, *Micrococcus, Pediococcus* and *Streptococcus*. Bacteria (*Leuconostoc pseudomesenteroides*, *Propionibacterium* spp., *Brevibacterium linens*,…), yeasts (*Geotrichum candidium*) and molds (*Penicillium camemberti*, *Penicillium roqueforti*) are sometimes required as ripening starters for specific cheese varieties. Microorganisms from the environment colonize cheese surfaces during draining, pressing, salting, brining and ripening.

Lactose fermentation produces lactic acid and secondary metabolites, including carbon dioxide and short chain fatty acids. Triacylglycerols can be clived and metabolized into plenty of organic compounds, including thioesters and secondary alcohols. However, lipolysis is generally limited in cheese. Proteins are clived into peptides and amino acids, which can be metabolized into various molecules contributing to cheese aroma and flavor.

During ripening, temperature, air flow and relative humidity of the ripening room have an influence on cheese final characteristics. Ripening duration depends on cheese type and can vary between two weeks and several years. A longer ripening period results in harder cheeses.

2. Cheese classification

Several factors can be considered to classify cheeses. Texture-based classification is the most common. Codex STAN 283-1978 defines rules for cheese labelling based on moisture on fat-free basis (MFFB) percentage (**Table** 1-2). This classification allows the distinction between soft, semi-hard, hard and extra-hard cheeses (Codex Alimentarius, 1978). However, suggested MFFB classes overlap, and cheeses manufactured using different processes can be pooled in a same MFFB class.

Table 1-2. Texture-based classification suggested by Codex STAN 283-1978.

% MFFB	Labelling
< 51	Extra hard
49-56	Hard
54-69	Semi-hard
> 67	Soft

Process-associated parameters should be considered for a more precise classification, including coagulation method (lactic or enzymatic curdling), cooking temperature, pressing or not, ripening or not and presence of a natural crust. However, a unanimously accepted classification has not been developed yet. Classification suggested by the magazine Profession Fromager is interesting, distinguishing lactic and enzymatic curds (**Erreur ! Source du renvoi introuvable.** and **Erreur ! Source du renvoi introuvable.**; Profession fromager, 2020).
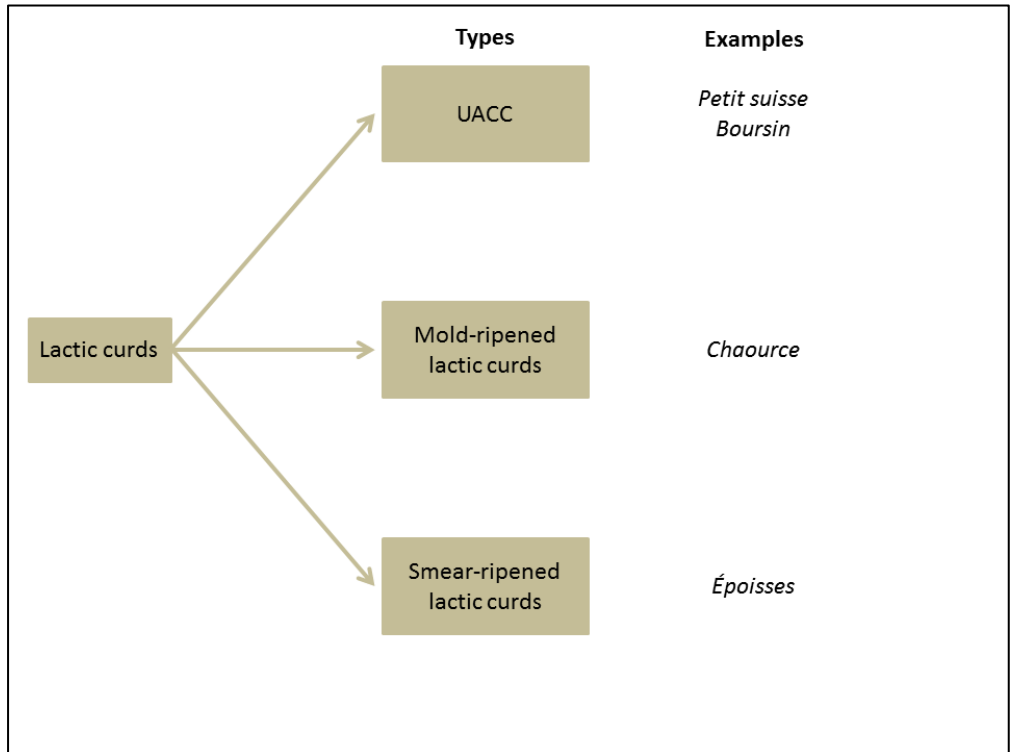
Figure 1-4. Classification of lactic curds (adapted from Profession Fromager, 2020).

Most lactic curds are not ripened and are called UACC in this thesis. UACC can be drained in cheesecloth or in shapes and have a high MFFB (80-90%). Respective examples are *Petit Suisse* and *Boursin*. Some ripened lactic cheeses can be found on the market, including *Chaource* and *Époisses*, two French PDO cheeses. *Chaource* is a mold-ripened soft cheese (MRSC), meaning that it has a typical bloomy crust composed of the white mould *Penicillium camemberti*, while *Époisses* is a smear-ripened soft cheese (SRSC) washed one to three times per week with *Marc de Bourgogne*, resulting in a yellow to red crust (Ministère de l'économie, de l'industrie et de l'emploi and Ministère de l'alimentation, de l'agriculture et de la pêche, 2010; Chaource, 2013).
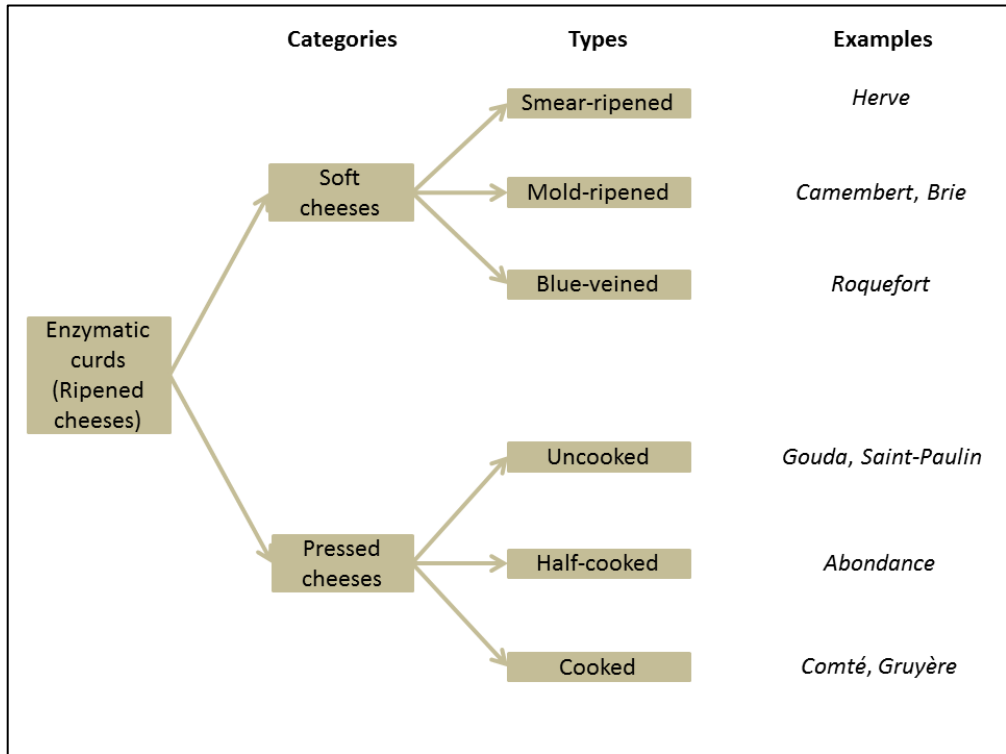
Figure 1-5. Classification of enzymatic curds (adapted from Profession Fromager, 2020).

Enzymatic curds are obtained by adding a significant amount of rennet to milk. Lactic starters are often added simultaneously. Due to enzymatic activity of rennet, curdling generally occurs in less than 1 h. The distinction between categories is based on the way of draining. For soft cheeses (or unpressed cheeses), draining is performed by gravity, while pressing allows an extended draining, resulting in semi-hard and hard cheeses.

Soft cheeses can be splitted into three main types, namely SRSC, MRSC and blue-veined cheeses. For all soft cheeses, curd is cut, but stirring is not compulsory. SRSC, including *Herve*, *Maroilles* and *Munster*, have a typical yellow to red rind. These cheeses are regularly washed during ripening, using water, brine, smear, wine or beer. These repeated washings allow the development of a complex surface microbiota, composed of bacteria, yeasts and moulds. *B. linens* is known to contribute to this red color. Dominant yeasts species are *Kluyveromyces lactis*, *Kluyveromyces marxianus* and *Debaromyces hansenii* (Irlinger and Spinnler, 2020). This microbiota is responsible for the development of specific aromas and flavors. In comparison, MRSC have a white rind composed of *G. candidum* and/or *P. camemberti*. Famous examples are *Brie de Meaux* and *Camembert*. Ripening period is shorter for MRSC (< 2 weeks) than for SRSC (3 to 6 weeks) (Goudédranche *et al.*, 2001b). Blue-veined cheeses have to be considered as soft

cheeses, as they are not pressed. Blue-veined cheeses are really diversified, but their production process comprises some common key steps. Curd grains have to be dehydrated on their surface during a procedure called "*coiffage*", consisting in several cycles of stirring and resting. The blue-green mould *P. roqueforti* has to be added to milk and develops in cheese cavities. This mold is strictly aerobic, requiring cheese piercing to create air channels. Examples of blue-veined cheeses are *Bleu d'Auvergne*, *Roquefort* and *Gorgonzola*.

Pressed cheeses involve curd cutting, stirring and pressing. A first criterion to distinguish pressed cheese is the maximal temperature reached during stirring. To produce uncooked pressed cheese, the maximal temperature must always remain under 40°C (Goudédranche *et al*., 2002). Considering pressing intensity and ripening duration, soft (*Reblochon*), semi-soft (*Saint-Paulin*, *Saint-Nectaire*, *Tome de Savoie*) or hard (*Gouda*, *Raclette*, *Trappiste*) uncooked pressed cheeses can be distinguished. *Cantal* and *Cheddar*, for which curd is milled and directly salted, belong to another family of uncooked pressed cheese. To produce half-cooked pressed cheeses, cheesemakers heat the curd up to 50°C. *Abondance*, *Leerdammer* and *Pecorino* are famous examples. Finally, curd is heated at temperatures higher than 50°C during cooked pressed cheeses manufacture, including *Emmental* and *Parmesan* (Profession Fromager, 2020). Various types of crusts can be observed, as well as artificial coatings.

Aside from these major cheese families, some specific products are considered as cheeses, according to the definition stated in Chapter 1:
-	Buttermilk cheese: heating of buttermilk up to 80-90°C and agglomeration of proteins;
-	*Mascarpone*: heating of cream to 100°C and addition of lemon juice or acid to fasten curdling;
-	Whey cheese, including *Ricotta*: heating of whey to 80-90°C to flocculate and agglomerate proteins;
-	Soft cheeses without crust and stored in brine (*Feta*);
-	*Pasta filata* cheeses, including *Mozzarella* and *Burrata*: dipping of cut curd into hot water (70-90°C), stretching and kneading, leading to a rubbery aspect (Kebchaoui, 2012).

State of the art on prevalence and survival of *L. monocytogenes* in cheese in 2018

Before starting new research on this topic, a literature review was performed, gathering available papers on the prevalence of *L. monocytogenes* in various types of cheeses from the whole world. Similarly, studies on the growth of the pathogen during cheese manufacture, ripening or storage were consulted and summarized in a review paper, published in *International Journal of Dairy Technology*. Provided that the review was written in 2018, an update of the scientific knowledge on this topic is also proposed in the present thesis.

The first part of this section is adapted from the following published review article:

Gérard A., El-Hajjaji S, Niyonzima E., … and Sindic M. (2018). Prevalence and survival of *Listeria monocytogenes* in various types of cheese – A review. *Int. J. Dairy Technol.*, 71(4), 825-843.

Amaury Gérard started to work on this paper directly after hiring. He has done the whole literature review on this topic and summarized the data to write the following review paper. He also contributed to the submission of the manuscript, and to the rewriting of the paper before publication in *International Journal of Dairy Technology*.

1. Abstract

Since the publication of Regulation (EC) No 2073/2005, RTE foods allowing the development of *L. monocytogenes*, including cheese, should be free of this pathogen in 25 g of product. This review was carried out to gather studies on the prevalence of the pathogen in various types of cheese in Europe, while also including data from other continents. Given that Regulation (EC) No 2073/2005 distinguishes cheeses allowing or not the survival of *L. monocytogenes* based on food pH and a_w, the review also focuses on the determinants of this growth/no growth in the same types of cheese.

Keywords: *Listeria monocytogenes*, Cheese, Product safety.

2. Introduction

Although listeriosis is not one of the most commonly occurring foodborne diseases, the increasing number of reported cases has led to a growing interest from scientists and authorities (Cabedo *et al*., 2008). Listeriosis, caused by the pathogenic bacterium *L. monocytogenes*, is generally a benign disease for immunocompetent people. Nevertheless, it can be deleterious for some of the population, including neonates, elderly people, pregnant women and immunocompromised patients, as well as people suffering from diabetes or liver and renal diseases (Doorduyn *et al*., 2006; Buchanan *et al*., 2017). Individuals aged over 65 years represent the majority of EU reported cases (EFSA-ECDC, 2016). For this age group, occurrence of listeriosis is two times higher for males than for females (Takkinen, 2017). Deaths linked with listeriosis occur in around 20.0-30.0% of cases for patients from vulnerable groups (Sanaa *et al*., 2004). In 2015, 2,206 cases of listeriosis were registered in EU, causing 270 deaths. Long-term data highlight an increase in reported cases during the last decade (EFSA-ECDC, 2016).

Almost all human listeriosis cases (99%) are attributable to food consumption (Takkinen, 2017). Various types of food that caused listeriosis outbreaks have clearly been identified, including cheese.

L. monocytogenes represents a noticeable threat in food because of its ability to survive under an impressive diversity of conditions. On the one hand, the bacterium is known to be psychrotrophic, *i.e.* able to grow below 7°C. Some strains of *L. monocytogenes* can survive at temperatures a few degrees under freezing point, but without proliferation (Carpentier and Cerf, 2011). On the other hand, the pathogen is also able to multiply at temperatures up to 45°C, with optimal growth between 30 and 37°C (Saltijeral *et al.*, 1999). *L. monocytogenes* also tolerates a wide pH range. For instance, Carpentier and Cerf (2011) reported that the bacterium can grow in environments with pH between 4.6 and 9.5. Therefore, with respect to pH, many foods are susceptible to the growth of *L. monocytogenes*. Tolerance of *L. monocytogenes* to pH is also linked with a_w. It is commonly reported that the bacterium is not capable of growth when $a_w < 0.92$ (Nolan *et al.*, 1992).

In addition, *L. monocytogenes* is halotolerant, able to grow in salt concentrations up to 10% (Ferreira *et al.*, 2014). Bacteria of the genus *Listeria* are facultative anaerobes, being able to grow in low levels of oxygen and high carbon dioxide conditions (Gandhi and Chikindas, 2007; Lungu *et al.*, 2009). Obviously, tolerance to salt, temperature, low oxygen concentrations, pH and a_w varies among the strains (Gandhi and Chikindas, 2007).

As detailed in the part 'Context' of this chapter, EC has established criteria to define the acceptability of a RTE food. As a reminder, the latter are based on available data on the presence/absence or enumeration of *L. monocytogenes* throughout the food supply chain for a given type of food. Regulation (EC) No 2073/2005 considers that the bacterium cannot grow in food when $pH \leq 4.4$ or $a_w \leq 0.92$. Moreover, a combination of $pH \leq 5.0$ and $a_w \leq 0.94$ is also inhibitory. If these criteria are not met, food is considered susceptible to the multiplication of *L. monocytogenes*. In this case, EC demands a total absence of *L. monocytogenes* in 25 g when food leaves producer's control. An alternative criterion can be applied when the producer can demonstrate that during the whole shelf life, contamination is lower than a threshold value of 100 cfu/g of product (EC, 2005).

As a RTE food, cheese must comply with Regulation (EC) No 2073/2005. This review focuses on the occurrence of the pathogen in various types of cheese worldwide since the publication of this regulation. The paper tries to put this prevalence in relation to physico-chemical conditions (pH and a_w) met in these cheeses and with survival of the pathogen during process, ripening and storage. Papers on the occurrence of *L. monocytogenes* published within the period 2005–2018 were gathered using Google Scholar, and with English and French keywords.

3. *Occurrence of* L. monocytogenes *in cheese*

A diversity of cheeses is now available on the market (Little *et al.*, 2008). Therefore, classification of these products is extremely difficult. No consensus has been established yet, and authors are inclined to use different vocabulary and criteria to describe cheeses, including maturation characteristics or moisture content (Martinez-Rios and Dalgaard, 2018). Several parameters must be taken into account to define a cheese, including milk origin (bovine, caprine, ovine, etc.), milk

treatment (raw, thermized, pasteurized or microfiltered), milk homogenization, the use of a microbial starter and/or rennet for curdling, cooking of the curd, moulding, pressing, method for salting, addition of spices or other specific ingredients and conditions of ripening (relative moisture, temperature, time, maturing medium, rind washing, etc.). All these factors have an impact on cheese final properties. According to Codex Alimentarius, a texture-based classification should be established following the percentage of MFFB. A decrease in MFFB results in a distinction between soft, semi-soft, semi-hard and hard cheeses (Codex Alimentarius Commission (CAC), 2013). This review will consider three main categories, namely fresh cheeses, which should be classified apart from other soft cheeses due to important manufacturing differences, soft and semi-soft cheeses, and semi-hard and hard cheeses.

Two types of analyses are generally performed to investigate the occurrence of *L. monocytogenes*: presence/absence in 25 g of product (qualitative data) and enumeration (quantitative data).

1. Fresh cheeses

Following the definition of Martinez-Rios and Dalgaard (2018), fresh cheeses are "curd-style cheeses which do not undergo any ripening". Manufacture generally involves lactic curdling and only a small concentration of rennet. Fresh cheeses, which can be shaped or not, are popular in Latin America and in the south of the United States (Soto Beltran *et al*., 2015). **Table** 1-3 summarises studies on the presence of *L. monocytogenes* in 25 g of various fresh cheese varities. The prevalence of contaminated samples substantially varies among studies and countries. Many of the published articles deal with Hispanic-style fresh cheese (also called Latin-style fresh cheese), such as *Minas Frescal* in Brazil or *Queso Fresco* in Mexico. The occurrence of contamination of Latin-style fresh cheese ranges from 0.0 to 37.5% (Kinde *et al*., 2007; Moreno-Enriquez *et al*., 2007; Brito *et al*., 2008; Cabedo *et al*., 2008; Torres-Vitela *et al*., 2012; Soto Beltran *et al*., 2015; Reda *et al*., 2016). *L. monocytogenes* can reach levels higher than 10^4 cfu/g in *Minas Frescal* (Brito *et al*. 2008). In Europe, the bacterium has also been isolated from Italian fresh cheeses (Rantsiou *et al*., 2008; Parisi *et al*., 2013). In Austrian fresh cheeses collected from retail stores, a percentage of contamination comparable to Latin-style fresh cheese has been observed (Wagner *et al*., 2007). Similar findings have also been reported for white cheese from Turkish bazaars (Arslan and Özdemir, 2008).

The use of raw milk is often cited as a major factor for the contamination of dairy products with *L. monocytogenes*. According to Federal Agency for the Safety of the Food Chain (FASFC) (2011), the bacterium was present in 2.2–10.2% of raw milk samples in EU. However, milk heat treatment was sometimes insufficient to guarantee the absence of *L. monocytogenes* in cheese. Indeed, at least one study reported that fresh cheeses made from pasteurized milk carried the pathogen (Rosas-Barbosa *et al*., 2014). Parisi *et al*. (2013) found that all 20 raw milk samples tested were free of the pathogen, but cheeses processed with milk from the same dairies were contaminated. This can be attributed to postprocessing contamination, which

represents the major cause of cheese spoilage with *L. monocytogenes* (Schvartzman *et al*., 2011; Ibarra-Sanchez *et al*., 2017). In factories, the pathogen has been isolated from floors, drains, conveyor belts, crates, brine and workers' equipment (Larson et al. 1999; Gudbjörnsdóttir *et al*., 2004; Pintado *et al*., 2005; Fox *et al*., 2011; Osaili *et al*., 2012; Parisi *et al*., 2013; Ferreira *et al*., 2014; Rosas-Barbosa *et al*., 2014; Ibarra-Sanchez *et al*., 2017). As highlighted in **Table** 1-3, *L. monocytogenes* can be isolated from cheeses taken at various points of distribution.

Handcrafted fresh cheeses were more frequently contaminated than cheeses from larger factories (Ibarra-Sanchez *et al*., 2017). Globally, an improved hygiene quality can be observed in relation with the level of industrialization.

Generally, samples with contamination higher than 100 cfu/g are scarce (Rantsiou *et al*., 2008). From **Table** 1-3, it can be observed that studies enumerating the pathogen are not frequent. It would, however, be highly interesting to focus on the levels of the pathogen to know the potential risk related to the consumption of such contaminated products.

The presence of *L. monocytogenes* in some fresh cheeses is not surprising. Unfortunately, only a few studies have reported a_w and pH of the considered samples. Nevertheless, physico-chemical properties of fresh cheese are generally ideal for the growth of the bacterium, *i.e.* high moisture content ($> 50\%$), average pH > 6 and relatively low salt content (0.85%) (Olarte *et al*., 1999; United Sates Department of Agriculture – Food Safety and Inspection Service (USDA-FSIS), 2003; Brito *et al*., 2008; Ibarra-Sanchez *et al*., 2017). Apart from a Swedish study, all cheeses from **Table** 1-3 with an average pH > 4.4 were found to be contaminated with *L. monocytogenes* (Rosengren *et al*., 2010; Torres-Vitela *et al*., 2012; Soto Beltran *et al*., 2015). Therefore, several large-scale listeriosis outbreaks due to the consumption of fresh cheese have been reported in the literature. Indeed, 12 outbreaks linked with fresh cheese have been identified since 2005, for a total of 139 cases, and causing at least 25 deaths (Martinez-Rios and Dalgaard, 2018). Due to these outbreaks, it is recommended in the United States that pregnant women avoid the consumption of fresh cheese (Torres-Vitela *et al*., 2012). As highlighted by Martinez-Rios and Dalgaard (2018), EFSA should analyze more fresh cheese samples to determine the prevalence of *L. monocytogenes* in European cheeses. Indeed, their panel included only 2% of fresh cheeses.

However, some fresh cheeses are less susceptible to *L. monocytogenes* survival. Indeed, exceptions are reported, such as *Ayib*, a cottage cheese from Ethiopia. *Ayib* is much more acidic than previously discussed Latin-style fresh cheeses, with an average pH of 4.0. A study on *Ayib* reported only 1.0% of contaminated samples (Gebretsadik *et al*., 2011). A *Cottage* cheese from Egypt, with pH around 4.2, was free of *L. monocytogenes*, as well as *Kareesh* cheese, another Egyptian fresh cheese (Ismaiel *et al*., 2014; Reda *et al*., 2016). Further, it can be expected that Walloon *Maquée*, a high moisture UACC from Belgium with low pH, would be less susceptible to *L. monocytogenes* contamination and growth. Studies on these acidic cheeses are rarer because it is expected that their pH prevents survival of the bacterium. Nevertheless, data from **Table** 1-3 demonstrate that a pH < 4.4 can

sometimes be insufficient to prevent survival of the bacterium (El Marnissi *et al*., 2013).

Although they require a heat treatment during processing, *Burrata*, cream cheese, *Ricotta* and *Mozzarella* comply with the definition of fresh cheeses. These products present physico-chemical conditions favorable for the multiplication of *L monocytogenes*. In two studies performed by Di Pinto *et al*. (2010) and Dambrosio *et al*. (2013), respectively, none of 186 *Mozzarella* and of 404 *Burrata* samples were contaminated. During *Burrata* and *Mozzarella* manufacture, curd is dipped in hot water (80–90°C) before thermoplastification (Ibarra-Sanchez *et al*., 2017). This treatment is sufficient to kill pathogens originating from milk, but the subsequent steps present possibilities for exogenous contamination to occur. Cream cheese was more susceptible to listerial contamination; nearly 2.0% of the 108 samples being contaminated (Di Pinto *et al*., 2010). This type of cheese also undergoes a heat treatment after curdling, but at lower temperatures, around 55°C. This seems to be insufficient to kill all *L. monocytogenes* cells. In addition to that, postprocessing contamination is likely. *Requeson*, a whey cheese from Mexico, showed a prevalence of 6.7% (Rosas-Barbosa *et al*., 2014). On the other hand, 30 samples of *Ricotta*, another whey cheese, were free of *L. monocytogenes* (Parisi *et al*., 2013). *Requeson* and *Ricotta* are, however, cooked up to 80–90 °C during processing. Again, postprocessing steps play a major role in contamination of the product with *L. monocytogenes* (Santorum *et al*., 2012).

Table 1-3. Occurrence of *L. monocytogenes* in fresh cheese.

Type of cheese	Country	Sampling	Number of samples	Detection (%)	pH	a_w	Levels (log_{10} cfu/g)	Id. method	Enum. method	Reference
Adobera	Mexico	Cheese factories	16	18.8	N.A.	N.A.	-	C+B	-	Rosas-Barbosa et al., 2014
	Mexico	Distribution centres	100	12.0	5.0-7.3	N.A.	-	C+B +API	-	Torres-Vitela et al., 2012
Burrata	Italy	Dairies	404	0.0	N.A.	N.A.	-	C+B	-	Dambrosio et al., 2013
Cream cheese	Italy	Supermarket	108	1.9	N.A.	N.A.	-	C+B	-	Di Pinto et al., 2010
Cottage cheese	Ethiopia	Retail stores	100	1.0	N.A.	N.A.	-	C+B	-	Gebretsadik et al., 2011
	Egypt	Retail markets, groceries and restaurants	50	0.0	4.2	N.A.	-	C+B	-	Reda et al., 2016
Jben	Morocco	Traditional dairies	96	4.2	4.3	N.A.	-	C+B	-	El Marnissi et al., 2013
Kareesh	Egypt	Supermarkets	30	0.0	N.A.	N.A.	-	C+B	-	Ismaiel et al., 2014
Minas Frescal	Brazil	Retail stores	55	11.0	N.A.	N.A.	2.5-4.3	C+B	PC	Brito et al., 2008

(Continued)

Table 1-3. (Continued)

Type of cheese	Country	Sampling	Number of samples	Detection (%)	pH	aw	Levels (log10 cfu/g)	Id. method	Enum. method	Reference
Mozzarella	Italy	Supermarket	186	0.0	N.A.	N.A.	-	C+B	-	Di Pinto et al., 2010
Undetermined fresh cheese	Italy	Schools' refectories, old poeple's home, restuarants and cafetarias	258	3.5	N.A.	N.A.	-	C+B	-	Pesavento et al., 2010
	United States	American border with Mexico	204	2.0	N.A.	N.A.	-	C+B+API	-	Kinde et al., 2007
	Spain	Retail stores and food industry	78	1.3	N.A.	N.A.	1.0-2.0	C+B	PC	Cabedo et al., 2008
	Italy	Cheese factories	15	6.7	N.A.	N.A.	-	C+B+API	-	Parisi et al., 2013
	Italy	Local producers and markets	31	12.9	N.A.	N.A.	0.0-3.6	PCR	qPCR	Rantsiou et al., 2008
	Sweden	Farm dairies	78	0.0	5.4-5.7	0.96	-	C+B	-	Rosengren et al., 2010
	Austria	Retail stores	25	4.0	N.A.	N.A.	0.0-2.0	C+PCR	PC	Wagner et al., 2007
	Austria	Household level	27	0.0	N.A.	N.A.	-	C+PCR	PC	Wagner et al., 2007

(Continued)

Table 1-3. (Continued)

Type of cheese	Country	Sampling	Number of samples	Detection (%)	pH	a_w	Levels (log_{10} cfu/g)	Id. method	Enum. method	Reference
Panela	Mexico	Processing plants	60	0.0	N.A.	N.A.	-	C+B+PCR	-	Alcázar Montañez et al., 2006
	Mexico	N.A.	16	37.50	N.A.	N.A.	-	C+B	-	Rosas-Barbosa et al., 2014
	Mexico	Distribution centres	100	6.0	4.9-6.5	N.A.	-	C+B+API	-	Torres-Vitela et al., 2012
Queso Fresco	Mexico	Farms, cheese processing plants and retail stores	149	3.4	N.A.	N.A.	-	C+B+PCR	-	Moreno-Enriquez et al., 2007
	Mexico	N.A.	16	6.3	N.A.	N.A.	-	C+B	-	Rosas-Barbosa et al., 2014
	Mexico	Retail stores	75	9.3	5.5±0.3	0.90±0.01	-	C+B+PFGE	-	Soto Beltran et al., 2015
Requeson	Mexico	N.A.	15	6.7	N.A.	N.A.	-	C+B	-	Rosas-Barbosa et al., 2014
Ricotta	Italy	Dairy plants	30	0.0	N.A.	N.A.	-	C+B	-	Parisi et al., 2013
White cheese	Turkey	Bazaars	142	9.2	N.A.	N.A.	-	C+B	-	Arslan and Özdemir, 2008

Legend: C, microbial culture; B, biochemical tests; API, analytical profile index; PFGE, pulsed-field gel electrophoresis; PC, plate count; N.A., unavailable information; -, no enumeration; pH range is given when available, otherwise average value is given (with SD when available); a_w is given with SD when available.

2. Soft and semi-soft cheeses

Ripened soft cheeses are manufactured without pressing, with a relatively short ripening time, and have a creamy texture. In contrast to fresh cheese, ripened soft cheese can be manufactured from enzymatic or lactic curd. Ripened soft cheeses can be divided into two main categories. On the one hand, MRSC have a typical white rind, composed of *P. camemberti* and/or *G. candidum*. *Camembert* and *Brie* are well known MRSC. On the other hand, SRSC, also called washed rind soft cheeses or bacterium-ripened soft cheeses, generally present red rinds. During ripening, they are brushed or washed with salted water containing or not specific starters. Rind is generally composed of coryneform bacteria, now classified as Actinobacteria (Rea *et al.*, 2007). Pressing is part of the production process of semi-soft cheese, but due to a limited ripening time, it remains creamy and foldable. A wide variety of semi-soft cheeses can be found in European countries, including *Saint-Paulin* and *Reblochon*. Blue-veined cheeses, containing *P. roqueforti* in their core, were considered as soft or semi-soft cheeses in this review.

The diversity of soft and semi-soft products and processes is much greater than for fresh cheeses. In a study conducted in Belgium, soft and semi-soft cheeses had pH from 4.16 to 7.47, and a_w from 0.93 to 0.99 (Lahou and Uyttendaele, 2017). However, many soft and semi-soft cheeses present physico-chemical conditions that are favourable for the survival and growth of *L. monocytogenes*.

Table 1-4 gathers studies published since 2005 on the occurrence of the bacterium in soft and semi-soft cheeses. Presence was always determined in 25 g of cheese. Several studies have revealed that soft cheeses, mainly MRSC and SRSC, are the most problematic in terms of contamination with *L. monocytogenes* (Choi *et al.*, 2016; EFSA-ECDC, 2016; Lahou and Uyttendaele, 2017). SRSC is more likely to be contaminated with the pathogen, due to the high amount of postprocessing handling, including rind washing and cheese turning (Izquierdo *et al.*, 2009). In Germany in 2000, 20 tons of SRSC were recalled (Rudolf and Scherer, 2001). In 2015, such a recall also occurred in Belgium with *Herve* cheese, another SRSC (Lahou and Uyttendaele, 2017). Finally, contaminated *Taleggio*, an Italian SRSC, was responsible for an outbreak in Italy in 2011 (Amato *et al.*, 2017).

As for fresh cheeses, it appears that the occurrence of *L. monocytogenes* in soft and semi-soft cheeses is quite variable. Globally, most of the studies reported percentages of incidence between 0.0 and 14.0% (Vitas *et al.*, 2004; Manfreda *et al.*, 2005; Colak *et al.*, 2007; Wagner *et al.*, 2007; Cabedo *et al.*, 2008; Prencipe *et al.*, 2010; Angelidis *et al.*, 2012; Osaili *et al.*, 2012; Rakhmawati *et al.*, 2013; Iannetti *et al.*, 2016; Ahmed *et al.*, 2017; Gelbicova *et al.*, 2017; Lahou and Uyttendaele, 2017). However, some of them reported extremely high rates of contamination among samples. The highest rate of contaminated samples was 46.0% in Portuguese *Castelo Branco* (Pintado *et al.*, 2005). Filiousis *et al.* (2009) focused on soft and semi-soft cheeses obtained from Greek markets and reported that 40.0% of samples were contaminated. Among dairy products, soft and semi-soft cheeses are often the most contaminated (Martinez-Rios and Dalgaard 2018). Unfortunately, physico-chemical data are not available for the

two surveys reporting the highest occurrence. Some studies reporting high prevalence of *L. monocytogenes* are nevertheless biased due to too small number of samples. In these cases, a single contamination has a huge impact on the final prevalence (Filiousis *et al.*, 2009; Rosas-Barbosa *et al.*, 2014).

While some soft cheeses present unfavorable conditions for the survival of *L. monocytogenes*, such as those with a low pH, most of them generally present favorable conditions. For instance, the pH of *Castelo Branco* rind and core was reported to be around 6.0 and 5.4, respectively, after 15 days of ripening (Pintado *et al.*, 2005). No further evolution in pH was observed during ripening and storage. Worse, pH levels may increase on the rind during the ripening of some SRSC (Rudolf and Scherer, 2001). Ripening and storage are thus critical steps. For instance, Manfreda *et al.* (2005) compared the occurrence of *L. monocytogenes* in *gorgonzola* just before packaging and at the end of shelf-life. The number of contaminated samples reaching the limit of detection grew from 2.1 to 4.8%. Regarding the type of milk, an older study from Rudolf and Scherer (2001) found no significant difference in contamination between cheeses made from ovine, bovine or caprine milk.

Although *L. monocytogenes* may not be present in a cheese, other *Listeria* species could be isolated, such as *Listeria innoccua* (Angelidis *et al.*, 2012). The presence of other species of the genus suggests that the conditions could be suitable for the growth of *L. monocytogenes*, and that specific measures should be implemented (Pintado *et al.*, 2005).

It is important to distinguish cheese rinds and cores. Rinds are much less acidic, and thus more favorable for the multiplication of the pathogen. For instance, *Camembert* or *Brie* rinds can have pH > 7 (Prencipe *et al.*, 2010). In blue-veined cheeses from Italy, 55.0% of the 120 samples showed a contamination of their rind, but not in their paste (Bernini *et al.*, 2013). Similar findings have been reported for *Taleggio* (Iannetti *et al.* 2016). Given that postprocessing contamination is the most common transmission route, more attention should be paid to cheese surfaces. *L. monocytogenes* was isolated on the surface of *Prato* cheese, a Brazilian semi-soft cheese, because of contaminated food contact surfaces (Barancelli *et al.*, 2014). Therefore, it is sometimes advised to remove rinds before consumption (Prencipe *et al.*, 2010). In addition, risk of transmission of the pathogen from rind to paste during cutting procedure should be given more consideration (Bernini *et al.*, 2016; Iannetti *et al.*, 2016).

Recent studies in EU were encouraging. Of 3,452 ripened soft cheeses from retail stores all over EU, only 0.5% were contaminated with *L. monocytogenes* (Rakhmawati *et al.*, 2013). Lahou and Uyttendaele (2017) isolated the bacterium from 3.1% of 32 RSC in Belgium, while only 0.4% of 525 samples were contaminated in Sweden (Lambertz *et al.*, 2012). Differences in contamination rates for any given cheese could be explained by the level of modernization of the process. Indeed, in small traditional dairies, automation and sanitary quality of the equipment are limited (Colak *et al.*, 2007). As for fresh cheese, the use of raw milk is not a key factor for the growth of *L. monocytogenes*. In EFSA report on zoonoses for the year 2015, noncompliances associated with cheeses made with pasteurized milk (1.3%) were just

slightly less common than noncompliance associated with cheeses made from raw milk (1.4%) (EFSA-ECDC, 2016). Based on seven EFSA reports covering the period 2005–2015, Martinez-Rios and Dalgaard (2018) found no significant differences of prevalence between raw milk and pasteurized milk soft/semi-soft cheeses.

Table 1-4. Occurrence of *L. monocytogenes* in soft and semi-soft cheeses.

Type of cheese	Country	Sampling	Number of samples	Detection (%)	pH	aw	Levels	Id. method	Enum. method	Reference
Blue-veined cheese	Italy	Cheese factory	120	55.0	6.0-6.8	0.93-0.97	0.0-4.0 $\log_{10}$ cfu/g	C+B	PC	Bernini et al., 2013
Brie	Italy	Diverse sale points	300	1.0	5.4-8.1	0.93-0.99	0.4-110.0 MPN/g	PCR	MPN	Prencipe et al., 2010
Camembert	Italy	Diverse sale points	178	0.0	5.5-7.9	0.96-0.99	-	PCR	-	Prencipe et al., 2010
Castelo Branco	Portugal	N.A.	63	46.0	46.0	N.A.	-	C+B	-	Pintado et al., 2005
Crescenza	Italy	Diverse sale points	437	0.2	4.9-7.3	0.93-1.00	<0.4 MPN/cm^2	PCR	MPN	Prencipe et al., 2010
Gorgonzola	Italy	Industrial plant	1489	2.1	N.A.	N.A.	-	C+B	-	Manfreda et al., 2005
	Italy	Diverse sale points	444	4.7	5.0-8.0	0.92-0.99	0.4-9.3 MPN/g	PCR	MPN	Prencipe et al., 2010
Panel	Greece	Retail stores	137	0.0	3.8-7.6	0.91-0.99	-	C+API	-	Angelidis et al., 2012
	Italy	Retail stores	894	2.1	N.A.	N.A.	0.0-2.0 $\log_{10}$ cfu/g	C+B	PC+MPB	Iannetti et al., 2016
	Spain	Retail stores and food industry	103	0.0	N.A.	N.A.	-	C+B+API	-	Cabedo et al., 2008

(Continued)

Table 1-4. (Continued)

Type of cheese	Country	Sampling	Number of samples	Detection (%)	pH	a_w	Levels	Id. method	Enum. method	Reference
Taleggio	Italy	Diverse sale points	324	6.5	5.4-8.0	0.92-0.99	0.4-460 MPN/g	PCR	MPN	Prencipe et al., 2010
Tulum	Turkey	Markets	250	4.8	N.A.	N.A.	-	C+B	-	Colak et al., 2007
Undefined soft cheese	Iraq	Supermarkets, resturants and veterinary quarantine centres	50	2.0	N.A.	N.A.	-	C+B+PCR	-	Ahmed et al., 2017
	Portugal	Retail stores	49	14.3	4.9-6.6	0.93-0.99	0.0-2.3 $\log_{10}$ cfu/g	C+VIDAS	PC	Almeida et al., 2007
	Greece	Markets	10	40.0	N.A.	N.A.	-	C+B+API+ PCR+PFGE	-	Filiousis et al., 2009
	Czech Republic	Retail market	387	5.2	N.A.	N.A.	0.0-2.0 $\log_{10}$ cfu/g	C+B	PC	Gelbíčová et al., 2017
	Bulgaria	Retail level	63	0.0	N.A.	N.A.	-	C+B	-	Gyurova et al., 2014
	Belgium	Retail stores	32	3.1	4.2-7.5	0.94-0.99	0.0-5.0 $\log_{10}$ cfu/g	VIDAS	PC	Lahou and Uyttendaele, 2017

(Continued)

Table 1-4. (Continued)

Type of cheese	Country	Sampling	Number of samples	Detection (%)	pH	aw	Levels	Id. method	Enum. method	Reference
Undefined soft cheese	European Union	Retail stores	3452	0.5	N.A.	N.A.	0.0-5.0 log10 cfu/g	N.A.	N.A.	Rakhmawati et al., 2013
	Sweden	Retail outlets	525	0.4	N.A.	N.A.	0.0-4.0 log10 cfu/g	C+B+VIDAS	PC	Thisted Lambertz et al., 2012
	Spain	Industries and markets	99	1.0	N.A.	N.A.	-	C+B	-	Vitas et al., 2004
	Austria	Retail stores and household levels	233	4.7	N.A.	N.A.	0.0-2.0 log10 cfu/g	C+PCR	PC	Wagner et al., 2007
White brined cheese	Jordan	N.A.	350	12.0	4.7-6.7	N.A.	-	C+PCR	-	Osaili et al., 2012

Legend: C, microbial culture; B, biochemical tests; API, analytical profile index; PFGE, pulsed-field gel electrophoresis; V, VIDAS; PC, plate count; MPN, most probable number; N.A., unavailable information; -, no enumeration; pH range is given when available, otherwise the average value is given (with SD when available); aw is expressed as a range.

3. Hard and semi-hard cheeses

Hard and semi-hard cheeses are characterized by a lower a_w compared to fresh, soft and semi-soft cheeses. This decrease is obtained by fast curdling, eventual cooking and intensive pressing of the curd, combined with an extended ripening period. Hard cheeses pH is rather variable, with values ranging from 4.9 to 8.0 (Saltijeral *et al*., 1999; Almeida *et al*., 2007). Hard cheeses present a_w values from 0.91 to 0.97 (Smukowski, 2013). Currently, no listeriosis outbreaks linked with hard cheeses are referenced (Martinez-Rios and Dalgaard, 2018). **Table** 1-5 summarises studies on the proportion of hard and semi-hard cheeses in which *L. monocytogene*s was detected (in 25 g of sample). Globally, the percentage of contaminated samples is close to 0.0 (Alcazar Montanez *et al*., 2006; Kongo *et al*. 2006; Gil *et al*. 2007; Cabedo *et al*. 2008; Little *et al*., 2008; Filiousis *et al*., 2009; Prencipe *et al*., 2010; Arrese and Arroyo-Izaga, 2012; Almeida *et al*., 2013). The low prevalence of the bacterium is explained by the lower a_w of hard and semi-hard cheeses, creating unfavourable conditions for survival and growth of *L. monocytogenes* (Kongo *et al*., 2006; Abrahao *et al*., 2008). According to Rudolf and Scherer (2001), hard cheeses made in the same dairies as contaminated soft cheeses, and with the same ripening microbiota, were not contaminated at the end of the ripening period, confirming that physico-chemical conditions in hard cheeses do not allow the survival of the pathogen. Nevertheless, Arrese and Arroyo-Izaga (2012) detected other species of the genus *Listeria* in *Idiazabal* cheese, an ovine milk hard cheese from Basque Country. One study detected a higher occurrence of the pathogen than the aforementioned studies. Almeida *et al*. (2007) observed an occurrence of 5.5%, but with a very limited sample size (18 cheeses), and only one sample was contaminated in that study. In fact, Almeida *et al*. (2013) observed an increase in the number of contaminated samples in relation with the decrease in the size of the dairies and the level of industrialization.

Table 1-5. Occurrence of *L. monocytogenes* in hard and semi-hard cheeses.

Type of cheese	Country	Sampling	Number of samples	Detection (%)	pH	a_w	Levels	Id. method	Enum. method	Reference
Asiago	Italy	Diverse sale points	449	0.2	5.2-7.7	0.92-0.99	<0.36 MPN/g	PCR	MPN	Prencipe et al., 2010
Chihuahua	Mexico	Processing plants	60	0.0	N.A.	N.A.	-	C+B+ PCR	-	Alcazar Montanez et al., 2006
Idiazabal	Spain	Retail stores	51	0.0	4.9-5.5	0.96	-	C+B	-	Arrese and Arroyo-Izaga, 2012
	Spain	Farmhouses	27	0.0	N.A.	N.A.	-	C+B	-	Gil et al., 2007
Sao Jorge	Portugal	Dairy-processing plants	66	0.0	5.5±0.2	N.A.	-	C+B+ PCR	-	Kongo et al., 2006

(Continued)

Table 1-5. (Continued)

Type of cheese	Country	Sampling	Number of samples	Detection (%)	pH	a_w	Levels	Id. method	Enum. method	Reference
Undetermined hard cheeses	Portugal	Retail stores	18	5.5	4.9-6.6	0.96-0.98	<1.0 log_{10} cfu/g	C+VID AS	PC	Almeida et al., 2007
	Portugal	Processing plant	3016	0.7	N.A.	N.A.	-	C+B+VI DAS	-	Almeida et al., 2013
	Spain	Retail stores and food industry	91	0.0	N.A.	N.A.	-	C+B+A PI	-	Cabedo et al., 2008
	Greece	Market	10	0.0	N.A.	N.A.	-	C+B+A PI+PCR +PFGE	-	Filiousis et al., 2009
	United Kingdom	Retail premises	1535	0.1	N.A.	N.A.	0.0-2.3 log_{10} cfu/g	C+B	PC	Little et al., 2008

Legend: C, microbial culture; B, biochemical tests; API, analytical profile index; PFGE, pulsed-field gel electrophoresis; V, VIDAS; PC, plate count; MPN, most probable number; N.A., unavailable information; -, no enumeration; pH range is given when available, otherwise the average value is given (with SD when available); a_w is expressed as a range.

4. *Survival of* L. monocytogenes *in cheese*

To understand the survival of *L. monocytogenes* in cheese during processing, ripening, packaging and storage, challenge studies can be performed. These consist in an inoculation of the pathogen during manufacture or storage. According to Bernini *et al*. (2013), "challenge testing evaluates if an inoculated organism can grow in a specific product and determines the point at which the growth reaches unacceptable levels in a specific product". The pathogen can also be directly injected into the final product. Alternatively, studies can focus on natural contaminations. This approach is called a "durability study". Both types of investigation have advantages and disadvantages. On the one hand, durability studies seem to be more realistic because contamination is natural. Indeed, it is difficult to mimic an adequate level of contamination when challenge testing a product. On the other hand, it is sometimes very hard to perform a durability study because of the low occurrence or low level of contamination of the concerned product (EURL *Lm*, 2014).

A wide variety of inoculation tests have recently been performed. These investigations focused on the influence of several parameters, including ripening duration, storage temperature and level of initial contamination. Inoculation can occur at different steps of the process, such as cheese processing, ripening, packaging or storage. Some authors also opted for the use of *L. innocua* to perform these experiments, due to its safety. However, in the latter case, researchers should choose a strain that behaves as similarly as possible to *L. monocytogene*s in order to mimic its growth (Samelis *et al*., 2009; Pinto *et al*., 2009). **Table** 1-6 summarises the main conclusions of papers focusing on the survival of *L. monocytogenes* in various types of cheese.

1. Fresh cheese

Fresh cheese a_w cannot prevent the survival and, in some cases, the growth of *L. monocytogenes*. Regarding low pH fresh cheese, such as Katiki (pH 4.3–4.5) or Galotyri (pH 3.8–4.4), a decrease is generally observed during storage at all temperatures (Rogga *et al*., 2005; Kagkli *et al*., 2009). A longer persistence is frequently observed at lower temperatures. However, Schoder *et al*. (2003) demonstrated that 7 days of storage at 7°C were unable to cause a decrease in the levels of *L. monocytogene*s in a cheese with pH 4.3. Fresh cheeses with a lower acidity are not able to reduce contamination. Kapetanakou *et al*. (2017) reported constant levels of *L. monocytogenes* (*i.e.* 100 cfu/g) in a *cottage* cheese with pH 5.0 during shelf life. In *Queso Blanco* (pH 6.8), *L. monocytogenes* was able to grow, irrespective of the storage temperature (Uhlich *et al*., 2006). In addition to pH, the level of the initial inoculum also had an influence (Schoder *et al*., 2003). Coatings of spices around fresh cheeses were not found to prevent listerial growth (Lobacz *et al*. 2016).

2. Soft and semi-soft cheese

Soft cheeses represent the riskiest category regarding *L. monocy*togenes, due to favorable pH and a_w. In terms of temperature, it was observed that the multiplication of *L. monocytogenes* is also slower at lower temperatures in soft and semi-soft

cheeses (Back *et al.*, 1993; Lahou and Uyttendaele 2017). *Camembert* is the most common soft cheese studied regarding growth of *L. monocytogenes* (Back *et al.*, 1993; Gay and Amgar, 2005; Linton *et al.*, 2008; Kapetanakou *et al.*, 2017). All studies on C*amembert* have reported the same observations: it is susceptible to the multiplication of *L. monocytogenes*. For soft cheeses, it is important to distinguish core and rind. Indeed, for MRSC and SRSC, microbiota on the rind and in the core is different. Pastes are rich in LAB, while rinds are mainly composed of moulds and yeasts (Back *et al.*, 1993; Kapetanakou *et al.*, 2017). In blue-veined cheeses, moulds are also observed in the core. In SRSC, no moulds are observed, but yeasts are found on the surface, predominantly from the genus *Debaryomyces* (Mounier *et al.* 2005; Irlinger *et a*l., 2015). Mounier *et al.* (2005) reported that these yeasts produce alkaline compounds leading to an increase in pH levels. As a result, less acid-tolerant bacterium can grow, including *B. linens* or species from the genus *Corynebacterium*.

Change in pH during cheese processing, ripening and storage is highly associated with this microbiota (Dalzini *et al.*, 2017). During the first hours after processing, LAB grow rapidly and produce organic acids from carbohydrates, resulting in a decrease of 1.5–2.0 pH units (Prieto *et al.* 2000; Florez *et al.*, 2006; Dalzini *et al.*, 2017). After a few days, moulds start to grow on the rind or in the paste, respectively for MRSC and blue-veined cheeses (Prieto *et al.*, 2000). Due to proteolytic activity of moulds, an increase in pH is generally observed in the concerned cheese part, associated with an increased concentration of free amino acids (Prieto *et al.*, 2000; Florez *et al.*, 2006; Dalzini *et al.*, 2017). Alkaline compounds resulting from lactate metabolism are also responsible for this increased pH (Dalzini *et al.*, 2017).

Consequently, a much higher pH is observed in the rind than in the core of MRSC and SRSC, sometimes increasing up to 7.0 during ripening of *Camembert* or *Brie* (Back *et al.*, 1993; Millet *et al.*, 2006; Schvartzman *et al.*, 2014; Bernini *et al.* 2016; Kapetanakou *et al.*, 2017). In blue-veined cheese pastes, pH can increase up to values higher than 6 (Prieto *et al.*, 2000; Florez *et al.*, 2006; Dalzini *et al.*, 2017). The behaviour of *L. monocytogenes* in soft cheese highly correlates with pH changes. While no increase in *L. monocytogenes* contamination in c*amembert* core was observed at refrigeration temperature, Back *et al.* (1993) observed an increase of 2 $\log_{10}$ cfu/g on the rind, where pH increases, during 40 days of storage. This dominant localisation of *L. monocytogenes* on the surface was also observed with the use of bioluminescent strains (Dalzini *et a*l., 2017). Furthermore, similar results have been reported for *Saint-Nectaire*, *Halloumi* and *Gorgonzola* (Millet *et al.*, 2006; Bernini *et al.*, 2016; Kapetanakou *et al.*, 2017). On the other hand, Dalzini *et al.* (2017) observed a growth of inoculated *L. monocytogenes* higher than the limit of 2 $\log_{10}$ cfu/g in *Gorgonzola* core, while the population of the pathogen remained stable on the rind. According to Corsetti *et al.* (2001), yeasts that develop in MRSC and blue-veined cheeses could sometimes enhance the ability of *L. monocytogenes* to grow, by producing growth factors.

The type of milk also has an influence. Pasteurized milk cheeses generally seem more susceptible to the multiplication of the pathogen in soft cheese, in case of

postpasteurization contamination. Endogenous microbiota of raw milk, composed among others of LAB, could play an inhibitive role on *L. monocytogenes* due to increased competition (Schvartzman *et al*., 2011; Tiwari *et al*., 2014). In soft cheese manufactured by direct acidification, that is, by adding lactic acid, the population of *L. monocytogenes* was increased by 2–3 $\log_{10}$ cfu/g in comparison with cheese including lactic starters (Naldini *et al*., 2009). Some enzymes found in raw milk, for instance lactoferrin and lactoperoxydase, which have bacteriostatic properties, can also prevent *L. monocytogenes* growth (Food and Agriculture Organization (FAO), 2005; Gay and Amgar, 2005; Tiwari *et al*., 2014; Lahou and Uyttendaele, 2017).

Ripening duration also plays a role. Indeed, a_w progressively diminishes during ripening and cheeses become harder. As a consequence, less growth was observed during storage of G*orgonzola* over 80 days of ripening ($a_w = 0.92$) in comparison with G*orgonzola* aged for 50 days ($a_w = 0.97$). Growth was also delayed by 30 days in a 80-day ripened cheese (Bernini *et al*., 2013). In a further study performed by Bernini *et al*. (2016), piquant G*orgonzola* ripened for 80 and 120 days did not enable the growth of the bacterium, while it was possible in sweet G*orgonzola* with a shorter ripening duration.

Regarding semi-soft cheese, studies suggest that it is more difficult for the pathogen to grow in this type of cheeses. Condoleo *et al*. (2016) found no growth of the bacterium during storage of an Italian raw ovine milk semi-soft cheese. Pinto *et al*. (2009) observed a decrease in the levels of *L. monocytogenes* in Minas traditional *Serro* cheese with inoculum levels ranging from 10 to 1,000 cfu/g. Overall, studies suggest that it is possible to detect *L. monocytogenes* in semi-soft cheese, but that its growth is limited.

3. Hard and semi-hard cheese

Studies on the occurrence of *L. monocytogenes* in hard and semi-hard cheese indicate that it is difficult for the bacterium to grow in these types. Inoculation studies have confirmed these findings. Although growth of the bacterium was observed during manufacture of *Swiss* hard cheese, it was no longer detectable after ripening (Buazzi *et al*., 1992; Bachmann and Spahr, 1995). No growth was observed in G*ouda*, *Parmesan*, *Cheddar*, *Cantal*, *Edam* and *Pecorino* (Ryser and Marth, 1987; Northolt *et al*., 1988; Yousef and Marth, 1990; Chatelard-Chauvin *et al*., 2015; Ortenzi *et al*., 2015; Kapetanakou *et al*., 2017).

Bachmann and Spahr (1995) reported that the pH of *Swiss* hard and semi-hard cheeses increased by 0.3–0.9 units during ripening. Thus, a_w is generally the most limiting factor for *L. monocytogenes* in hard or semi-hard cheese. For instance, $a_w < 0.90$ in *cantal* or < 0.92 in *Gouda* rinds have been reported (Wemmenhove *et al*., 2013; Chatelard-Chauvin *et al*., 2015). In naturally contaminated C*heddar* (pH 5.5), the bacterium never reached the threshold value of 100 cfu/g and disappeared during the storage period (Dalmasso and Jordan, 2014). For *Chihuahua* and *Manchego*, two Mexican cheeses, levels of the bacterium remained at the initial level (*i.e.* 10^6 cfu/g) during storage (Solano-Lopez and Hernandez-Sanchez, 2000). Both natural and artificial contaminations lead to the same observations for hard and

semi-hard cheeses. In *Cheddar*, *Pecorino* and *Parmesan*, pH could be a limiting factor. Specifically, pH values were found to decrease to 5.0 during ripening and storage, while a_w remained above 0.94 (Ryser and Marth, 1987; Yousef and Marth, 1990; Ortenzi *et al*., 2015). Sodium chloride percentage in these types of cheese seems to have no influence on the behaviour of the pathogen, while decreasing the salt content of *Cheddar* cheese did not change the survival of *L. monocytogenes* (Hystead *et al*. 2013).

Contrary to soft cheeses, surveys report that hard cheeses made from pasteurized or thermized milk are not more likely to support listerial growth than raw milk cheese (Ryser and Marth, 1987; Solano-Lopez and Hernandez-Sanchez, 2000; Samelis *et al*., 2009). If the starter culture probably plays a role in the inhibition of *L. monocytogenes*, the key step explaining this is ripening duration (Kandarakis *et al*., 1998; Cetinkaya and Soyutemiz, 2004). Indeed, ripening period for hard cheeses is generally from 6 months up to several years.

The effect of storage temperature on the behaviour of *L. monocytogenes* in hard cheese is complex. Overall, it appears that storage at room temperature could favour a decrease in the population of *L. monocytogenes* (Valero *et al*., 2014). According to Giannou *et al*. (2009), "the lower the storage temperature, the higher and longer the survival of *L. monocytog*enes was". Refrigerated storage could even permit the levels of contamination to be maintained or grown (Bellio *et al*., 2016; Moosavy *et al*., 2017). However, scientists expect negative effects of an increased storage temperature on the appearance and physico-chemical characteristics of the cheeses (Moosavy *et al*., 2017).

Surprisingly, *L. monocytogenes* was found to disappear during storage of G*raviera*, a cheese with pH 5.6 and a_w 0.95, on average. These physico-chemical values are usually considered as insufficient to prevent the multiplication of the pathogen (Giannou *et al*., 2009). LAB seem to play a major role in this inhibition (Kagkli *et al*., 2009). It is well established that LAB are more active when the temperature is higher, *i.e.* at room temperature (Valero *et al*., 2014). Samelis *et al*. (2009) observed that a decrease in *L. monocytogenes* contamination was linked with an increase in LAB populations during the early stages of ripening and storage. These raw milk endogenous bacteria are responsible for increased competition for nutrients. They can also produce bacteriocins (Reis *et al*., 2012; Kapetanakou *et al*., 2017). Brining time could also be of interest in the prevention of *L. monocytogenes* contamination. Indeed, Wemmenhove *et al*. (2016) showed that G*ouda* a_w decreased with brining time (0.96, 0.93 and 0.90 for 0.33, 2.10 and 8.90 days of brining, respectively).

Regarding cheese weight, no influence on the behavior of the bacterium has been reported (Chatelard-Chauvin *et al*., 2015). Finally, according to Wemmenhove *et al*. (2018), the behaviour of *L. monocytogenes* in hard cheese could also be influenced by the concentration of undissociated lactic acid. They showed that *L. monocytogenes* was unable to grow in *Gouda* when undissociated lactic acid concentration is higher than 6.35 mM.

To our knowledge, only a single study has reported the growth of *L. monocytogenes* in a hard cheese, g*ruyere*, made from pasteurized milk (Leong *et al.*, 2014). The fact that this cheese was stored at an abuse temperature of 25°C could explain the growth of *L. monocytogenes*.

Table 1-6. Studies on the survival of *L. monocytogenes* in various types of cheese.

Type of cheese	Studied parameter(s)	Name of the cheese(s)	Type of milk (R/T/P/HP)	Natural contamination (Y/N)	Organism (Lmo/Li)	Main conclusions	References
Fresh	Storage temperature	*Galotyri*, *Katiki*	P	N	Lmo	Fastest decrease at higher temperature (e.g. 20 °C) allowed by low pH (3.8-4.5), long survival	Rogga et al., 2005 ; Kagkli et al., 2009
		Queso blanco	R	N	Lmo	Increase at all temperature, higher growth rate with increasing temperature, allowed by higher pH (4.8-6.8)	Uhlich et al., 2006
	Inoculated strain(s)	*Katiki*	P	N	Lmo	Longer survival with a single strain in comparison with a cocktail	Kagkli et al., 2009
	Specific ingredients	*Gusto* cheese	N.A.	N	Lmo	No influence of spices or fresh herbs on the survival	Lobacz et al., 2016
	Starter cultures	*Minas frescal*	P	N	Lmo	Stable contamination in cheeses with starters, increase of 2-3 $\log_{10}$ cfu/g when direct acidification	Naldini et al., 2009
	No specific parameter	*Cottage* cheese	P	N	Lmo	Survival close to the level of the inoculum (2 $\log_{10}$ cfu/g)	Kapteanakou et al., 2017
		Unspecified	R	Y	Lmo	Survival close to the level of the initial contamination (2.3 $\log_{10}$ cfu/g)	Schoder et al., 2003

(Continued)

Table 1-6. (Continued)

Type of cheese	Studied parameter(s)	Name of the cheese(s)	Type of milk (R/T/P/HP)	Natural contamination (Y/N)	Organism (Lmo/Li)	Main conclusions	References
Soft/Semi-soft	Temperature of storage	*Camembert*	P	N	Lmo	No growth at 3, 6 and 10°C, growth at 15°C, faster on the surface	Back *et al.*, 1993
		Unspecified	R/P	N	Lmo	Increasing growth rate at higher temperature,	Tiwari *et al.*, 2014 ; Lahou and Uyttendaele 2017
	Type of milk	*Camembert*	R/P/HP	N	Lmo	Growth twice as slow with raw in comparison with pasteurized milk; high pressure treatment of inoculated milk sufficient to eradicate the pathogen	Gay and Angar 2005 Linton *et al.*, 2008
		Smeared-cheese	R/P	N	Lmo	No growth during process in raw-milk cheese, but well in pasteurized-milk cheese	Schvartzman *et al.*, 2011
	Level of contamination	Minas Traditional *serro* cheese	N.A.	N	Li	No difference of survival, remains at the same level with all inocula	Pinto *et al.*, 2009
	Ripening duration	*Gorgonzola*	P	N	Lmo	Smaller growth in cheeses with 80 days of ripening (in place of 50 days) ; growth delayed by 30 days (linked with lower aw)	Bernini *et al.*, 2013

(Continued)

Table 1-6. (Continued)

Type of cheese	Studied parameter(s)	Name of the cheese(s)	Type of milk (R/T/P/HP)	Natural contamination (Y/N)	Organism (Lmo/Li)	Main conclusions	References
Soft/ Semi-soft	No specific paramter	*Saint-Nectaire*	R	N	Lmo	No growth in the core, but well on the surface between day 8 and 18 of ripening	Millet *et al.*, 2006
		Semi-soft sheep milk chreese	R	N	Lmo	Growth during early stages of manufacture, but followed by a drecrease	Condoleo *et al.*, 2016
Semi-hard/Hard	Temperature of storage	*Graviera, Lighvan,* unspecified	R	N	Lmo	Long term survival at 4°C, faster decrease at higher temperatures	Giannou *et al.*, 2016 ; Bellio *et al.*, 2016 ; Moosavy *et al.*, 2017
	Temperature of ripening	*Cantal, Cheddar, Parmesan*	R/P	N	Lmo	Observed increase during cooking, pressing and early ripening, but decrease and disappearance during ripening at all temperatures	Ryser and Marth 1987 ; Yousef and Marth 1990 ; Chatelard-Chauvin *et al.*, 2015
	Starter culture	*Graviera*	T	N	Li	Growth delayed when starter culture is added	Samelis *et al.*, 2009

(Continued)

Table 1-6. (Continued)

Type of cheese	Studied parameter(s)	Name of the cheese(s)	Type of milk (R/T/P/HP)	Natural contamination (Y/N)	Organism (Lmo/Li)	Main conclusions	References
Semi-hard/Hard	No specific parameter	*Cheddar, Edam, Gouda, Kashar, Pecorino, Swiss*	R/P	Y/N	Lmo	Contamination decreases during ripening and falls under the LOQ	Buazzi *et al.*, 1992 ; Northolt *et al.*, 1988 ; Bachmann and Spahr 1995 Cetinkaya and Soyutemiz 2004 ; Wemmenhove et al. 2013 ; Dalmasso and Jordan 2014 Ortenzi *et al.*, 2015
		Chihuahua, Edam, Gouda, Manchego	P	N	Lmo	Contamination remains just under the intial level during storage	Solano-Lopez and Hernander-Sanchez 2000 ; Kapetanakou et al. 2017
		Gruyere	N.A.	N	Lmo	Growth when stored at 25°C	Leong *et al.*, 2014

Legend: R/T/P/HP, Raw/Thermised/Pasteurized/High Pressure treated milk; Y/N, Yes/No; Lmo/Li, *L. monocytogenes*/*L. innocua*; N.A., unavailable data.

5. Conclusion

Occurrence and survival of *L. monocytogenes* in cheese are important research topics, listeriosis being the only foodborne disease for which an increase was observed for the period 2012–2018. Globally, it is well established in the literature that some categories of cheese are more susceptible to the growth of *L. monocytogenes*. For instance, soft, semi-soft cheeses and nonacidic fresh cheeses are the riskiest. If the pathogen can sometimes be found in UACC and SH/HC, its growth is generally not possible, due to lower pH or moisture conditions. The trend that favors the use of pasteurized milk for cheese production does not seem to be backed by available literature. Indeed, no obvious differences can be observed in the prevalence of *L. monocytogenes* in raw compared to pasteurized milk cheese. Worse, pasteurized milk could favour the survival of the pathogen, cheese being free of competitive natural lactic microbiota. Moreover, most cheese contaminations are not linked to the microbial quality of milk but to a lack of hygiene during postpasteurization or postprocessing steps. Another important factor to take into account when considering prevalence and survival of *L. monocytogenes* is the physico-chemical differences between cheese rind and core, as surface pH is generally more favorable. A further factor to consider regarding prevalence of *L. monocytogenes* is its heterogeneous distribution in a single batch, but also in a single piece.

6. Future research and recommendations

This review revealed that most studies focused on cheese from Hispanic countries or from France. Data from other EU countries, such as Belgium, are currently scarce although there is a wide diversity of typical cheeses in these regions. Therefore, it would be of interest to perform a large-scale investigation on the occurrence of *L. monocytogenes* in these countries, for instance in Belgium. This study should be followed by inoculation and shelf-life studies for a panel of Belgian cheeses. In these studies, the pathogen should be inoculated either in the core or on the surface, depending on the physico-chemical conditions. Furthermore, many of the studies presented in this review used high initial contaminations, which do not reflect the reality. It is indeed suggested by EURL *Lm* (2014) to target an initial inoculum of $2 \log_{10}$ cfu/g. In addition, EURL *Lm* (2014) also advises that the temperature should vary during storage of inoculated cheeses during shelf-life studies, to mimic the different steps of the food supply chain. Very few papers have considered these changes in storage temperatures. The purpose of such a large-scale investigation would be to extrapolate the results to all cheeses presenting the same properties. Afterwards, producers could take advantage of the conclusions without being forced to perform their own challenge-tests. In addition to physico-chemical parameters, the microbial richness of cheeses can also play an important role in the survival of *L. monocytogenes*. Combining investigation of these factors within a single survey could provide interesting and important information.

Updated state of the art for the period 2018-2021 on prevalence and survival of *L. monocytogenes* in cheese

The above review article was written in 2017 and published in 2018. An update on the knowledge on the prevalence and on the behavior of *L. monocytogenes* in various cheese varieties is thus presented hereafter.

1. Prevalence of *L. monocytogenes* in various cheese varieties

Consumption of products made from raw milk remains a debate in some countries. Sonnier *et al.* (2018) reported that a majority of listeriosis outbreaks related to dairy products in the United States between 1993 and 2006 were associated with food produced from raw milk. Nevertheless, the interest of consumers for artisanal raw milk cheeses is increasing. Minimizing the risk for food safety associated with the consumption of such dairy products remains thus essential.

Papers on the prevalence of *L. monocytogenes* were not abundant during recent years, especially in high level scientific journals. Papers from national or low impact journals were not included in the following summary.

A study on 245 Italian raw milk cheeses did not identify *L. monocytogenes* (Costanzo *et al.*, 2020). Similarly, the pathogen was not detected in 40 cheeses sampled in Turkish supermarkets and delicatessen shops, although *L. innocua* and *Listeria ivanovii* were isolated (Arslan and Ozdemir, 2020). It is an interesting improvement in comparaison with figures reported in the review paper. Nevertheless, no precisions on types of cheese were available. In developing countries, prevalence of the pathogen is still a concern. In this part of the world, most cheeses are produced in an artisanal way, using raw milk, and in small processing plants, where hygiene can be dubious. In Chile, 19 out of 168 (*i.e.* 11.3%) semi-hard *Chanco* cheeses, were contaminated with *L. monocytogenes* (Barria *et al.*, 2020). In the latter study, seasonality in the proportion of contaminated cheese was observed, with the highest proportion in fall. A study on 120 artisanal cheeses from Iran showed nine contaminated samples (*i.e.* prevalence of 7.5%), while eight extra samples contained other *Listeria* species. Nevertheless, precisions on cheese varieties were missing (Akrami-Mohajeri *et al.*, 2018). A meta-analysis gathering 31 Iranian studies on dairy products found a prevalence of 17% in traditional cheeses (Hamidiyan *et al.*, 2018). In comparison, another meta-analysis on the prevalence of *L. monocytogenes* in ripened soft cheeses was published by Churchill *et al.* (2019). Combining 100 papers on RSC from both developed and developing countries, an estimated prevalence of 4.4% was obtained. Only seven out of these 100 papers did not identify positive samples. Keba *et al.* (2020) reviewed the prevalence of *L. monocytogenes* in Ethiopian cheeses. In *Ayib*, the Ethiopian version of *Cottage* cheese, prevalence of the pathogen ranged from 0.0 to 5.0%, although pH was around 3.7, so under the threshold value provided by Regulation (EC) No 2073/2005.

2. Fate of L. monocytogenes in different cheese varieties

Comparisons between papers remain difficult to perform, as important variations in protocols are observed (Hunt *et al.*, 2018):
- Natural or artificial contamination of cheese;
- Inoculation of *L. monocytogenes* or *L. innocua*;
- Inoculation in milk, in curd or in cheese after ripening;
- Variable levels of contamination;
- Enumeration during production and/or ripening and/or storage.

Studies strictly following EURL *Lm* (2014) guidelines for assessing the growth potential of *L. monocytogenes* in cheese were not identified. Nevertheless, some interesting papers were published recently.

Mozzarella is a particular type of unripened cheese, which should be considered as an eventual threat for food safety. Indeed, as for unacidified unripened cheeses, natural hurdles to the growth of *L. monocytogenes* are limited. *Mozzarella* has a high pH (6.42-6.50) and low levels of natural microflora, as cheeses are dipped into hot water during manufacture (Tirloni *et al.*, 2019a). This step caused a decrease in the levels of the pathogen from 5 to less than 1 $\log_{10}$ cfu/g during challenge studies. Nevertheless, survivor cells were able to grow during refrigerated storage (Murru *et al.*, 2018). Furthermore, concentrations of undissociated short-chain organic acids able to act against *L. monocytogenes* are too low. During challenge tests on artificially contaminated *Mozzarella* (2-3 $\log_{10}$ cfu/g as initial contamination), $\delta > 3$ $\log_{10}$ cfu/g were observed during refrigerated storage, and up to 4.7 $\log_{10}$ cfu/g at higher temperatures (Tirloni *et al.*, 2019a). The situation is comparable for *Ricotta*, a whey cheese heated at 75-80°C during manufacture, and susceptible to post-processing contamination (Tirloni *et al.*, 2019b).

Salazar *et al.* (2020) showed that *L. monocytogenes* was able to grow during *Gouda* manufacture, from 1 $\log_{10}$ cfu/mL of artificially contaminated milk to more than 2 $\log_{10}$ cfu/g in curd before ripening. During curdling, *L. monocytogenes* was more concentrated in curd (1.7 $\log_{10}$ cfu/g) than in whey (0.3 $\log_{10}$ cfu/g). During ripening, a long-term persistence of the pathogen was observed. Similarly, the pathogen was still detected at levels < 10 cfu/g after 6 months of *Cheddar* ripening, irrespective of the initial milk contamination level (*i.e.* 1, 3 or 5 $\log_{10}$ cfu/mL) (Chon *et al.*, 2020).

A challenge study with *L. innocua* was performed on *Fossa di Sogliano*, a traditional Italian SHC (Giacometti *et al.*, 2020). In this study, pasteurized milk was inoculated at a level of 4.5 $\log_{10}$ cfu/mL and the evolution of the contamination was monitored during 5 months of ripening. A significant decrease in the levels of *L. innocua* was observed, with contamination between 2.3 and 2.9 $\log_{10}$ cfu/g at the end of ripening. The authors mentioned that, in addition to cheese pH and a_w and to Jameson effect, cheese microstructure could also inhibit the growth of *Listeria* species, by limiting the diffusion of essential compounds.

Centorotola *et al.* (2020) performed a challenge study on *Pecorino di Farindola*, by inoculating two strains of *L. monocytogenes* in raw ewe milk (10^5 cfu/mL).

Manufactured samples had pH 5.54 and a_w 0.97 after pressing, decreasing to 0.83 at the end of ripening (150 days). A progressive decrease in the levels of the pathogen was observed during ripening, correlated to the decrease in a_w. δ during storage of *Pecorino di Farindola* was not investigated in this paper.

3. Novel approaches

Aside from classic inoculation studies, assessing the ability of particular bacterial strains, molecules or treatments to inhibit the growth of *L. monocytogenes* in cheese is now a hot topic.

1. High pressure processing

One of these novel approaches is to assess the anti-listerial impact of high-pressure treatments (Linton *et al.*, 2008; Hereu *et al.*, 2012; Bleoanca *et al.*, 2016; Ferreira *et al.*, 2016). High pressure processing consists in the application of pressure between 100 and 1,000 MPa to destroy pathogenic bacteria in food. Contrary to heat treatments which could alter milk/cheese organoleptic and nutritional properties, high pressure processing is less destructive. Nevertheless, the efficiency of such treatments depends on cheese types and on *L. monocytogenes* strains (Ferreira *et al.*, 2016; Evert-Arriagada *et al.*, 2018). In addition to that, Morales *et al.* (2006) reported that natural cheese constituents, including lactose, galactose and glucose, could have a baroprotective effect on *L. monocytogenes*. In *Queso Fresco*, a pressure of 600 MPa during 20 minutes decreased *L. monocytogenes* under the detection level. However, after a lag time, the pathogen was able to grow again during storage (Tomasula et al., 2014). On the opposite, Evert-Arriagada *et al.* (2018) reported that pressure higher than 600 MPa should be sufficient to guarantee food safety during normal storage of fresh cheeses. Linton *et al.* (2008) compared *Camembert* made from inoculated raw milk and inoculated milk treated by high pressure (500 Mpa, 10 minutes). While the pathogen reached 3.85 $\log_{10}$ cfu/g during ripening and storage of raw milk cheese, it did not grow in pressure-treated samples. Similar observations were reported for *Serra da Estrela*, a SRSC treated at 600 MPa for 3 minutes (Inácio *et al.*, 2014).

2. Essential oils

Another option is the addition of essential oils to cheese or packaging. Essential oils have recognized antimicrobial properties. Cheese is a suitable matrix for their immobilization, due to high fat and protein contents (Gayán *et al.*, 2012). Bleoanca *et al.* (2016) demonstrated that the addition of thyme extract in Latin-style fresh cheese allowed a decrease in the intensity of high-pressure treatment necessary to decrease *L. monocytogenes* levels. Similarly, Lim *et al.* (2020) shown that a biodegradable packaging including grapefruit seed extract could be used at retail in order to inhibit the development of *L. monocytogenes*. Nevertheless, assessment of the impact of these essential oils on cheese sensorial properties was not included in the papers.

3. Supercritical carbon dioxide and hydrogen peroxide

The use of supercritical carbon dioxide as an alternative to heat treatment for *Mozzarella* preservation has also been investigated. Supercritical carbon dioxide alone was insufficient to lower *L. innocua* contamination under the limit of detection, but its effectiveness could be improved by adding low concentrations of peracetic acid (Sikin *et al.*, 2016). A protocol for the industrial use of hydrogen peroxide to control the growth of *L. monocytogenes* in ripened soft cheeses is currently developed in the USA (Robinson and D'Amico, 2020).

4. Modified atmosphere

Modified atmosphere packagings are also investigated, in relation to the growing demand for RTE foods free of preservative agents. Brown *et al.* (2018a) performed a study on *Queso Fresco*, a Latin-style fresh cheese, considering thus a worst case; this type of product being known for allowing the growth of *L. monocytogenes*. In this experiment, authors inoculated the pathogen on the surface of *Queso Fresco* at a level of 4 $\log_{10}$ cfu/g. Cheese were stored under seven atmospheres, including air, vacuum and various ratios of nitrogen and carbon dioxide. Conclusion was that packagings with the highest amount of carbon dioxide, *i.e.* 70 and 100%, limited *L. monocytogenes* growth extent, in comparison with traditional packaging.

5. Bacteriocins or bacteriocin-producing strains

To limit the development of *L. monocytogenes*, two other solutions are favoured: the addition of bacteriocin-producing strains, or the direct use of purified antimicrobial compounds. According to Silva *et al.* (2018), bacteriocins are "peptides or proteins ribosomal synthesized by bacteria that inhibit or kill other related or unrelated microorganisms".

Up to now, performances of many strains have already been assessed. For instance, Giannou *et al.* (2009) combined commercial LAB with an enterocin-producing strain to manufacture g*raviera*. Unfortunately, *L. monocytogenes* did not grow but was able to survive for a long time. Martinez *et al.* (2015) observed an inhibition of the growth of the pathogen in cheese spread when the bacteriocin-producing strain *Latilactobacillus sakei* subsp. *sakei* 2a was used as starter. Other authors have been interested in the role of *Carnobacterium divergens*, *Enterococcus faecium*, *Enterococcus mundtii*, *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus plantarum, Latilactobacillus curvatus*, *L. lactis* or *Staphylococcus equorum*, with promising results (Izquierdo et al., 2009; Mojgani *et al.*, 2010; Dal Bello *et al.*, 2012; Pingitore *et al.*, 2012; Aspri *et al.*, 2017; Bockelmann *et al.*, 2017; Lourenço *et al.*, 2017; Morandi *et al.*, 2019; Morandi *et al.*, 2020; El-Sayed *et al.*, 2021; Sameli *et al.*, 2021). The choice of strains of interest is not restricted to organisms isolated from dairy products (Ho *et al.*, 2017; Lawton *et al.*, 2020). Wan *et al.* (1997) directly added piscicolin 126, a bacteriocin, to milk during manufacture of *Camembert*, and observed an inhibitive activity on *L. monocytogenes*. Nisin and bacteriocin-like substance P34 have also been used extensively (da Silva Malheiros *et al.*, 2012; Cui *et al.*, 2016). Nevertheless, the efficiency of bacteriocins is sometimes limited. In *Minas Frescal*, nisin and bacteriocin-like peptide cerein 8A

only allowed exponential growth phase to be delayed (Bizani *et al.*, 2008; da Silva Malheiros *et al.*, 2012). A combination of several antimicrobial compounds could be more efficient (Lakicevic and Nastasijevic, 2017; Mills *et al.*, 2017, Morandi *et al.*, 2020). Exhaustive tables of bacteriocin-producing strains and bacteriocins assessed in cheese are available in a review paper written by Silva *et al.* (2018). The inclusion of endolysin from bacteriophages, or of bacteriophages themselves, into cheese products is another alternative (Lakicevic and Nastasijevic, 2017; Van Tassell *et al.*, 2017).

6. Smart packaging

The direct use of bacteriocins in cheese manufacture present some drawbacks, as they can easily be degraded (Silva *et al.*, 2018). According to Kristo *et al.* (2008), it could be more effective to incorporate antimicrobial compounds into packaging or coating rather than directly into the product. Essential oils and bacteriocins can directly be added into packaging in order to exert their antimicrobial activities. Such packaging could destroy the pathogen, increase duration of its lag phase, or limit its growth (Brown *et al.*, 2018b). Suppakul *et al.* (2008) prepared polyethylene films containing basil oil, showing an inhibitive action on artificially contaminated *Cheddar*. Ahmed *et al.* (2017) performed a challenge study on *Cheddar* packaged with film containing cinnamon. During 11 days of storage, the load of *L. monocytogenes* was decreased by 2.5 $\log_{10}$ cfu/g. Edible coatings (galactomannans, starch, halloysite) including antimicrobial compounds like nisin or natamycin, are of increasing interest (Martins *et al.*, 2010; Dalzini *et al.*, 2016; Meira *et al.*, 2016; Ollé Resa *et al.*, 2016). Such films could be able to protect cheeses against *L. monocytogenes*. For instance, during seven days of storage of *ricotta*, a coating of galactomannans with nisin allowed to decrease listerial load by 2.2 $\log_{10}$ cfu/g after seven days of storage, in comparison with uncoated cheese (Martins *et al.*, 2010). During manufacture of *Ricotta*, heat treatment is applied, which is generally sufficient to eradicate *L. monocytogenes*. Nevertheless, chances of contamination during post-processing handlings are well real. Consequently, applying an edible coating directly after manufacture could be an interesting strategy. These emerging approaches could become interesting alternatives to heat treatment, by making cheese a functional food that is able to prevent or limit the growth of *L. monocytogenes*.

State of the art on DNA sequencing approaches

During this thesis, to investigate cheese microbial diversity and its possible influence on the growth of *L. monocytogenes*, various DNA approaches and techniques were used. Uncultural approaches, including DNA sequencing, allowed new insights in the understanding of microbial communities in the environment, but also in various food matrices. This type of analyses is now performed routinely and for limited costs. The following part of the manuscript aims at providing keys to understand methods used during Chapter 6 and Chapter 7 of this thesis.

1. DNA sequencing technologies

First methods allowing DNA sequencing were developed during the seventies, especially one of the most renowned sequencing methods, called Sanger (Sanger, 1977). This technology is based on DNA synthesis from the complementary template. Briefly, Sanger sequencing technology is based on four reactions occurring simultaneously. In each tube, DNA to sequence and deoxyribonucleotides triphosphates (dNTP, *i.e.* dATP, dTTP, dCTP and dGTP) are added, but with a fraction of a dideoxyribonucleotide triphosphates (ddNTP): ddATP, ddTTP, ddCTP or ddGTP. DNA polymerase allows elongation of strand complementary to DNA template, by incorporating dNTP. Randomly, ddNTP are incorporated to elongated strand, resulting in elongation termination, due to missing hydroxyl group on the ribose. Fragments from all tubes are then separated using gel electrophoresis, allowing to know DNA sequence (Anton Leberre, 2014).

Since 2005, a new range of sequencing techniques emerged, generally described as next-generation sequencing (NGS) techniques.

The first one is known as pyrosequencing. In this case, dNTP are added in a defined order. When incorporated to elongated strand by DNA polymerase, a pyrophosphate is liberated. The latter one is used by enzyme ATP sulfurylase to produce ATP. Detection is then based on luciferine-luciferase reporting system, with oxidation of luciferin resulting in a light signal recorded by a camera. Prior to sequencing, this method requires amplification of DNA using emulsion PCR, using spheres on which template DNA sequences are attached (Margulies *et al.*, 2005).

More recently, sequencing methods based on reversed dye terminators, including Illumina, gained in popularity, as this firm became the world leader in DNA sequencing market. Prior to analysis, template DNA must be fragmented, and Illumina adapters are added at both fragments ends. This technology also requires the generation of DNA sequences clusters prior to sequencing. In this case, clusters are not generated on spheres but on a flow cell, *i.e.* a solid support containing short DNA sequences complementary to Illumina adapters. After formation of bridges on the flow cell, complementary strands are synthetized. Then, new cycles of denaturation-elongation are performed, allowing cluster formation. When clusters are formed, the four dNTP, each marked with a specific fluorescent dye coupled to a reversible terminator, are added together. This terminator allows to temporarily blocate elongation, permitting a detection of emitted fluorescence. Once done, terminator is cleaved and a new cycle (generally up to 300 cycles) can be performed (Bentley *et al.*, 2008).

In parallel to Illumina, another technology is commonly used, named Ion Torrent. Sequencing is based on the liberation of a proton when a dNTP is added to elongated strand, resulting in pH variations. Ion Torrent does not require fluorescent dyes (Marsaud, 2019).

Recently, new methods, considered as third or fourth generation sequencing techniques, emerged, including Oxford Nanopore and PacBio RS. The description of these techniques is out of the scope of this thesis.

2. Metagenetics

Metagenetics, also known as DNA barcoding, allows the study of whole communities from environmental or food samples, especially communities of microorganisms, including bacteria, archaea, yeasts and molds. Metagenetics does not require sequencing of all DNA from the sample. Prior to sequencing, PCR amplification steps are required, targeting DNA fragment specific to the studied population. For bacteria and archaea, common targets are hypervariable regions of 16S rDNA gene (**Figure** 1-6). Commonly used primers target the amplification of V1-V3 or V3-V4 regions. Regarding eukaryotic microorganisms, *i.e.* yeasts and molds, targets are generally 18S rDNA gene or internal transcribe spacers (ITS). Considering an Illumina approach, a library of sequences must be prepared after amplification of the target(s), notably by adding adapters at both fragments ends, allowing fixation of strands on the flow cell.
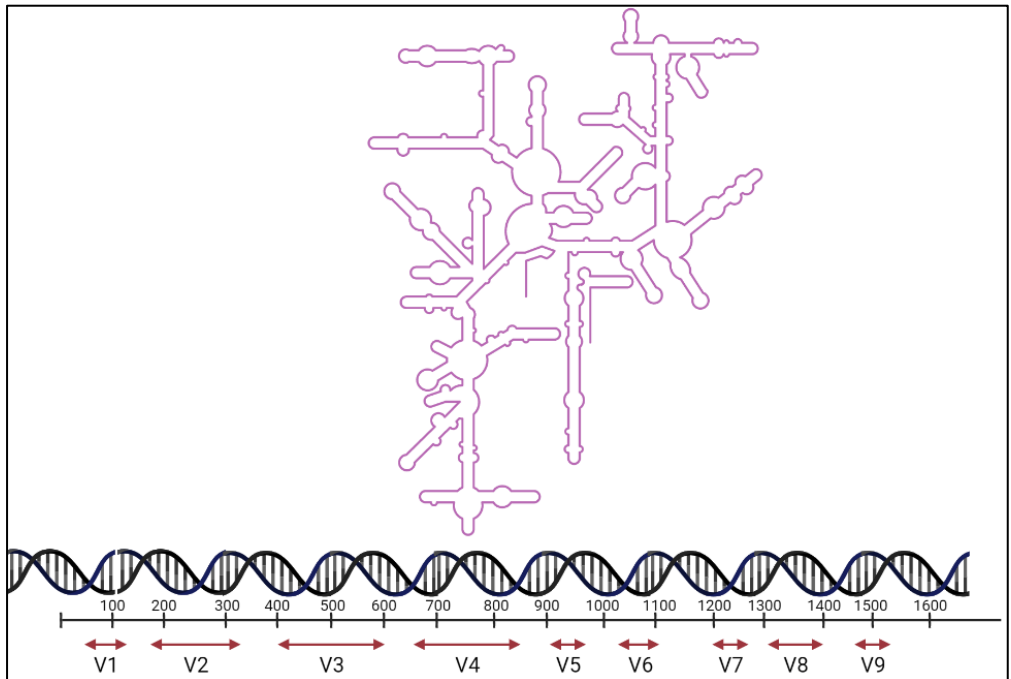
Figure 1-6. Bacterial 16S rRNA gene and localization of variable regions.

Illumina sequencing produces paired end reads, provided to the user under FASTQ format. Bioinformatic treatment of this data is a primordial work. Various open access pipelines are available, including QIIME and Mothur (Schloss *et al*., 2009; Caporaso *et al*., 2010). Reads are always paired, as sequencing occurs in both reverse and forward senses. The first bioinformatic step is to merge paired reads in a unique sequence, called a contig. Various quality control steps are then required, including:

- checking that contigs have the expected number of nucleotides;
- checking for ambiguous nucleotides;
- removing of duplicated contigs.

Next step is to align contigs with reference sequences, available in databases, including SILVA bacteria (Quast *et al*., 2012). Using VSEARCH, it is then required to remove potential chimeric sequences, *i.e.* sequences obtained when algorithm paired two reads orginating from different organisms (Rognes *et al*., 2016). Finally, sequences are clustered into operational taxonomic units (OTU), amplicon sequence variants (ASV) or phylotypes. Generally, an OTU gathers sequences not differing from each other by more than 3%. ASV approach allows a more precise distinction between sequences. Phylotype is an approach based on phylogeny (Schloss, 2019).

Interpretation of the results involved some concepts of ecology. Microbial diversity can be described at several scale (**Figure** 1-7). α-diversity allows to consider diversity at small scale, *e.g.* within a sample. It can be described using species richness, *i.e.* the number of observed species in an environment, or species evenness, considering relative abundances of species in the sample. Two indicators commonly used to assess species evenness are Simpson index and Shannon index. β-diversity considers diversity between two samples or ecosystems. Finally, γ-diversity concerns larger scale, including many ecosystems, and can for instance study the impact of a gradient. β- and γ-diversities are generally described based on dissimilarity matrices (Jaccard matrix, Bray-Curtis matrix or Yue and Clayton θ matrix) or UniFrac matrices. Jaccard matrix is a dissimilarity matrix only gathering absent (0) or present (1) species within studied communities, not considering their relative abundances, while Bray-Curtis and Yue and Clayton θ dissimilarity matrices take the latter into account. UniFrac matrix involves aspects associated to phylogenetic distances between species. Matrices are then graphically visualized using Principal Coordinates Analysis (PCoA) or Non-metric MultiDimensional Scaling (NMDS).
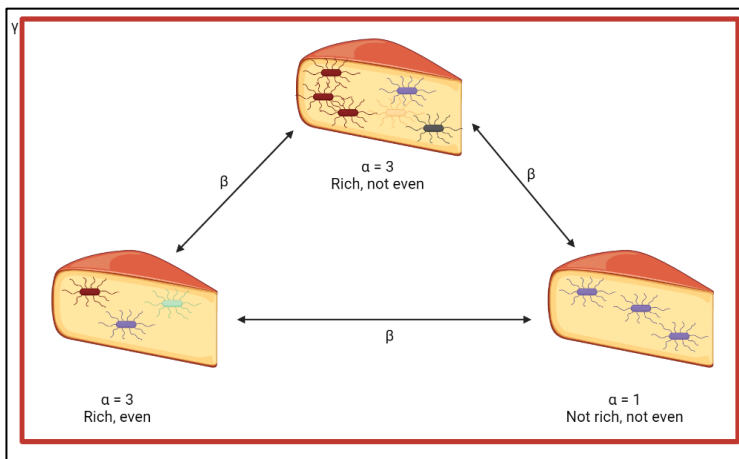
Figure 1-7. α-, β- and γ-diversities for cheese bacterial communities.

3. Metagenomics

Contrary to metagenetics, for which only specific genetic targets are amplified by PCR and sequenced, metagenomics, also known as shotgun sequencing, considers total genetic material present within a given sample. While metagenetics only identifies who is present in the samples, metagenomics also provides a functional potential.

The first important step of this approach is random DNA shearing, resulting in multiple fragments. In this case, libraries for sequencing can be PCR-free, meaning that preparation does not involve PCR amplification steps. Sequencing occurs using NGS, as previously detailed (Illumina, 2021).

When performing metagenomics, bioinformatic work can be trickier. For some applications, sequence reads can be assigned to respective taxa, aiming to characterize global diversity in an environment (including bacteria, archaea, virus and eukaryotes). For other applications, a step of genome assembly is necessary, consisting in reuniting sequences which were fragmented prior to sequencing. Genomes can be assembled based on an available reference, or *de novo* when sequenced for the first time. Prior to assembly, a quality control stage is required, resulting in an eventual trimming, *i.e.* removing of Illumina adapters and of bad quality bases and filtering of raw reads (Dominguez Del Angel *et al*., 2018; Liao and Shi, 2020). Various assemblers are available, including Geneious, SPAdes and Velvet (Zerbino and Birney, 2008; Bankevich *et al*., 2012; Kearse *et al*., 2012). Algorithms behind these assemblers are out of the scope of this manuscript. The mission of these assemblers is to create contigs as long as possible, in terms of number of nucleotide pairs, and to scaffold these contigs, *i.e.* putting contigs in relationship to each other (Dominguez Del Angel *et al*., 2018).

Once assembly is satisfying, genomes have to be annotated, *i.e.* identifying coding sequences and promoters and genes location on the genome as well as their respective function(s). For bacteria, genome annotation is relatively easy, as most parts of the genome, *i.e.* 90%, code for proteins. Regarding structural annotation, the tricky point is to determine the correct reading frame among the six possible, namely three on the sense strand and three on the antisense strand (**Figure** 1-8; Salzberg, 2019). Once genes are located based on available annotated genomes from databases, functions of encoded protein can be predicted based on homologies. This approach is particularly efficient when closely related genomes are already available but is much trickier when working on new species or new genes (Beckloff *et al*., 2012).

When genome is annotated, comparative genomics can be performed, *i.e.* comparing genome in order to explain or predict biological differences. Nowadays, integrated online platforms are available and allow to perform all steps required for genome assembly, genome annotation and comparative genomics, including Pathosystems Resource Integration Center (PATRIC; Wattam *et al*., 2014). The latter platform was used during this thesis (see Chapter 7).
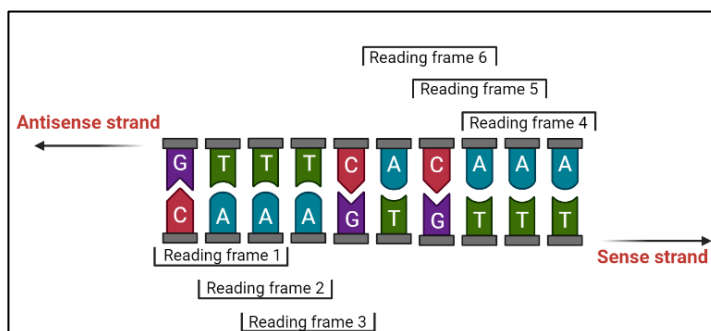
Figure 1-8. All possible reading frames.

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Chapter 2

Objectives

Global introduction of this thesis demonstrated that the presence of *L. monocytogenes* in cheese is still an issue nowadays. Indeed, various listeriosis outbreaks associated to contaminated cheese consumption have been identified during the last 30 years, in all parts of the world. Due to acute danger for people at risk, food safety criteria regarding the presence of *L. monocytogenes* in food are strict, and the non-detection of the pathogen in food allowing its growth is expected before sales. Initially, all cheeses are considered as allowing this growth. Nevertheless, already available conclusions of growth experiments performed with *L. monocytogenes* in cheese are really variety-dependent. In addition to the risk for food safety, the detection of *L. monocytogenes* in food has dangerous moral and economic consequences for producers. Identifying more precisely food representing an effective risk for food safety in case of contamination with *L. monocytogenes* is thus a prior topic. It is important to go beyond absence/presence studies and to focus on the general behavior of *L. monocytogenes* in diverse cheese varieties. Although Belgian cheeses are relatively unknown, cheese manufacture is not a marginal activity in Belgium. The following question directly rises:

"What is the fate of *Listeria monocytogenes* in Belgian artisanal cheeses, and what are the factors explaining its growth or no growth?"

Other questions directly arise from this global research that must be answered before solving this major concern:
- **What are major Belgian artisanal cheese varieties and types?**
- **How are major artisanal cheeses manufactured?**
- **Do physicochemical characteristics of major Belgian artisanal cheeses favorize *L. monocytogenes* growth?**
- **What is the current prevalence of *L. monocytogenes* in major Belgian artisanal cheeses?**
- **What is the growth potential of *L. monocytogenes* in major Belgian artisanal cheeses?**
- **What are bacterial ecosystems of Belgian artisanal cheeses?**
- **Could cheese microbiota exert an inhibiton on the growth of *L. monocytogenes* in some Belgian artisanal cheese varieties?**

The following chapters of the present thesis aim to provide potential answers to questions and objectives listed hereabove. Chapter 3 proposes the results of surveys performed among Belgian artisanal cheese producers, allowing identification of major cheese families and providing global statistics on production processes. The fourth chapter is dedicated to the deeper characterization of a panel of Belgian artisanal cheeses, and to investigations on the prevalence of *L. monocytogenes* in these products. Chapter 5 describes challenge studies performed in order to describe the fate of artificially inoculated cells of the pathogen in representative cheese varieties. Next chapter focuses on cheese microbiota and looks for bacterial species potentially inhbiting the growth of *L. monocytogenes*. The goal of the seventh

chapter was to isolate and characterize a new *Fusobacterium* sp. which has a high relative abundance in three samples of a *Herve* cheese. Finally, Chapter 8 proposes a conclusion and a global discussion on the work performed and on the obtained results.

Chapter 3

Belgian artisanal cheeses and producers

Outline

Before starting investigations on the fate of *L. monocytogenes* in diverse varieties of Belgian artisanal cheese, it was necessary to acquire knowledge on artisanal manufacture in Belgium. Although cheeses from this country are less famous than neighboughring products, traditional cheese production has been rooted in Belgium for centuries, especially associated with monastery, including the famous *Abbaye* cheese (Androuet, 2020). Nevertheless, not many data on the practices for this sector are publicly available. The aim of this chapter is thus to report results of surveys perfomed among artisanal cheese producers. Collected data concerned: dairy farms, milking, cheese manufacture and major cheese families. This preliminary work was necessary to provide an accurate picture of artisanal cheese production in Belgium.

Design of the surveys

1. Survey on breeding and milking practices in Walloon dairy farms

A first survey was conducted among Walloon dairy producers listed in the directory of DiversiFerm, a structure aiming to guide producers wanting to diversify their activities and included in the Laboratory of Quality and Safety of Agro-Food Products of Gembloux Agro-Bio Tech – University of Liège (DiversiFERM, 2020). This work was performed as part of a broader study project aiming to focus on raw milk butter, but collected all data on breeding, stalling, feeding and milking practices in Wallonia, which are of interest for the present thesis.

2. Survey among Belgian artisanal cheese producers

A second survey was conducted among Belgian artisanal cheese producers, listed from a directory provided by FASFC and from the book "Le grand guide des fromages de Wallonie" (Agence wallonne pour la Promodtion d'une Agriculture de Qualité (APAQ-W), 2016). Were considered:

- Dairy farmers directly transforming their own milk;
- Cheese producers buying milk to one or several neighbouring farms.

Globally, 246 producers were listed, from which 177 were from Wallonia and 69 from Flanders, respectively. All of them were contacted by phone to answer a survey on cheeses, manufacture, ripening, packaging and sales. Statistical analyses were performed using Minitab 18 (State College, PA, USA).

Walloon dairy Farms and milking

Results of the first survey are fully detailed by El-Hajjaji *et al.* (2019). Only main outcomes are summarized in the present thesis.

The participation rate was 70% (147 dairy producers out of 211 contacted). **Table 3-1** presents data on breeding and milking practices. These factors are important, as milk is the main ingredient for cheese manufacture. Milk composition, microbiota and properties are influenced by breeding and milking practices.

A majority of farmers only reared one cow breed (70.0%). Major breeds found in farms were Prim'Holstein, Belgian blue and Jersey. More than 71.0% of producers had less than 60 dairy cows. Milking parlours and pipelines were the most used milking equipments. A milking parlour is a room dedicated to milking. Pipelines are used in smaller dairy farms and allow milking directly in housing area (Reinemann and Rasmussen, 2011). Housing areas were generally partly mulched or composed of duckboards. Loose housing was the major stalling system observed in Belgium (72.5%). Most producers (90.0%) used silage as feed. The use of silage, especially of poor quality (*i.e.* pH > 5.0), has been recognized for a long time as a risk factor concerning milk contamination with *L. monocytogenes* (Sanaa *et al.*, 1993; Nucera *et al.*, 2016). However, nearly all cows were brought to pastures during summer seasons.

Table 3-1. Data on breeding and milking in Walloon dairy farms (derived from El-Hajjaji *et al.*, 2019).

Factors	Number of producers and percentage
Cows in production	(n farms = 142)
< 20	*11 (7.7%)*
20-40	*34 (23.9%)*
40-60	*56 (39.4%)*
> 60	*41 (28.9%)*
Milking equipment	(n farms = 142)
Pipeline	*31 (21.8%)*
Bucket milker	*8 (5.6%)*
Robot	*6 (4.2%)*
Milking parlour	*97 (68.3%)*
Stalling system	(n farms = 138)
Loose	*100 (72.5%)*
Tied up	*28 (20.3%)*
Cubicles	*10 (7.2%)*
Housing area	(n farms = 142)
Fully mulched	*26 (18.3%)*
Partly mulched	*88 (62.0%)*
Duckboard	*28 (19.7%)*

Profile of Belgian artisanal cheese producers

Results of this survey were not published. Among 246 Belgian artisanal cheese producers listed, 33 were not contacted, as their phone numbers were not found. Among 213 contacted producers, 21 did not produce cheese anymore. Participation rate to the survey was 74.0% (142 complete answers). Thirty-two of these producers were from Flanders; the remaining 110 being based in Wallonia. Together, they produced 98 and 326 cheese varieties, respectively. A map of Belgian artisanal cheese producers contacted during the suvey was built using QGIS 3.0 (**Figure** 3-1).

Most producers were found in the provinces of West Flanders, Hainaut, Namur and Luxemburg.
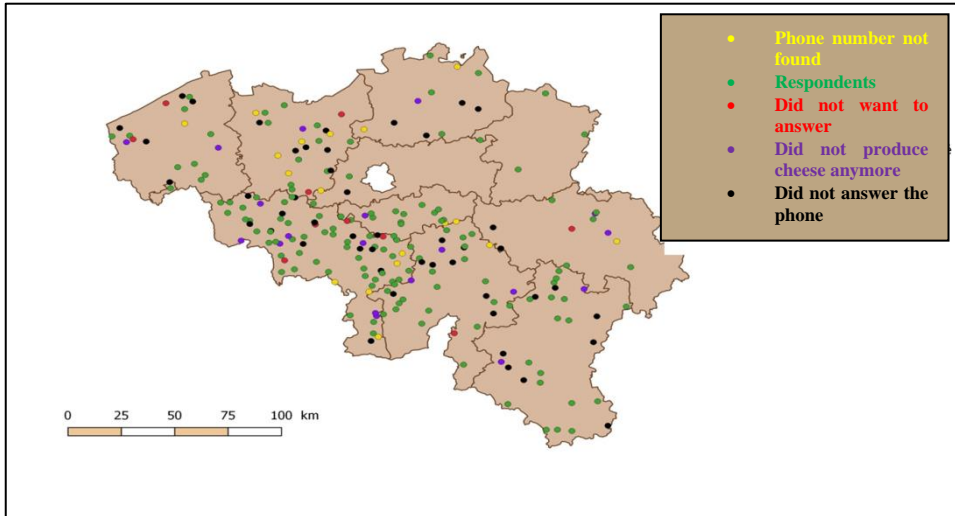
Figure 3-1. Map of Belgian artisanal cheese producers listed during the survey.

The 21 producers who stopped their cheese-related activities were asked for the reasons. A major cause was financial issues, but some producers mentioned the constraints imposed by food safety regulations and by FASFC, including the stress represented by the presence of *L. monocytogenes* in cheese. Such answers highlighted the importance of performing studies to acquire a broader knowledge on the effective threat that *L. monocytogenes* can represent in various types of Belgian artisanal cheeses.

The average age of Belgian artisanal producers was 48.9 ± 11.5 years old. In Flanders, more than 70.0% of producers had more than 20 years of experience in cheese manufacture, while they were only 36.0% in Wallonia. In the latter region, cheese production is thus more recent, and is a new way for farmers to diversify their activities and to generate higher benefits (Lefébure *et al*., 2021). At a national level, 65.0% of the producers followed one or more trainings in cheese manufacture. Less than 20.0% of cheeses were manufactured in facilities where more than two people were working. Finally, only 18.0% of cheese producers were certified organic; a lot of them being afraid of extra administrative constraints.

Milk for cheese production

Milk is the main ingredient in cheese manufacture. An important part of the survey was dedicated to milk (**Figure** 3-2). Among 142 cheese producers who answered the survey, only 10% bought milk to neighbouring farms. It means that most of them were dairy farmers who transformed their milk into products with a higher added value, including cheese, butter and yoghurt. Proportions of the 434 artisanal cheeses produced with cow's, goat's, ewe's or buffalo's milk were 73.8, 18.0, 5.7 and 0.2%,

respectively. Remaining cheeses were made from mixture of milk from two or more animal species. Whole milk was used to produce 70.0% of these 434 cheeses, and 87.0% were made from raw milk.

In Wallonia, 79.0% of the producers transformed milk directly after milking, without cooling and storage, avoiding unnecessary energetic costs associated with milk heating. In Flanders, 60.0% of the producers favoured the use of tank milk.
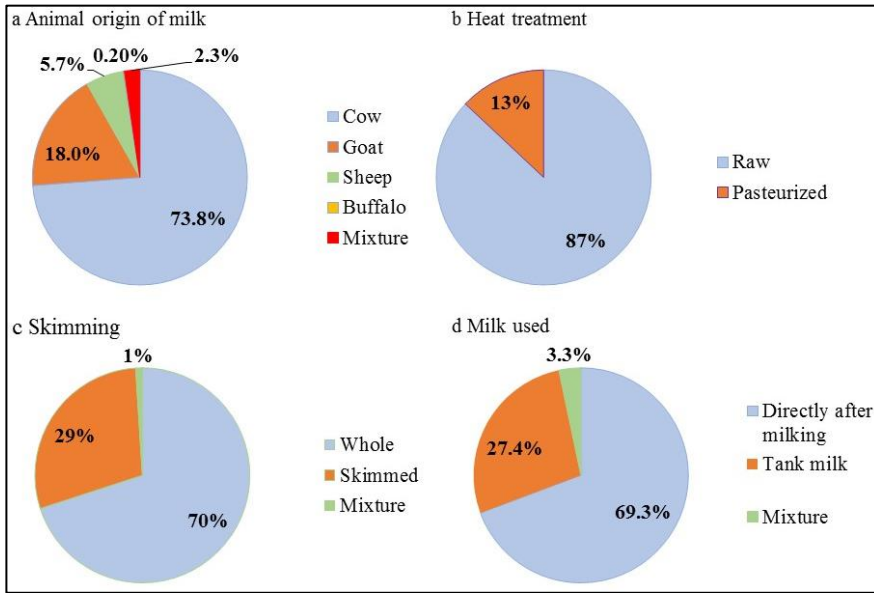
Figure 3-2. Type of milk used for artisanal cheese manufacture in Belgium (animal origin, heat treatment, skimming and eventual refrigerated storage).

Huge variations were observed concerning milk volume used for each cheese manufacture (**Table** 3-2). Wilcoxon-Mann-Whitney's test revealed significant differences between regions (p-value = 1.21 x 10^{-14}), Flemish farmers generally using higher volumes.

Table 3-2. Descriptive statistics related to milk volumes used for one cheese production (L).

Region	Minimum	Median	Average ± s.d.	Maximum
Wallonia	4	100	205 ± 341	3,500
Flanders	25	400	900 ± 903	3,000

Cheese manufacture

1. General aspects

Annual cheese production was highly variable between factories, but no significant differences in the distribution were observed between Flanders and

Wallonia (p-value = 0.28; **Table** 3-3). Most observed frequencies of production were once a week, twice a week, and daily.

Table 3-3. Descriptive statistics related to annual cheese production (kg).

Area	Minimum	Median	Average ± s.d.	Maxium
Belgium	80	1,800	8,010 ± 22,138	160,000
Wallonia	80	1,650	5,828 ± 18,821	160,000
Flanders	150	2,300	16,146 ± 30,815	120,000

Figure 3-3 shows all families of artisanal cheeses produced in Belgium. Major families (in terms of occurrence) met during the survey were uncooked pressed cheeses, mainly *Saint-Paulin*-type SHC (SPSHC) in Wallonia, and *Gouda*-type SHC (GSHC) in Flanders. UACC, including low and full fat *Maquée* and shaped unripened cheeses, represented one third of the products. MRSC and SRSC were also common (more than 12.0% each), contrary to half-cooked and cooked pressed cheeses (< 3.5% together). Only six blue-veined cheeses were identified (*i.e.* 1.6% of all cheeses). Various minor varieties were observed, namely *Ricotta*, *Feta*, *Boulette*, *Mozzarella*, *Mascarpone* and *Halloumi*.
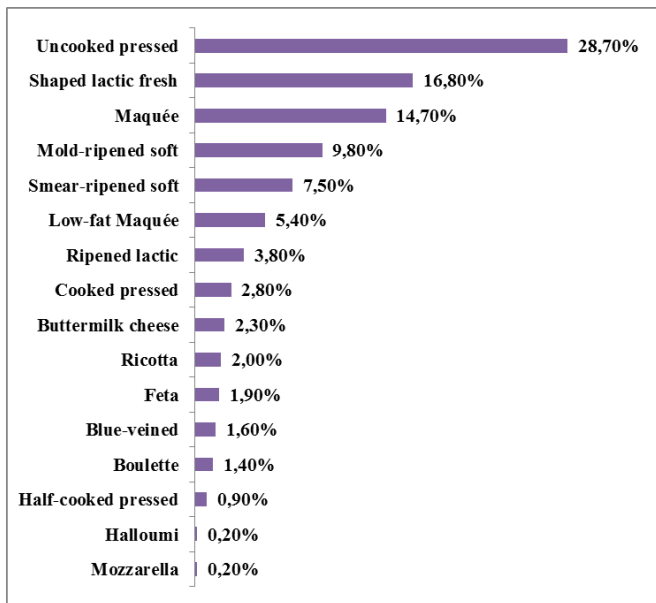
Figure 3-3. Major types of Belgian artisanal cheeses.

2. Curdling

In 92.0% of the 424 cheeses, starter cultures were used. When no starters were added, financial reasons were often mentioned by producers. All commercial starters met during the survey are summarized in **Table** 3-4. Although the number of

commercial starters available is important, they often shared common bacterial species or even subspecies. For curdling, most producers used *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* as mesophilic starters, and *S. thermophilus* as thermophilic starter. *Leuconostoc* spp. were also commonly met. Ripening starters were sometimes added to milk, including *G. candidum* (influence on cheese color), *P. roqueforti* (for ripened blue-veined cheeses), *P. camemberti* (surface microbiota of MRSC) and *B. linens* (surface microflora of SRSC). It is important to note that some producers could have deliberately omitted to mention some starters to preserve manufacturing secrets. Rennet was added in 94.9% of the cheeses. Remaining products are specific varieties which did not required rennet nor starters addition, including buttermilk cheese or *Ricotta*. Rennet dose was often lower than 10 mL/100 L of milk (43.7% of cheeses) or around 30 mL/100 L (40.6% of cheeses), corresponding to lactic and enzymatic curds, respectively.

Table 3-4. List of starters identified during the survey.

Commercial starters	Types	Lactococcus lactis subsp. cremoris	Lactococcus lactis subsp. lactis	Lactococcus lactis subsp. lactis biovar diacetalys	Brevibacterium linens	Streptococcus thermophilus	Leuconostoc mesenteroides	Lactobacillus helveticus	Geotrichum candidum	Penicillium camemberti	Penicillium roqueforti
Choozit GEO17	R								x		
Choozit MA4001	M+T	x	x	x		x					
Choozit MA4002	M+T	x	x	x		x					
Choozit LM57	R						x				
Choozit Probat 222	M+R	x	x	x			x				
Choozit RM32	M+T	x	x	x		x					
Choozit RM34	M+T	x	x	x		x					
Choozit SR3	R				x						
Choozit TA52	T					x					
Flora Danica	M+R	x	x	x			x				
GEO68	R								x		
Hansen PR4	M										
Hansen R-704	M+T	x	x								
PAL Cheese 114D	M+T	x	x			x		x			
PAL 122	R	x	x	x							
PAL LH56	R							x			
PC Neige	R									x	
PC SAM3	R									x	x

Legend: R, ripening starters; M, mesophilic starters; T, thermophilic starters.

3. Draining

As a reminder, the goal of draining is to separate curd from whey, using various techniques, namely curd cutting, heating, stirring, natural draining and pressing.

Half of the 424 cheeses required curd to be cut. Curd was not cut during production of UACC, and some producers of soft cheeses mentioned that this step was not required. Whey was removed and replaced by hot water in 42.0% of the cheese varieties, and the mixture was then stirred to increase syneresis and to decrease lactose content. One third of cheeses were not moulded.

4. Ripening

Half of the cheeses from the survey were unripened. Matured cheeses were ripened in cellar or specific rooms (60.0%) or in modified fridges (40.0%). Ripening duration was majoritary shorter than one month (54.0% of ripened cheeses) or comprised between one and two months (30.0%). Other cheeses (mainly GSHC) were ripened for a longer period, up to several years. One out of five cheeses required the addition of specific ripening starters. Beer was used during ripening to wash 8.0% of cheese varieties met during the survey. Artificial coatings were not used in Wallonia but were placed around all pressed cheeses in Flanders.

Cheese packaging and sales

Whole cheeses are rarely sold, especially in the case of direct sale to consumers. In this case, slices or pieces are preferred. It was difficult to collect data on packaging, as producers were not able to provide enough precision on used materials.

Around 95 % of artisanal cheese producers were concerned by "Business to consumers", with multiple channels, including shops at farms, street markets, agricultural cooperatives and buying groups. "Business to business" also concerned two thirds of the producers. More than the half of the latter delivered their customers themselves. Most producers sell their cheeses in a radius of less than 50 km around the factory, but the survey identified two producers selling cheese up to 500 km from their farm.

Conclusions

The main objective of this chapter was to identify Belgian artisanal cheese producers and major cheese varieties, in order to select representative samples during next steps of this thesis.

Various types of artisanal cheese factories were found in Belgium: some of them were small and only produced UACC, while bigger factories produced up to 160 tons of cheese every year. In the latter, a nearly fully automated production process was implemented, while everything was handmade in smaller factories, including curd cutting and shaping. Huge differences were observed between cheese production practices in Wallonia and in Flanders. Variations started at the beginning of the process, with the use of raw milk directly after milking in Wallonia, and of pasteurized tank milk in Flanders. The major cheese family was the same in both

regions, namely uncooked pressed cheese. However, Flemish cheesemakers produced GSHC, for which cheese wheels were bigger and artificially coated. In Wallonia, SPSHC were found, with a smaller weight and a natural crust, requiring care during ripening.

Based on collected data on cheese manufacture, clustering methods were used to develop an improved classification tool for Belgian artisanal cheeses. However, the resulting classification was really close to the one proposed by Profession Fromager (2020) and detailed in Chapter 2.

The major types of cheese considered during the following chapters of this thesis were:

- Unripened acid-curd cheeses, both *Maquée* and moulded: UACC;
- Ripened soft cheeses, including smear-ripened soft cheeses and mold-ripened soft cheeses: RSC, including SRSC and MRSC;
- Semi-hard cheeses, including *Gouda*-type semi-hard cheeses and *Saint-Paulin*-type semi-hard cheeses: SHC, including GSHC and SPSHC.

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Chapter 4

Characterization of Belgian artisanal

cheeses

Outline

Previous chapter provides the state of the art of artisanal cheese production in Belgium. Following phone surveys, it was possible to identify major cheese families, *i.e.* UACC, GSHC and SPSHC. Before being able to assess the growth potential of *L. monocytogenes* in artisanal cheese varieties, it was necessary to collect more data on their physico-chemical characteristics, especially pH and a_w values. As a reminder, referring to Regulation (EC) No 2073/2005, if cheese has $pH \leq 4.4$, or $a_w \leq 0.92$, or $pH \leq 5.0$ and $a_w \leq 0.94$, it should be considered as not allowing the growth of *L. monocytogenes*. In the latter case, performing expensive challenge studies would be useless.

In order to characterize manufacturing process of major varieties of Belgian artisanal cheeses, 65 factories were visited. For this purpose, three tracking sheets were designed, respectively for UACC, RSC and SH/HC (*i.e.* pressed cheeses). Qualitative and quantitative data on production processes were compiled. Initially, the goal was to use this information to build decision trees allowing the classification of Belgian artisanal cheeses. Rapidly, it was noted that it was not possible to improve existing classifications, especially the one suggested by Profession Fromager (2020).

Following visits in cheese factories, and for one year, samples from 134 batches were collected in 65 artisanal factories. Selection was based on major families identified during the survey presented in Chapter 3, to consider a sample group representative of varieties produced in Belgium.

Another objective was to confirm that the presence of *L. monocytogenes* in Belgian artisanal cheese remains a current issue in Belgium. Prevalence of the pathogen in the 134 batches was evaluated by performing *L. monocytogenes* detection tests. Enumeration was performed in case of detection. Samples were collected during all seasons, as food spoilage by *L. monocytogenes* can be seasonal (Dalzini *et al*., 2016).

Paper: Survey on the prevalence of *Listeria monocytogenes* in Belgian artisanal cheeses

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Contribution of Amaury Gérard to the paper:

Physico-chemical and microbiological analyses were performed by ILVO and QP, respectively. During the study, Amaury Gérard was responsible for contacting artisanal cheese producers, for planning samples collection, as well as for compiling and interpreting results. He was also the main person in charge of the redaction of this paper.

1. Abstract

Description of the subject. Cheese is a vector of *L. monocytogenes*. By default, EC imposes its absence in cheese before sales, but fixes pH and a_w thresholds below which it cannot grow.

Objectives. To study pH and a_w of Belgian artisanal cheeses and the prevalence of *L. monocytogenes* in these products.

Method. Salt content, pH and a_w of 134 cheeses were determined. Absence of *L. monocytogenes* in 25 g of cheese was also checked.

Results. Three samples had pH or a_w under threshold values from Regulation (EC) No 2073/2005. Nevertheless, all unripened cheeses were acidic in comparison with data from foreign countries. *L. monocytogenes* was isolated from 1.49% of the samples.

Conclusions. Belgian artisanal cheeses could allow the growth of *L. monocytogenes*, and the bacterium was isolated from two samples. Further experiments should be performed to understand the fate of the pathogen in these products.

Keywords. Cheese, Physico-chemical properties, *Listeria monocytogenes*, Surveys, Belgium, Regulations.

2. Introduction

Each Belgian eats 14.5 kg of cheese per year (Agriculture et Agroalimentaire Canada, 2018). Cheese can be the vector of *L. monocytogenes*, which is responsible for listeriosis, a foodborne disease of which 2,549 cases were reported in Europe in 2018. The same year, case fatality of listeriosis was 15.6%. This foodborne disease is thus dangerous, especially for people at risk, including neonates, pregnant women and old or immunocompromised people (EFSA-ECDC, 2019).

Considering the risk for food safety, criteria regarding the presence of *L. monocytogenes* in RTE foods are strict, especially before sales by producer (EC, 2005). This bacterium is known to be able to survive or even to grow into a lot of cheeses, including *Brie*, *Camembert*, *Cottage* cheese, *Gorgonzola* and S*aint-Nectaire* (Gérard *et al.*, 2018). Currently, only a few cheeses available on the market can be considered as not allowing the growth of *L. monocytogenes* (*i.e.* as belonging to category 1.3 from Regulation (EC) No 2073/2005, *i.e.* pH $\leq$ 4.4 or $a_w \leq$ 0.92 or pH $\leq$ 5.0 and $a_w \leq$ 0.94), namely fresh cheeses with a sufficiently low pH and hard cheeses with $a_w <$ 0.92. All other cheeses are considered as allowing the growth of the pathogen and belong thus to category 1.2. Consequently, producers must guarantee that *L. monocytogenes* remains undetected in cheese before it is put on the market (EC, 2005).

Provided that *L. monocytogenes* is a ubiquitous bacterium, the latter criterion is not easy to fulfil. The consequence is an intense pressure on artisanal producers, for which the presence of the pathogen in cheese can have harmful moral and financial consequences. It seems thus important to focus more on the issue of the presence of

L. monocytogenes in Belgian artisanal cheeses. Belgian cheeses, including *Herve* and *Maquée*, remain unstudied. Given the lack of knowledge regarding these products, they cannot be classified with precision into categories from Regulation (EC) No 2073/2005. A first step was to focus on the prevalence of *L. monocytogenes* in Belgian artisanal cheeses. Their pH and a_w were also investigated and put in relation with threshold values from European Regulation.

3. Methods

1. Sampling

Between January and December 2018, 134 cheeses were sampled in 65 Belgian farmhouses. Each of these farmhouses was visited between one and four times. Animal origin of milk, milk heat treatment, and type of cheese were considered (**Table** 4-1). Classification of cheeses was based on texture and ripening, considering UACC, ripened soft cheeses and SH/HC (Codex Alimentarius, 2006). As presented in **Table** 4-2, sampling covered a whole year for each type of cheese. Sampling was based on the results of a survey conducted on 130 Belgian artisanal producers (see Chapter 4). From this survey, major cheese types were identified. For UACC, considered subtypes were: (a) full-fat *Maquée*, a UACC obtained from whole milk after at least 24 h of lactic curdling and packaged in plastic punnets, (b) low-fat *Maquée*, a *Maquée* produced from skimmed milk, and (c) other unripened cheeses, including shaped UACC. Three main subtypes of RSC were distinguished, namely (a) SRSC, unpressed cheeses regularly washed during ripening, resulting in a typical red rind, (b) MRSC, unpressed cheeses with a typical white rind composed of *P. camemberti* and/or *G. candidum* and (c) blue-veined cheeses, presenting *P. roqueforti* in their core. Considered subtypes of SH/HC were: (a) GSHC, unpressed SHC surrounded by an artificial coating and ripened for several month, (b) SPSHC, also known as *Abbaye*, unpressed SHC with a natural rind and ripened for a shorter period (*i.e.* at least three weeks) and (c) other SH/HC comprising half-cooked and cooked pressed cheeses.

Table 4-1. Sampling plan (n=134).

Factor	Number of samples
Type of milk	
Bovine	*105*
Caprine	*20*
Ovine	*9*
Milk treatment	
Raw	*103*
Pasteurized	*31*
Type of cheese	
UACC	*38*
Full-fat Maquée	*16*
Low-fat Maquée	*10*
Others	*12*
Ripened soft cheeses	*40*
SRSC	*16*
MRSC	*23*
Blue-veined	*1*
SH/HC	*56*
GSHC	*21*
SPSHC	*23*
Others	*12*
Province	
Flanders	*38*
Antwerp	*8*
East Flanders	*13*
Flemish Brabant	*4*
Limburg	*3*
West Flanders	*10*
Wallonia	*96*
Hainaut	*30*
Liège	*19*
Luxemburg	*13*
Namur	*27*
Walloon Brabant	*7*

Table 4-2. Monthly distribution of sampling by type of cheese.

Type of cheese	J	F	M	Ap	Ma	Ju	Jl	Au	S	O	N	D	Tot
UACC	1	8	11	0	4	0	5	1	2	2	3	1	38
RSC	6	2	4	2	0	0	4	1	3	6	8	4	40
SH/HC	2	4	10	3	2	0	2	5	9	10	4	5	56

2. *L.* monocytogenes detection and enumeration

To detect *L. monocytogenes*, a pre-enrichment step was performed by incubating 25 g of cheese diluted in half-Fraser broth (Led Techno, Heusden-Zolder, Belgium) for 24 h at 24°C, followed by isolation on RAPID'*L.mono* plates, after incubation at 37°C for 24 h (± 2 h). Suspect colonies were confirmed on ALOA (Bio-Rad, Hercules, USA). For enumeration, samples were diluted (1:10) in buffered peptone water (BPW; Led Techno, Heusden-Zolder, Belgium) and incubated at 20°C for 1 h. Then, 100 µL and 1 mL of this suspension were spread on RAPID'*L.mono* plates and incubated at 37°C for 24 h (± 2 h).

3. Physico-chemical analyses

For all samples, pH was measured in the core using InLab Surface Pro-ISM electrode (Mettler Toledo, Colombus, OH, USA) and a_w using Aqualab 4TE water activity meter (Decagon Devices Inc., Pullman, WA, USA). For ripened cheeses, pH was also measured on the crust. Salt content was determined following ISO 5943:2006 method (International Organization for Standardization (ISO), 2006).

4. Statistical analyses

All statistical analyses were performed using Minitab 18 (State College, PA, USA). The number of cheeses differed between types. Generalized linear model (GLM) were built to look for significant differences. Tukey's test was performed for pairwise comparisons. Kruskal-Wallis test was performed when variance homogeneity or data normality were not fulfilled, and Dunn's test was used for multiple comparisons. Boxplots were built using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA).

4. Results

Figure 4-1 summarizes physico-chemical analyses performed on the 134 samples. In cores, pH range of UACC was limited (4.2–4.9) while this was more variable for other categories (**Table** 4-3). Significant differences (p-value < 0.001) were observed between UACC and all ripened cheeses. On the surface, pH was always higher than in the core. Variability of a_w was limited but the averages differed significantly between all categories (p-value < 0.001). Salt content was comparable between RSC and SH/HC (p-value = 0.394), while that of UACC was significantly lower (p-value < 0.001). *L. monocytogenes* was isolated from two samples, resulting in a prevalence of 1.49%. Both samples were made from raw milk and were collected in two cheese factories. One of the contaminated samples was a SPSHC made from bovine milk (pH = 5.32, a_w = 0.98), with contamination under 1 $\log_{10}$ cfu/g. The second sample was a MRSC made from ovine milk (pH = 7.57, a_w = 0.97). Contamination level of the latter sample was 4.68 $\log_{10}$ cfu/g.
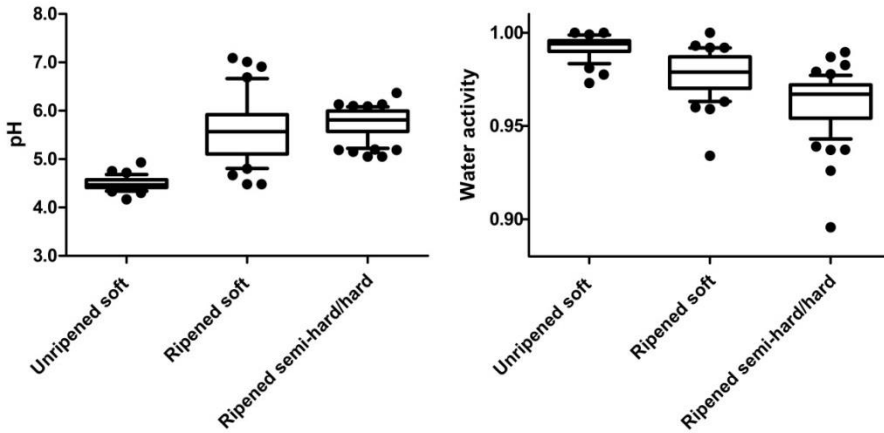
Figure 4-1. Boxplots of core pH and a_w for UACC, ripened soft cheeses and SH/HC.

Table 4-3. Physico-chemical characteristics of the collected cheeses (average ± standard deviation).

Type of cheese	No. of samples	pH core[a]	pH surface[a]	a_w[b]	Salt content (%)[c]
UACC	38	4.5±0.3	/	0.99±0.01	0.4±0.4
RSC	40	5.6±0.1	6.9±0.7	0.98±0.01	1.8±0.7
SH/HC	56	5.6±0.3	6.8±0.7	0.96±0.02	1.7±0.6

Legend: [a]electrode; [b]chilled mirror dew point electrode; [c]ISO 5943; /, pH was not measured on the surface of UACC.

5. *Discussion*

UACC had pH values lower than those commonly reported for unripened cheeses. UACC studied in this paper were prepared by adding starters to milk and by maturing this mixture for at least one day, resulting in a pH between 4.4 and 5.0 (Goudédranche *et al.*, 2001). Many available papers focused on Hispanic unripened cheeses, generally having pH > 5.0 (Torres-Vitela *et al.*, 2012; Soto Beltran *et al.*, 2015). To our knowledge, only one paper studied UACC and reported a prevalence of *L. monocytogenes* of 0% (Reda *et al.*, 2016). It was also the case during the present study. In comparison, prevalence above 10.0% was commonly reported in Hispanic unripened cheeses (Torres-Vitela *et al.*, 2012; Soto Beltran *et al.*, 2015). For other types of cheeses, pH and a_w were similar to data found in the literature, excepting one SH/HC with a_w 0.89 (Gérard *et al.,* 2018). Such a low value has never been reported, although $a_w \leq 0.92$ has already been observed (Prencipe *et al.*, 2010). Salt contents measured during this study (< 2% of salt) were comparable to values provided by Gobbetti *et al.* (2018) in their book on Italian cheeses. Ibarra-Sanchez *et al.* (2018) found comparable salt contents for *Chihuahua*, *Manchego*, *Adobera* and *Queso Fresco* but *Sao Jorge* and *Cotija* were more salted (> 4.0% of salt) (Kongo *et*

al., 2006). According to Irlinger and Spinnler (2020) percentage of salt in cheese can sometimes be as high as 7.0%. In vitro, *L. monocytogenes* coud be able to grow at salt concentrations such as 10% (Ferreira *et al.*, 2014). Furthermore, it has already been observed that *L. monocytogenes* was able to survive for several months in cheese brines with more than 20% of salt (Larson *et al.*, 1999; Brown *et al.*, 2018). Consequently, salt content of Belgian artisanal cheeses, as well as pH and a_w, cannot be considered as natural hurdles to the growth of *L. monocytogenes*.

The prevalence of *L. monocytogenes* observed during this study (1.49% of 134 samples) is higher than data reported by some papers on *Asiago* (0.2% of 449), *Chihuahua* (0.0% of 60) or *Sao Jorge* (0.0% of 66). However, the latter studies only considered SH/HC (Alcazar *et al.*, 2006; Kongo *et al.*, 2006; Prencipe *et al.*, 2010). The prevalence of *L. monocytogenes* observed for Belgian artisanal cheeses in this survey was also higher than figures reported by EFSA-ECDC for EU cheeses in 2018, *i.e.* 0.3% (EFSA-ECDC, 2019). More than 30,000 samples were considered by the latter report. In comparison, more than 200 times less samples were considered by the present paper, which could explain the higher prevalence. Indeed, in case of reduced sampling size, a single contaminated cheese has an increased impact on the percentage. In 2018, 247 artisanal cheese producers were listed in Belgium. By collecting samples in 65 factories, around one out of four cheese producers were concerned by the study. It could have been interesting to focus more on production volume of each cheese subtype in order to properly take this factor into account when designing the sampling plan. Nevertheless, it seems important to focus on all types of cheeses found on the Belgian market, provided that they are susceptible to be contaminated by *L. monocytogenes* and eaten by consumers.

In the present survey, both contaminated samples were made from raw milk. However, a meta-analysis based on recent EFSA reports showed no significant differences in the occurrence of *L. monocytogenes* between cheeses produced from raw or pasteurized milk (Martinez-Rios and Dalgaard, 2018). The contaminated SH/HC sample was a SPSHC produced by a farmer in a shared processing facility. Enumerated *L. monocytogenes* levels were < 10 cfu/g. The pathogen was not isolated from other cheeses produced in the same workshop. A hypothesis could be that the bacterium was already present in raw milk or that it was transmitted to cheese during post-processing steps. Indeed, re-contamination during post-processing handlings or during ripening is a frequent transmission route (Schvartzman *et al.*, 2011; Ibarra-Sanchez *et al.*, 2017). Levels under the enumeration limit of method ISO 11290-2 (*i.e.* < 10 cfu/g) have already been reported during prevalence studies on SH/HC (Gérard *et al.*, 2018). During random controls performed in 2017-2018 by FASFC, all identified contaminated SH/HC had *L. monocytogenes* levels under 10 cfu/g (unpublished results). Such a contamination at the beginning of the storage of a SH/HC sample should not necessary be considered as a threat for food safety. Indeed, if the production process of some SH/HC, including *Cantal*, is known to allow the growth of *L. monocytogenes*, the extended ripening period has an inhibiting effect on the pathogen (Chatelard-Chauvin *et al.*, 2015). During refrigerated storage, no growth was observed anymore

in *Chihuahua*, *Edam*, *Gouda* and *Manchego* (Gérard *et al.*, 2018). Nevertheless, as each cheese has its proper characteristics, further investigations should be performed to know the fate of *L. monocytogenes* in this sample, for instance using challenge studies.

The second contaminated sample identified during this study was a MRSC made from raw ovine milk. In this case, the producer bought milk from a dairy farmer and transformed it in its own facility. Observed *L. monocytogenes* level was 4.68 $\log_{10}$ cfu/g. Such a high contamination is worrying regarding food safety but is not unprecedented for RSC (Bernini *et al.*, 2013; Rakhmawati *et al.*, 2013, Thisted Lambertz *et al.*, 2012). For instance, levels of 4 $\log_{10}$ cfu/g were identified in a RSC involved in a Canadian listeriosis outbreak, while levels up to 6 $\log_{10}$ cfu/g have been observed during an outbreak associated with *Camembert* in Norway (Johnsen *et al.*, 2010; Gaulin *et al.*, 2012). Ripened soft cheeses are generally considered as the riskiest cheese family regarding *L. monocytogenes*, due to their highly favourable a_w and pH, especially on their rind. For instance, pH higher than 7.0 has been reported on the surface of *Brie* and *Camembert* (Gérard *et al.*, 2018). A contamination of cheese surface generally results from a transfer of *L. monocytogenes* during post-processing steps. Nevertheless, during this study, the contamination was identified in cheese core, meaning that this hypothesis was not the most suitable. Further investigations showed that the ovine milk used to produce this cheese contained 3.48 $\log_{10}$ cfu/mL of *L. monocytogenes*. Milk was analyzed again one week later and was not contaminated anymore.

6. *Conclusion*

Given that most Belgian artisanal cheeses have pH > 4.4 and/or a_w > 0.92, they should be considered as allowing *L. monocytogenes* to grow, following Regulation (EC) No 2073/2005. Nevertheless, it is known that other factors can inhibit its growth in cheese, including concentration of organic acids and endogenous microbiota, with some species producing antimicrobial compounds like bacteriocins. Each cheese has its own physico-chemical and microbiological characteristics. To avoid the intense pressure on Belgian cheese producers in case of detection of *L. monocytogenes*, further studies should be implemented, including challenge studies. The goal of such experiments is to know the fate of *L. monocytogenes* in artificially contaminated cheese samples. The initial contamination should be 100 cfu/g. Samples are stored in the fridge until end of shelf-life. The pathogen is then enumerated and δ can be calculated by comparing levels at end of shelf-life and day-0. If δ ≤ 0.5 $\log_{10}$ cfu/g, cheese is considered as not suitable for the growth of *L. monocytogenes* (category 1.3 from Regulation (EC) No 2073/2005). Levels up to 100 cfu/g are then tolerated before cheese is put on the market, decreasing pressure on artisanal producers. However, despite this tolerance, good hygiene practices and HACCP still must be applied in order to minimise the presence of *L. monocytogenes* in cheeses and workshops.

Complementary information

In addition to pH and a_w, other physico-chemical parameters were also determined for all cheese samples, namely dry matter and fat contents, but these measurements were not included in the article presented hereabove. These data are summarized in **Table** 4-4. Dry matter was determined according to ISO 5534 method. Fat content was assessed following ISO 3433 method. Briefly, fat was treated with chlorhydric acid and extracted with petroleum ether and diethyl ether. Dry matter content was statistically different between all types of cheese (all p-values < 0.001). Nine low-fat cheeses were included in the panel, skewing average fat content of UACC, which was significantly lower than for other cheese types. Considering only products made from whole milk, no statistical differences were observed between cheese types (p-value > 0.050). Fat contents measured during this study were comparable to values provided by Gobbetti *et al.* (2018) in their book on Italian cheeses.

Table 4-4. Physico-chemical characteristics of the collected cheeses (average ± standard deviation).

Type of cheese	No. of samples	Average dry matter content ± s.d. (%)[a]	Average fat content ± s.d. (%)[b]
UACC	38	27.4 ± 10.4	38.0 ± 21.9
RSC	40	48.8 ± 5.8	51.3 ± 3.7
SH/HC	56	60.1 ± 5.5	52.3 ± 2.2

[a]ISO 5534, [b]ISO 3433.

As described in the article, two contaminated batches were identified. All samples from the contaminated SH/HC batch had *L. monocytogenes* levels under 10 cfu/g. However, a great heterogeneity was observed concerning MRSC batch. For the latter one, ten cheeses were analyzed in triplicate (**Table** 4-5). Levels between < 10 and 3,400,000 cfu/g were observed. This phenomenon was already described, but it raises questions regarding sampling for detection of *L. monocytogenes* in routine analyses. Indeed, differences were observed between cheese pieces, but also within a given piece (for instance piece n°6).

Table 4-5. Enumerations in triplicate for each of the ten cheeses from the contamination MRSC batch, performed on 3 x 25 g (cfu/g).

Cheese	Enumeration 1	Enumeration 2	Enumeration 3
1	< 10	< 10	< 10
2	< 10	< 10	< 10
3	< 10	< 10	< 10
4	< 10	< 10	< 10
5	< 10	< 10	< 10
6	< 10	16,000	17,000
7	20,000	120,000	3,400,000
8	150,000	170,000	810,000
9	2,100,000	2,300,000	2,900,000
10	3,000,000	3,300,000	3,400,000

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Chapter 5

Assessment of the growth potential of *L. monocytogenes* in Belgian artisanal cheeses

Outline

During previous chapters, a better knowledge of various types of Belgian artisanal cheeses and of their production processes has been acquired. A sampling plan was thus designed to select the most representative cheese families, namely UACC and SHC. SRSC and MRSC were also included in the panel. The aim was to assess the growth potential of *L. monocytogenes* in these artisanal products using challenge studies, *i.e.* from artificial contamination of cheese and comparison of the levels at day-0 and at end of shelf-life. The following article will describe in detail the protocol followed during these challenge studies as well as the main results. Aside from the article, durability studies, based on naturally contaminated samples, were also performed. Results are presented in the section "Complementary information" of this chapter.

Paper: Determination of the growth potential of *Listeria monocytogenes* in various types of Belgian artisanal cheeses by challenge tests

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Contribution of Amaury Gérard to the paper:

Physico-chemical and microbiological analyses were performed by ILVO and QP, respectively. During the study, Amaury Gérard was responsible for the coordination of the work (including contacts with artisanal cheese producers, planification of cheese collection, planification of analysis, management of cheese storage) and for compilation, analysis and interpretation of results. He was also the main writer of the paper.

1. Abstract

Cheese potentially allowing the growth of *L. monocytogenes* must be free of the pathogen in 25 g before being put on the market, while a level of 100 cfu/g is tolerated when the pathogen is unable to grow during shelf-life. Challenge studies were performed in order to assess the growth potential of *L. monocytogenes* in at least one batch of 32 Belgian cheese varieties from 32 factories. All varieties were grouped in four categories: UACC, MRSC, SRSC and SHC (comprising GSHC and SPSHC). Associated microbiota and cheese physico-chemical characteristics were also studied. A cocktail of three strains was used to inoculate cheese at day-0, and samples were stored until end of shelf-life at 7-9°C. Growth potential was considered as the difference (a) between median contamination at the end and at the beginning of the test or (b) between the highest value at the end of the test and the lowest value at its beginning. *L. monocytogenes* always decreased in UACC but showed extended growth in 21 out of 25 batches of ripened soft cheeses. Contrasting results were obtained for SHC, as important intra- and inter-batch variability was observed. For the latter, the recommended method based on medians to calculate the growth potential led to erroneous food safety considerations, and it should always be advised to focus on absolute levels.

Keywords: Challenge test, *Listeria monocytogenes*, Cheese, Growth potential, Intra-batch variability, Inter-batch variability.

2. Introduction

L. monocytogenes is a Gram-positive, facultative anaerobic bacterium belonging to the Firmicutes phylum. This pathogen is responsible for a foodborne disease called listeriosis. During 2018, 2,549 cases of listeriosis were reported by EU member states. Listeriosis is thus the fifth most prevalent foodborne disease in EU, after campylobacteriosis (246,571 cases), salmonellosis (91,857 cases), STEC infections (8,161 cases) and yersiniosis (6,699 cases). More worrying, an increase in the number of cases has been observed in the past few years (EFSA-ECDC, 2019). In addition to that, the mortality rate of listeriosis can be as high as 20 to 30%. The majority of the population would only face diarrhea in case of contamination with *L. monocytogenes*, but for people at risk, including neonates, pregnant women and immunocompromized or elderly people, much more harmful consequences can be expected. Symptoms include septicaemia, abortion, stillbirth, meningitis and damage to nerves (Buchanan *et al*., 2017; Ibarra-Sanchez *et al*., 2017; Sanaa *et al*., 2004). Various foods have already been identified as potential vectors of *L. monocytogenes*, especially RTE foods, including cheese. As listed by Martinez-Rios and Dalgaard (2018), several foodborne outbreaks linked to contaminated cheese have already been identified. These outbreaks are mainly associated with contaminated unripened cheese, mainly from Hispanic countries, or with contaminated RSC (Ibarra-Sanchez *et al*., 2017; Martinez-Rios and Dalgaard, 2018).

Criteria regarding the presence of *L. monocytogenes* in RTE foods are strict. Following Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs,

L. monocytogenes should not reach a contamination level > 100 cfu/g during shelf-life. Furthermore, before placing a RTE food allowing its growth on the market, the pathogen must remain undetected in 25 g. Based on data available on the growth/no growth of *L. monocytogenes* in food, this regulation also identifies three situations in which one can consider that the growth of *L. monocytogenes* is not permitted. Consequently, pH ≤ 4.4, a_w ≤ 0.92, or a combination of pH ≤ 5.0 with a_w ≤ 0.94 are considered sufficient to prevent growth of the pathogen. When a RTE food is not considered as allowing this growth, a contamination level of 100 cfu/g is tolerated before placing the food on the market.

Cheese is generally consumed without any preparation and is thus considered as RTE food. Consequently, it must comply with Regulation (EC) No 2073/2005. Numerous cheese varieties exist worldwide. Products vary in terms of production process, but also in terms of physicochemical properties (Ibarra-Sanchez *et al*., 2017). Indeed, in their review, Gérard *et al*. (2018) reported for instance pH from 4.2 to 7.3 in unripened cheeses, combined with a_w > 0.99. For crusts of RSC and SH/HC, including A*siago*, *Brie*, *Camembert* and *Gorgonzola*, pH > 7.5 has been reported, due to the development of the surface microflora and to its metabolic activities (Irlinger *et al*., 2015; Prencipe *et al*., 2010).

Cheese samples presenting conditions unfavourable for the growth of *L. monocytogenes* are very scarce (Gérard *et al*., 2018). As *L. monocytogenes* is a ubiquitous bacterium, to produce cheese free of the pathogen remains a topical challenge. Nevertheless, the presence of the bacterium in cheese does not necessary mean that it will be able to grow or even to survive. A decrease in the contamination with *L. monocytogenes* was, for instance, observed during ripening of *Minas* traditional *Serro* cheese, a SHC from Brazil, with pH comprised between 4.5 and 4.9 (Pinto *et al*., 2009). The same phenomenon was reported during storage of *Graviera* cheese with pH 5.6 and a_w 0.95. In this study, a decrease in *L. monocytogenes* viability was observed when storage temperature was increased to 12 and 25°C (Giannou *et al*., 2009).

Besides the physico-chemical characteristics of cheese, predictive models and comparison with the scientific literature also allow estimation of the fate of *L. monocytogenes* in a given cheese. Nevertheless, traditional and/or artisanal cheeses are sometimes obtained by a particular production process, or present specific characteristics. In Belgium, more than 230 artisanal cheesemakers have been identified during a survey, producing some specific traditional products like *Maquée*, *Boulette*, *Abbaye* and *Herve* (unpublished results). It is thus difficult to use growth models or the literature to assess if these cheeses could permit the growth of *L. monocytogenes* (Alvarez-Ordonez *et al*., 2015). Regulation (EC) No 2073/2005 allows cheesemakers to demonstrate, to the satisfaction of the competent authority, that *L. monocytogenes* is not able to grow and exceed a contamination of 100 cfu/g in their products. In this case, contamination up to 100 cfu/g before sales is tolerated (EC, 2005). Several studies can be performed by the producers to reveal the fate of *L. monocytogenes* in cheese, including challenge studies and durability studies. In EU, various documents are available for food business operators in order to perform

challenge studies, namely guidance documents published by DG SANCO (2008) and EURL *Lm* (2014). In Belgium, FASFC (2016) also published a scientific opinion related to challenge studies and shelf-life studies for *L. monocytogenes* in cheese.

Challenge studies allow to assess δ of *L. monocytogenes* in artificially contaminated cheeses under abuse conditions of storage (Beaufort, 2011; Alvarez-Ordonez *et al.*, 2015). Durability studies represent an alternative to challenge studies; they are more realistic, but also more difficult to implement. Indeed, such experiments require naturally occurring contaminations. Another alternative is to manufacture cheese using artificially contaminated milk. One of the drawbacks of this option is the challenge of adjusting the level of the inoculum to reach a final contamination of around 100 cfu/g of cheese. In addition to that, a pilot-scale laboratory fully equipped for cheese production is required, with biosafety level 2 (FASFC, 2016).

The goal of this study was thus to assess the growth potential of *L. monocytogenes* in a sample group of artisanal cheeses by performing challenge studies.

3. Methods

1. Sampling

Previously, a survey of artisanal cheese producers allowed the identification of major cheese types produced in Belgium. A sampling plan was designed in order to select 32 cheeses, representative of the diversity of products found in Belgium, from 32 farmhouses. All batches were collected between July 2018 and March 2019. The classification of cheeses was based on texture and/or ripening, as suggested by the Codex Alimentarius (2006). The study considered (a) UACC, acidified cheeses consumed without any ripening, (b) MRSC, unpressed cheeses with a typical white crust mainly composed of *P. camemberti*, (c) SRSC, unpressed cheeses regularly washed with water, brine or smear (a solution including water, salt and specific starters) during ripening and with a typical red crust, and (d) SHC, pressed cheeses with MFFB > 54. Hard cheeses (MFFB < 54) are uncommon in Belgium and were not included in the sampling plan. For each type of cheese, products made from pasteurized milk and from raw milk were considered.

2. Determination of the number of batches

Before collection of whole batches, isolated samples of each cheese were collected to measure their pH and a_w. Teoretical growth potential (δ_{th}) of *L. monocytogenes* in each cheese was predicted using Sym'Previus (Leporq *et al.*, 2005). Selected storage conditions were the same as described in detail in section 3.4 for challenge studies. As advized by EURL *Lm* (2014), it was decided to collect one batch if $\delta_{th} \leq 0$, and three batches if $\delta_{th} > 0$. For each batch, at least 12 samples were collected directly after production or after ripening, for unripened and ripened cheeses, respectively.

3. Cocktail of strains

To avoid bias associated with the use of a unique strain of *L. monocytogenes*, a cocktail of three strains was used to inoculate cheeses. The three selected strains,

namely 12MOBO53LM, 12MOBO96LM and 12MOBO98LM, were isolated from dairy products and were provided by EURL *Lm* for use during challenge studies (EURL *Lm*, 2013). Cryobeads containing individual strains were provided by EURL *Lm*. The latter were suspended separately in 9 mL of brain heart infusion (BHI broth) and incubated at 37 °C for 18 h. One hundred µL of this culture was diluted into 9.9 mL of BHI broth and incubated at 7 °C for 7 days. Equal quantities of the subculture containing each strain were mixed in a unique tube.

4. Inoculation

Among the 12 samples of each batch, six were inoculated with the cocktail of strains. This moment was considered as day-0. Remaining samples were used as controls. The targeted inoculum level was 100 cfu/g of cheese, as advised by FASFC (2016). The inoculation procedure varied between types of cheese. White cheeses were homogenized directly after inoculation. Other UACC were more solid but had no crust and were considered as homogeneous. The cocktail of strains was thus only inoculated in the core, in a single injection. Crusts of SRSC and MRSC are generally eaten by consumers. It was decided to inoculate both core and surface for these types of cheese, by dividing the global inoculum. *L. monocytogenes* was only inoculated in the core of SHC. Some SHC have an artificial and inedible coating on their surface and discerning the difference between artificial and natural crusts could sometimes be tricky for consumers. The volume of inoculum did not exceed 1% of the cheese mass (EURL *Lm*, 2014). Depending on the samples and on the concentration of the mixed cultures, proper dilutions of the latter were thus required. Cheeses were cut into pieces of at least 50 g. Cores were inoculated with a single injection. For inoculation on the surface, the volume was divided into small droplets on the surface and spread with a sterile spreader. Inoculation was judged as satisfactory when standard deviation of triplicate counts of *L. monocytogenes* for inoculated samples at day-0 was < 0.5 $\log_{10}$ cfu/g.

5. Storage

Three inoculated samples and three controls were directly analyzed at day-0 (see sections 3.6 and 3.7). White cheese was stored in its original container. All other types of cheese were wrapped in polyethylene film. Given that the term 'cheese' includes a huge variety of products, it was not possible to use the same storage scheme during all challenge studies. As an example, UACC can generally not be stored for more than 14 days, while SRSC, MRSC and SHC can be kept for at least 30 days at refrigeration temperature. During challenge studies, storage duration followed the recommendations provided by each producer. As advised by EURL *Lm* (2014) and FASFC (2016), samples with a shelf-life ≤ 21 days were always stored at 7 °C for two-thirds of shelf-life, before being stored at 9 °C for the remaining third of shelf-life. When shelf-life was > 21 days, samples were stored at 7 °C for the first half of shelf-life, and at 9 °C for the second half. At the end of shelf-life, all remaining inoculated and control samples were analyzed.

6. Physicochemical analyses

At day-0 and end of shelf-life, physico-chemical characteristics of cheese samples were studied. In cheese cores, pH and a_w were measured with InLab Surface Pro ISM electrode (Mettler Toledo, Columbus, OH, USA) and Aqualab 4TE water activity meter (Decagon Devices Inc., Pullman, WA, USA). ISO method 5534 was used to determine dry matter content (ISO, 2004b). Salt and fat contents were only tested at D0, since it was assumed that these parameters stayed the same relative to the dry matter content during shelf-life. Potentiometric titration of chloride ions with 0.1 M silver nitrate allowed to determine salt content (ISO, 2006). Fat was treated with hydrochloric acid and extracted with petroleum ether and diethyl ether (ISO, 2004a).

7. Microbiological analyses

Microbiological characteristics of all products were studied at D0 and at ESL. To detect and enumerate *L. monocytogenes* in cheese samples, RAPID'*L. mono* methods were used. Briefly, after pre-enrichment by diluting whole cheese pieces 10-fold in Half-Fraser broth (Led Techno, Heusden-Zolder, Belgium) and incubation at 30°C for 24 h, *L. monocytogenes* colonies were isolated on RAPID'*L. mono* plates (Bio-Rad, Hercules, CA, USA) and incubated at 37°C for 24 h. To confirm suspect colonies, a subculture was performed on Agar *Listeria* accoarding to Ottaviani and Agosti (ALOA; Bio-Rad, Hercules, CA, USA). For enumeration, after dilution (1:10) of the samples in BPW (Led Techno, Heusden-Zolder, Belgium) and incubation at 20°C for 1 h, volumes of 100 µL and 1 mL of this suspension were spread on the surface of three RAPID'*L. mono* plates. These Petri dishes were incubated at 37°C for 24 h before enumeration.

For all other microbiological analyses, 25 g of control cheeses was suspended in 225 mL of BPW. Pour-plate inoculation was performed with 1 mL of this suspension and 15 mL of plate count agar (PCA; Bio-Rad, Hercules, CA, USA) or 15 mL of MRS agar (Tritium Microbiologie, Eindhoven, Netherlands) that were incubated at 22°C for 72 h, to determine total psychrotrophic microbiota and psychrotrophic LAB counts, respectively. For total microflora, 1 mL of the suspension was also spread on the surface of three PCA plates. Pour-plate inoculation of 1 mL of the suspension into tryptone bile X-glucuronide (TBX) agar (Led Techno, Heusden-Zolder, Belgium) was used to enumerate *E. coli*, after incubation at 44°C for 18 h. Yeast and moulds counts were obtained by pour-plate inoculation of 1 mL of suspension in yeast glucose chloramphenicol (YGC) agar (Led Techno, Heusden-Zolder, Belgium) and incubation of plates at 25°C for 3 days.

8. Challenge test interpretation

For each batch, two methods were compared to calculate growth potential (δ). The first one was based on EURL *Lm* (2014) guidelines. δ was considered as the difference between the median contamination at the end of shelf-life and the median contamination at day-0, expressed as $\log_{10}$ cfu/g. Otherwise, δ was calculated as the difference between the highest contamination at the end of shelf-life and the lowest

value at day-0 (FASFC, 2019). The latter method is more stringent and allows intra-batch variability to be taken into account, as suggested by Lahou and Uyttendaele (2017). For both calculation methods, the highest δ of the three batches was used to conclude the fate of *L. monocytogenes*, in order to consider the worst case. Results were compared with δ_{th} and considered by type of cheese. When $\delta > 0.5$ $\log_{10}$ cfu/g, the product was considered as potentially suitable for the growth of *L. monocytogenes*. On the opposite, food was recognized as not suitable for the pathogen when $\delta \leq 0.5$ $\log_{10}$ cfu/g (EURL *Lm*, 2014).

9. Statistical analyses

All statistical treatments were performed using Minitab 18 (State College, PA, USA). Provided that the number of samples varied between cheese families, GLM were used to look for potential significant differences for each physico-chemical or microbialogical factor. Tukey's HSD test was used to perform multiple comparisons. Kruskal-Wallis test was performed when variance homogeneity or data normality were not fulfilled, and Dunn's test was used for multiple comparisons.

4. Results and discussion

1. Characterization of cheeses

Table 5-1 summarizes physico-chemical parameters measured for all cheeses. Statistical differences between cheese families are also presented. Globally, at D0, for all types of cheese, the variability in pH was limited. Regarding UACC, average pH was just above the threshold value of 4.4 provided by Regulation (EC) No 2073/2005. Other types of cheeses had less acidic pH. All pH measurements were performed in cheese pastes. Values for ripened cheeses, in the case of natural crusts, would have been higher if pH was measured on the surface, due to the metabolic activity of the ripening microbiota (Mounier *et al*., 2005). Variability in a_w was limited, but averages were significantly different for all categories, except between MRSC and SRSC (p-value < 0.001). However, no samples had sufficiently low a_w to theoretically prevent the growth of *L. monocytogenes*, *i.e.* $a_w \leq 0.92$. Globally, pH and a_w of ripened cheeses were like those found in the literature (Gérard *et al*., 2018). Variations were more important regarding dry matter and salt and fat contents. Average fat content of UACC was much lower because four out of 12 samples were made from skimmed milk. UACC were not salted during their production, but an average salt content of $0.4 \pm 0.4\%$ was observed. No significant differences in dry matter content were observed between day-0 and end of shelf-life (all p-values > 0.050). During storage, a_w did not vary significantly (all p-values > 0.050). Regarding pH, a significant increase was observed for all types of cheese. In soft cheeses, average pH increased by more than one unit.

Total psychrotrophic microbiota, psychrotrophic LAB, *E. coli* and yeasts and moulds were enumerated at day-0 and at the end of shelf-life. Enumerations and statistical differences are presented in **Table** 5-2. *E. coli* is an indicator of hygiene during cheese production. For all cheese families, average *E. coli* loads at day-0 were between 1.9 and 2.5 $\log_{10}$ cfu/g. These levels are lower than those observed by

Lahou and Uyttendaele (2017) for Belgian artisanal cheeses. In 38% of the samples, *E. coli* levels did not exceed $1 \log_{10}$ cfu/g. Average *E. coli* counts decreased significantly during shelf-life of UACC (p-value = 0.045); however, that was not the case in SRSC, MRSC and SHC.

Given that cheese is a fermented product, total microbial load was generally very high, reaching $8.3 \log_{10}$ cfu/g in some samples. Comparable levels were observed by Lahou and Uyttendaele (2017) in MRSC, SRSC and SHC. Total microbiota remained at the same level during shelf-life (all p-values > 0.050). Standard deviations were limited, meaning that microbial load was comparable between cheeses made from pasteurized milk and from raw milk. This is in accordance with observations of Delcenserie *et al*. (2014). LAB represent the majority of total microbiota, whether coming from starters or not (Gobbetti *et al*., 2018). At D0, yeasts and moulds counts were lower in UACC and SHC (p-value < 0.001), in comparison with both types of soft cheese. At the end of shelf-life, yeasts and moulds counts increased by $2 \log_{10}$ cfu/g in UACC and SHC (p-value < 0.001), while they remained at the same level in soft cheese (p-value > 0.700).

Table 5-1. Physico-chemical characteristics of the four cheese families at day-0 and end of shelf-life (average ± standard deviation).

Cheese families	pH Day-0	pH End of shelf-life	Statistical letters	a_w Day-0	a_w End of shelf-life	Statistical letters	Dry matter (%) Day-0	Dry matter End of shelf-life	Statistical letters	Fat (%) day-0	Salt (%) day-0
UACC	4.4 ± 0.1^a	4.7 ± 0.4^a	$H_{1,72}=16.3$ $p<0.001$	0.99 ± 0.01^a	0.99 ± 0.01^a	$F_{1,72}=0.0$ $p=0.938$	25.9 ± 9.7^a	23.8 ± 7.8^a	$F_{1,23}=0.3$ $p=0.577$	10.5 ± 8.4^a	0.4 ± 0.4^a
MRSC	$5.8\pm0.6^{b,c}$	7.0 ± 0.7^b	$H_{1,90}=44.9$ $p<0.001$	0.97 ± 0.02^b	0.98 ± 0.02^b	$F_{1,90}=0.0$ $p=0.958$	50.1 ± 3.0^b	49.9 ± 8.5^b	$F_{1,24}=0.0$ $p=0.942$	26.3 ± 2.0^b	1.6 ± 0.8^b
SRSC	5.7 ± 0.3^c	7.0 ± 0.7^b	$H_{1,54}=37.1$ $p<0.001$	0.97 ± 0.01^b	0.97 ± 0.01^b	$F_{1,54}=0.1$ $p=0.748$	49.9 ± 4.1^b	47.8 ± 3.8^b	$F_{1,22}=1.6$ $p=0.227$	24.6 ± 3.4^b	2.0 ± 0.4^c
SHC	5.8 ± 0.2^b	6.0 ± 0.3^c	$H_{1,186}=30.5$ $p<0.001$	0.96 ± 0.01^c	0.96 ± 0.02^c	$F_{1,186}=0.2$ $p=0.696$	59.7 ± 4.8^c	61.4 ± 5.7^c	$H_{1,61}=0.8$ $p=0.382$	31.1 ± 2.7^c	1.6 ± 0.5^b
Statistical analyses	$H_{3,204}=99.4$	$H_{3,198}=135.4$		$H_{3,204}=110.3$	$H_{3,198}=102.0$		$H_{3,71}=53.12$	$F_{3,65}=98.7$		$H_{3,71}=51.9$	$H_{3,70}=31.5$
P-values	<0.001	<0.001		<0.001	<0.001		<0.001	<0.001		<0.001	<0.001

Legend: H, H-statistic corresponding to Kruskal-Wallis test; F, F-statistic corresponding to ANOVA; p-values written in italic bold are statistically significant; superscript letters allow identification of significantly different groups.

Table 5-2. Microbial counts at day-0 and at the end of shelf-life for all types of cheese (average ± standard deviation).

Cheese families	Total microbiota at 22°C			LAB			E. coli			Yeasts and moulds		
	Day-0	End of shelf-life	Statistical letters	Day-0	End of shelf-life	Statistical letters	Day-0	End of shelf-life	Statistical letters	Day-0	End of shelf-life	Statistical letters
UACC	8.2±0.5[ab]	7.7±0.5[a]	$H_{1,23}$=3.1 p=0.079	8.1±0.4	7.9±0.5	$F_{1,23}$=0.9 p=0.347	2.5±1.6	1.4±0.6	$H_{1,23}$=4.0 **p=0.045**	3.2±1.6[a]	5.8±0.4[a]	$F_{1,23}$=12.3 **p<0.001**
MRSC	7.9±0.5[ab]	7.9±0.6[ab]	$H_{1,24}$=0.2 p=0.686	7.6±0.8	7.6±0.8	$F_{1,24}$=0.0 p=0.961	2.3±1.3	1.9±1.5	$F_{1,24}$=0.7 p=0.402	5.8±0.5[b]	5.8±0.4[a]	$H_{1,24}$=0.1 p=0.729
SRSC	8.1±0.2[a]	8.2±0.1[b]	$F_{1,24}$=4.0 p=0.059	7.8±0.2	7.7±0.6	$H_{1,24}$=0.3 p=0.583	2.1±1.3	2.2±1.3	$F_{1,24}$=0.0 p=0.861	6.0±0.0[b]	6.0±0.0[a]	$H_{1,34}$=0.2 p=0.707
SHC	7.6±0.8[b]	7.7±0.5[a]	$H_{1,62}$=0.2 p=0.678	7.6±0.7	7.5±0.7	$F_{1,62}$=0.5 p=0.486	1.9±1.3	1.5±0.9	$H_{1,62}$=1.5 p=0.223	3.3±1.5[a]	5.1±1.3[b]	$H_{1,62}$=17.9 **p<0.001**
Statistical analyses	$H_{3,67}$=4.9	$H_{3,66}$=12.8		$F_{3,67}$=2.2	$H_{3,66}$=5.2		$H_{3,67}$=2.5	$H_{3,66}$=2.2		$H_{3,67}$=34.1	$H_{3,66}$=10.4	
P-value	0.176	**0.005**		0.099	0.159		0.468	0.542		**<0.001**	**0.016**	

Legend: H, H-statistic corresponding to Kruskal-Wallis test; F, F-statistics corresponding to ANOVA; p-values written in italic bold are statistically significant; superscript letters allow identification of significantly different groups (by column).

2. Study of the growth potential of L. monocytogenes

Table 5- and **Table** 5- summarize characteristics of UACC considered during challenge studies and calculated growth potentials, respectively. Similarly, **Table** 5- and **Table** 5-6 present the results for soft cheese varieties, and **Table** 5- and **Table** 5-8 for SHC varieties. All initial contaminations ranged from 30 to 300 cfu/g and were thus satisfactory regarding available guidelines (FASFC, 2016). Globally, real δ was always lower than δ_{th}, except for challenge study SH10 with the most stringent calculation method. This is not surprising given that current models are only based on data obtained *in vitro* (Kapetanakou *et al*., 2017). Growth models on cheese matrices remain unavailable on major online modelling platforms, including Sym'Previus and ComBase (Baranyi and Tamplin, 2004; Leporq *et al*., 2005). Aside from pH and a_w, some cheese matrix intrinsic factors are not taken into account by current models, including cheese microbiological characteristics. As a consequence, growth models often overestimate the growth of *L. monocytogenes*, and this enlightens the importance of performing challenge studies in order to obtain more realistic growth data, which could then be useful for the development of more accurate predictive models.

Results were contrasted between types of cheese. In UACC, the pathogen was never able to grow, regardless of the method of calculation. In 20 out of 36 samples analyzed at the end of shelf-life, *L. monocytogenes* levels dropped under the limit of enumeration (*i.e.* < 10 cfu/g). No samples had a contamination > 100 cfu/g at the end of shelf-life. With the most stringent method of calculation, all δ were between -1.45 and 0.00 $\log_{10}$ cfu/g. δ were comparable between UACC produced from raw milk and from pasteurized milk. Belgian unripened cheeses are produced by extended lactic acidification, before shaping or not, and cannot be compared with Hispanic-style unripened cheeses, including *Queso Fresco*, which is mainly obtained by adding rennet to milk, and which has already been extensively studied (Ibarra-Sanchez *et al*., 2017). *Queso fresco* has high a_w, salt content of approximately 1.0 % and nearly neutral pH. This RTE food is thus favorable for the growth of *L. monocytogenes* (Ibarra-Sanchez *et al*., 2017). Whey cheeses and buttermilk cheeses are also considered as unripened cheeses but cannot be compared with UACC studied in this paper. UACC analyzed during this study had $a_w > 0.99$ and low salt content (0.4% on average), but had a much more acidic pH, slightly higher than the threshold value for no growth of *L monocytogenes* (*i.e.* 4.4). For *Galotyri*, a product more comparable to Belgian UACC, a similar decrease of *L. monocytogenes* levels was observed, although the inoculum levels were higher, *i.e.* 3 to 7 $\log_{10}$ cfu/g (Rogga *et al*., 2005). In contrast, the pathogen remained at 2 $\log_{10}$ cfu/g during 7 days of storage at 4 °C of an Irish UACC with pH 4.3 (Schoder *et al*., 2003). Similarly, in *Cottage* cheese with pH 5.03, a_w 0.99 and 1.0% salt, levels of the pathogen remained constant during the whole storage period at 7 °C (Kapetanakou *et al*., 2017).

Table 5-3. Individual characteristics of UACC varieties considered during challenge studies.

Challege study ID	Milk	pH	a_w	δ_{th}
UACC1	R	4.5	0.99-1.00	≤0.0
UACC2	R	4.4	0.98-1.00	≤0.0
UACC3	R	4.5	0.98	≤0.0
UACC4	R	4.4	0.99	≤0.0
UACC5	R	4.4-4.5	0.99	≤0.0
UACC6	R	4.5	0.99-1.00	≤0.0
UACC7	P	4.4	0.99	≤0.0
UACC8	R	4.5	0.98-1.00	≤0.0
UACC9	P	4.4-4.9	0.97-0.98	≤0.0
UACC10	R	4.4	0.99	≤0.0
UACC11	R	4.3-4.4	0.99-1.00	≤0.0
UACC12	R	4.4	0.97-0.99	≤0.0

Legend: R, raw milk; P, pasteurized milk; δ_{th}, theoretical growth potential assessed using Sym'Previus, expressed as $\log_{10}$ cfu/g; only 1 batch considered for each variety.

Table 5-4. Results of challenge studies on UACC varieties artificially contaminated with *L. monocytogenes*.

ID	Storage (days)	EURL *Lm* (2014)		FASFC (2019)		Range of final contamination
		δ	Growth (Yes/No)	δ	Growth (Yes/No)	
UACC1	19	**-1.04**	No	**-0.42**	No	0.95-1.48
UACC2	7	**-1.43**	No	**-0.92**	No	0.95-1.48
UACC3	10	**-1.16**	No	**-0.30**	No	0.95-1.60
UACC4	10	**-0.68**	No	**-0.63**	No	1.00-1.60
UACC5	10	**-0.48**	No	**0.00**	No	1.00-1.48
UACC6	14	**-0.95**	No	**-0.60**	No	0.95-1.00
UACC7	16	**-0.53**	No	**-0.53**	No	0.95
UACC8	12	**-1.59**	No	**-1.45**	No	0.95
UACC9	10	**-1.04**	No	**-0.42**	No	0.95-1.48
UACC10	15	**-0.95**	No	**-0.55**	No	0.95-1.30
UACC11	8	**-1.19**	No	**-1.08**	No	0.95-1.00
UACC12	14	**-1.05**	No	**-0.95**	No	0.95

Legend: δ, growth potential of *L. monocytogenes* calculated during challenge studies; EURL *Lm* (2014), growth potential considered as the difference between medians of the contamination, expressed as $\log_{10}$ cfu/g at the end of shelf-life and at day-0; FASFC (2019), growth potential considered as the difference between the highest contamination at the end of shelf-life and the lowest contamination at day-0, both expressed as $\log_{10}$ cfu/g; growth of *L. monocytogenes* is considered as possible if δ > 0.5 $\log_{10}$ cfu/g; range of contamination is also expressed as $\log_{10}$ cfu/g.

Table 5-5. Individual characteristics of soft cheese varieties considered during challenge studies.

Challege study ID	Milk	pH	a_w	δ_{th}	N batches
MRSC1	P	5.6-7.1	0.97-0.99	8.0	3
MRSC2	R	5.6-6.7	0.97-0.99	8.0	3
MRSC3	R	4.7-7.0	0.93-0.98	8.0	3
MRSC4	R	5.5-6.1	0.97-0.99	5.8	3
SRSC1	R	5.1-5.8	0.96-0.97	8.0	3
SRSC2	R	5.2-5.9	0.96-0.97	5.1	3
SRSC3	R	5.2-5.9	0.96-0.98	8.0	3
SRSC4	R	5.6-6.0	0.97-0.98	5.0	3

Legend: R, raw milk; P, pasteurized milk; δ_{th}, theoretical growth potential assessed using Sym'Previus, expressed as $\log_{10}$ cfu/g.

Table 5-6. Results of challenge studies soft cheese varieties artificially contaminated with *L. monocytogenes*.

ID	Storage (days)	EURL *Lm* (2014)				FASFC (2019)				Range of final contamination
		δ_{B1}	δ_{B2}	δ_{B3}	Growth (Yes/No)	δ_{B1}	δ_{B2}	δ_{B3}	Growth (Yes/No)	
MRSC1	30	**4.70**	3.35	2.20	Yes	4.98	3.54	**5.31**	Yes	1.78-6.79
MRSC2	30	4.44	**4.45**	3.84	Yes	4.53	**5.36**	4.27	Yes	5.38-7.26
MRSC3	28	**0.93**	-0.68	-0.20	Yes	**1.05**	1.03	0.83	Yes	1.48-3.23
MRSC4	30	**1.53**	1.33	0.70	Yes	**1.63**	1.53	1.19	Yes	1.70-3.23
SRSC1	40	**-0.68**	-0.99	-1.05	No	-0.30	**0.17**	-0.35	No	0.95-1.95
SRSC2	30	0.83	**1.59**	-0.14	Yes	1.47	**2.24**	2.15	Yes	2.11-4.53
SRSC3	30	1.04	**2.68**	-0.30	Yes	1.80	**2.93**	0.15	Yes	1.60-4.53
SCRC4	40	1.29	**2.16**	1.33	Yes	1.91	**2.68**	1.38	Yes	3.15-4.68

Legend: δ_{Bx}, growth potential of *L. monocytogenes* calculated during challenge studies for batch x; EURL *Lm* (2014), growth potential considered as the difference between medians of the contamination, expressed as $\log_{10}$ cfu/g at the end of shelf-life and at day-0; FASFC (2019), growth potential considered as the difference between the highest contamination at the end of shelf-life and the lowest contamination at day-0, both expressed as $\log_{10}$ cfu/g; growth of *L. monocytogenes* is considered as possible if $\delta > 0.5 \log_{10}$ cfu/g; range of contamination is also expressed as $\log_{10}$ cfu/g.

Table 5-7. Individual characteristics of SHC varieties considered during challenge studies.

Challege study ID	Milk	pH	a_w	δ_{th}	N batches
SHC1	P	5.8-6.1	0.96-0.97	6.4	3
SHC2	P	5.5-5.9	0.95-0.97	8.0	3
SHC3	P	5.8-6.0	0.92-0.96	8.0	3
SHC4	R	5.8-6.1	0.96-0.97	8.0	3
SHC5	R	5.6-5.8	0.94-0.95	4.2	3
SHC6	R	5.6-6.1	0.94-0.96	8.0	3
SHC7	R	5.4	0.95-0.96	≤0.0	1
SHC8	R	5.8-6.0	0.96-0.97	8.0	3
SHC9	R	5.5-5.9	0.96-0.98	8.0	3
SHC10	R	5.6-5.7	0.96-0.97	≤0.0	1
SHC11	R	5.8-6.0	0.95-0.96	6.1	3
SHC12	R	5.0-6.0	0.95-0.98	8.0	1

Legend: R, raw milk; P, pasteurized milk; δ_{th}, theoretical growth potential assessed using Sym'Previus, expressed as $\log_{10}$ cfu/g.

Table 5-8. Results of the challenge studies performed on SHC varieties artificially contaminated with *L. monocytogenes*.

ID	Storage (days)	EURL *Lm* (2014)				FASFC (2019)				Range of final contamination
		δ_{B1}	δ_{B2}	δ_{B3}	Growth (Yes/No)	δ_{B1}	δ_{B2}	δ_{B3}	Growth (Yes/No)	
SHC1	21	-1.49	-0.74	-0.89	No	-1.36	-0.30	-0.83	No	0.95-1.48
SHC2	30	-0.12	-0.38	-0.07	No	0.30	0.77	0.04	Yes	0.95-3.11
SHC3	21	-1.13	-0.60	-0.23	No	-0.63	-0.07	0.04	No	1.30-2.08
SHC4	21	1.19	-0.90	-0.18	Yes	1.23	0.10	0.28	Yes	0.95-3.23
SHC5	14	-0.35	-0.41	-0.08	No	-0.14	0.04	0.04	No	1.30-2.40
SHC6	14	-0.52	0.12	-0.41	No	-0.10	0.33	0.05	No	1.30-2.18
SHC7	21	-0.48	/	/	No	-0.45	/	/	No	1.70-1.85
SHC8	14	0.04	-0.94	0.93	Yes	0.50	-0.70	1.38	Yes	0.95-3.08
SHC9	21	-0.27	-0.48	-0.20	No	0.87	0.10	0.24	Yes	1.78-3.23
SHC10	30	-0.53	/	/	No	0.45	/	/	No	1.48-2.60
SHC11	14	-0.37	-0.05	0.23	No	-0.22	0.28	0.70	Yes	1.48-2.30
SHC12	21	-0.05	-0.08	-0.12	No	0.36	2.75	1.18	Yes	1.78-4.60

Legend: δ_{Bx}, growth potential of *L. monocytogenes* calculated during challenge studies for batch x; EURL *Lm* (2014), growth potential considered as the difference between medians of the contamination, expressed as $\log_{10}$ cfu/g at the end of shelf-life and at day-0; FASFC (2019), growth potential considered as the difference between the highest contamination at the end of shelf-life and the lowest contamination at day-0, both expressed as $\log_{10}$ cfu/g; growth of *L. monocytogenes* is considered as possible if $\delta > 0.5$ $\log_{10}$ cfu/g; range of contamination is also expressed as $\log_{10}$ cfu/g.

Contrary to UACC, MRSC and SRSC are suitable for the growth of *L. monocytogenes*. For this type of product, three batches were always studied, since δ_{th} was always > 0. δ up to 4.7 $\log_{10}$ cfu/g have been observed, even with EURL *Lm* (2014) calculation based on median enumerations. During storage of similar cheeses at 7°C for 14 days, Lahou and Uyttendaele observed δ up to 1.92 $\log_{10}$ cfu/g. These δ are lower than those found in the present study, but the shelf-life was longer during the latter, and *L. monocytogenes* had more time to grow. This type of product has to be considered as dangerous for food safety, even in case of low initial contamination with the bacterium. During this study, the cocktail of *L. monocytogenes* strains was distributed between core and crust. It is well known that the surface of MRSC and SRSC represents a highly favourable medium for growth of the pathogen (Dalzini *et al*., 2017). For instance, Back *et al*. (1993) observed that *L. monocytogenes* did not grow in *Camembert* core during 40 days of refrigerated storage, but its levels increased by 2 $\log_{10}$ cfu/g on the rind. Furthermore, yeasts could favour the growth of *L. monocytogenes* (Corsetti *et al*., 2001). Surprisingly, for challenge study SRSC1, all batches had $\delta < 0$. As a consequence, this product had to be considered unsuitable for the growth of *L. monocytogenes* (EURL *Lm*, 2014). By investigating this cheese in detail, it was observed that it did not differ significantly from other SRSC in terms of pH, a_w, dry matter, salt content, fat content and microbial counts. A potential hypothesis would be that the microbiota of this cheese included particular NSLAB able to act against *L. monocytogenes*. In cheeses contaminated with 100 cfu/g of *L. monocytogenes*, Morandi *et al*. (2019) observed an inhibitive action of some NSLAB species, including *Carnobacterium* spp., *L. sakei* and some strains of *L. lactis*. This hypothesis should be confirmed using metagenetics.

Regarding SHC, contrasting results were observed. *L. monocytogenes* levels decreased during storage of all pasteurized milk SHC batches, following EURL *Lm* (2014) calculation. This was not the case for all samples made from raw milk. For raw milk SHC, huge intra- and inter-batch variability was observed. Four out of nine cheeses showed at least one positive δ among the three batches studied, with EURL *Lm* (2014) method of calculation. During challenge studies SHC4, SHC6, SHC8 and SHC11, opposite tendencies were observed between batches regarding growth of the pathogen (**Table** 5-6). For instance, during challenge study SHC8, a decrease of approximately 1 $\log_{10}$ cfu/g was observed in the first batch; *L. monocytogenes* remained at a level close to the inoculum in a second batch, while an increase of 1 $\log_{10}$ cfu/g was observed in the last batch. No significant inter-batch differences were identified regarding pH and a_w. These differences could be associated with bias introduced by direct inoculation of the pathogen, including variation of inoculum dispersion in cheese.

Considering EURL *Lm* (2014) method for δ calculation, 30 out of 32 batches of SHC did not show substantial growth (*i.e.* $\delta \leq 0.5$ $\log_{10}$ cfu/g), meaning that these products would not represent a threat for food safety in case of low contamination, *i.e.* < 10 cfu/g, at day-0. Regarding remaining batches, with $\delta > 0.5$ $\log_{10}$ cfu/g, the absence of *L. monocytogenes* in 25 g must remain compulsory. Positive δ has already been reported in Belgian SHC stored at 7°C for 14 days (Lahou and

Uyttendaele, 2017). In contrast, inoculation studies on *Edam* and *Gouda* contaminated after ripening did not show any growth of *L. monocytogenes* during storage (Kapetanakou *et al*., 2017).

As a reminder, the goal of a challenge study is to classify RTE food as suitable or not for the growth of *L. monocytogenes*, depending on whether δ is $>$ or $\leq 0.5 \log_{10}$ cfu/g. Nevertheless, looking at absolute contamination levels in SHC, five extra batches must be considered as potentially allowing the growth of the pathogen. Indeed, contamination of up to more than $4.0 \log_{10}$ cfu/g was observed (challenge study SHC12). These high levels are totally ignored when δ is calculated considering median values, remaining $< 0.5 \log_{10}$ cfu/g. While this method of calculation had no influence on the results of challenge studies for UACC, MRSC and SRSC, it led to underestimated growth in SHC. According to the chosen approach, food safety considerations were thus totally changed. The issue of intra-batch variability has already been pointed out by Lahou and Uyttendaele (2017) and FASFC (2019), for SHC and butter, respectively. A hypothesis could be that the method of inoculation in cheese cores could introduce bias responsible for this intra-batch variability. In the case of Lahou and Uyttendaele's (2017) study, using extreme values would not have changed the conclusion regarding the potential growth of *L. monocytogenes* in the concerned cheese samples. The only effect would have been an increased extent of growth. In contrast, in the present survey, giving more attention to absolute contamination levels sometimes changed the conclusions on potential growth of the pathogen.

5. *Conclusion*

The number of cases of listeriosis has increased during the last decade, as well as pressure on artisanal producers, who are supposed to guarantee the absence of *L. monocytogenes* in 25 g of cheese before it is placed on the market. It remains important to precisely identify RTE food allowing or not the growth of this bacterium. As a first approach, growth models remain an interesting solution, but they present extensively described drawbacks. Comparison with the literature is an alternative. Nevertheless, due to high variability between studies regarding inoculation level (1 to 6 $\log_{10}$ cfu/g), storage temperature (from refrigeration to room temperature) or shelf-life duration, it is often difficult to make a proper comparison between cheeses and between studies. Appropriate advice for producers would be to perform challenge studies for their products, with a standardized protocol, allowing them to make a more accurate comparison and to make a decision on the potential growth of *L. monocytogenes*. Indeed, as demonstrated by the present paper, each cheese has its own characteristics, and two products with similar pH, a_w, dry matter and microbial counts can lead to opposite behaviors of the pathogen. A surprising example is the SRSC from the present study, which combined all conditions favorable for the bacterium, as did all cheeses of the same type, but which did not allow its growth during challenge studies. Challenge studies on SHC indicated the issue of inter- and intra-batch variability, as well as eventual bias linked to the choice of inoculation method. A growth potential calculated with median values

does not guarantee that *L. monocytogenes* will not be able to reach levels > 100 cfu/g. Due to these phenomena, it should be logical to consider these cheeses as at-risk products. In the opposite way, a global conclusion was possible for UACC, obtained by lactic acid production by LAB or by direct acidification. None of the samples studied allowed the growth of *L. monocytogenes*. FASFC was invited to revise the current classification of these cheeses following Regulation (EC) No 2073/2005. Notwithstanding this, the presence of *L. monocytogenes* in RTE food should always be avoided, and a good cleaning and disinfection protocol, as well as hazard analysis and critical control points (HACCP), must be implemented. Similarly, in case of contamination, proper investigations must be implemented to identify its origin.

Although the goal of challenge studies is to assess δ of *L.* monocytogenes during RTE food storage, it is important to note that the conclusions of this study could be improved by monitoring the evolution of the contamination during shelf-life. In further experiments, microbiological analyses, including *L. monocytogenes* enumeration, could be performed daily or weekly in order to identify an eventual early growth of the pathogen in some cheese varieties. Alternatively, a more realistic way to predict the growth of *L. monocytogenes* in cheese manufactured from contaminated milk would be to inoculate the pathogen in milk, and to produce cheese with this raw material. However, this method has a lot of drawbacks which make it difficult to implement, including the necessity of performing preliminary studies to determine cheese-specific inoculum to reach 100 cfu/g at the end of ripening. Another tricky point is to be able to mimic ripening conditions found in artisanal cheese factories at a laboratory scale. Finally, it does not allow to consider cheese contamination from manufacturing environment.

Complementary information

While challenge studies were performed, natural *L. monocytogenes* contamination of diverse cheese varieties made from ovine milk occurred in a Walloon factory. In these circumstances, it is compulsory for the producer to destroy the whole batch and to recall cheeses which were already sold (FASFC, 2019b). Instead, whole contaminated batches were collected in order to perform durability studies.

The concerned batches included (a) *Feta*-type cheese, (b) MRSC, (c) SRSC, (d) blue-veined cheese and (e) SPSHC. Numbers of physico-chemical and microbiological analyses performed on these batches are summarized in **Table** 5-. All methods and enumeration media were the same as reported in the paper presented hereabove. A major difference with challenge studies was the number of enumerations of *L. monocytogenes* that were performed. Indeed, in case of natural contamination, levels of the pathogen are generally lower, and a greater variability is observed, in comparison to artificial inoculum. An increased number of replicates allows to take into account this inherent variability (FASFC, 2016).

Table 5-9. Physico-chemical and microbiological analyses performed during durability studies (all analyses were performed at both day-0 and end of shelf-life).

Analyses	Number of replicates for each batch
a_w in the core	3
pH in the core	3
Total aerobic microbiota (22 °C)	1
LAB (22 °C)	1
E. coli	1
Staphylococcus coagulase +	1
Yeasts and moulds	1
L. monocytogenes	30

Table 5- and **Table** 5-11 gather results of physico-chemical and microbiological analyses performed during durability studies. Again, none of the samples presented pH nor a_w allowing to inhibit the growth of *L. monocytogenes*, following criteria established by Regulation (EC) No 2073/2005. This agrees with results presented in Chapter 4. Total microbiota and LAB levels were comparable to values found in the literature and in the abovementioned paper (Delcenserie *et al*., 2014; Lahou and Uyttendaele, 2017). *E. coli* levels were particularly high in these batches, generally higher than averages mentioned earlier in this chapter. As *E. coli* is a hygienic indicator, it could be suggested to improve hygiene in this cheese factory and/or in the dairy farm providing ovine's milk. Although criterion exists regarding levels of *E. coli* in raw milk butter, it is currently not the case for raw milk cheeses. It was observed that *E. coli* levels decreased during storage, as these were under the enumeration limit at the end of shelf-life in MRSC, SRSC, blue-veined cheese and SHC, and decreased by 1.7 $\log_{10}$ cfu/g in *Feta*-type cheese. *Staphylococcus* coagulase + were only detected in *Feta*-type samples at the end of shelf-life.

Table 5-10. Physicochemical characteristics of the five batches naturally contaminated with *L. monocytogenes* at day-0 and at the end of shelf-life (average ± standard deviation).

Cheese families	Day-0		End of shelf-life	
	a_w	pH	a_w	pH
Feta-type	0.979 ± 0.002	5.22 ± 0.06	0.967 ± 0.002	5.32 ± 0.05
MRSC	0.970 ± 0.002	6.64 ± 0.45	0.968 ± 0.006	6.25 ± 0.00
SRSC	0.969 ± 0.005	6.92 ± 0.14	0.960 ± 0.008	7.10 ± 0.54
Blue-veined	0.975 ± 0.001	7.46 ± 0.01	0.940 ± 0.015	7.92 ± 0.00
SPSHC	0.980 ± 0.002	5.79 ± 0.02	0.953 ± 0.003	6.97 ± 0.10

Table 5-11. Microbiological characteristics of the five batches naturally contaminated with *L. monocytogenes* at day-0 and at the end of shelf-life ($\log_{10}$ cfu/g).

Cheese family	Day-0					End of shelf-life				
	Total aerobic microbiota	LAB	E. coli	Coagulase + *Staphylococcus*	Yeasts and moulds	Total aerobic microbiota	LAB	E. coli	Coagulase + *Staphylococcus*	Yeasts and moulds
Feta-type	>8.0	>8.0	4.5	<1.0	>5.9	8.1	8.0	2.8	2.3	>5.9
MRSC	>8.0	>8.0	3.1	<1.0	>5.9	>8.0	>8.0	<1.0	<1.0	>5.9
SRSC	>8.0	>8.0	5.0	<1.0	>5.9	7.6	7.8	<1.0	<1.0	>5.9
Blue-veined	>8.0	>8.0	3.6	<1.0	>5.9	7.6	7.1	<1.0	<1.0	4.6
SHC	8.1	7.6	1.6	<1.0	3.9	5.6	4.5	<1.0	<1.0	3.7

Results of the five durability studies are summarized in **Table** 5-. It can be observed that contamination decreased by around $1.5 \log_{10}$ cfu/g in *Feta*-type cheese. At the end of shelf-life, none of the 30 analyzed samples had level > 100 cfu/g. As this type of cheese was not considered during challenge studies, it is not possible to make a comparison between artificial and natural contaminations with *L. monocytogenes*. However, these results are not in accordance with those reported by Ehsani and Mahmoudi (2013), who observed an increase in the levels of the pathogen during 60 days of storage of an Iranian white-brined cheese (from 3.4 to $6.4 \log_{10}$ cfu/g). Similarly, such an increase was reported by Papageorgiou and Marth (1989).

Blue-veined cheese was not included in the sampling plan designed for challenge studies. During this shelf-life study, it was observed that the levels of *L. monocytogenes* at day-0 were extremely high, namely $7.6 \log_{10}$ cfu/g. This contamination remained stable during storage. Regarding MRSC, SRSC and SHC, initial levels of *L. monocytogenes* were comprised between 6.7 and $7.8 \log_{10}$ cfu/g. To our knowledge, such high natural contaminations in cheese were unprecedented. During storage, these levels remained stable. It is difficult to draw conclusions based on these studies, as behavior of the pathogen at such levels is probably different to what would be observed in case of initial contamination around $2.0 \log_{10}$ cfu/g.

Table 5-12. Results of durability studies performed on batches naturally contaminated with *L. monocytogenes*.

Cheese family	Median day-0 ($\log_{10}$ cfu/g)	Median end of shelf-life ($\log_{10}$ cfu/g)	δ	Samples with enumeration > 100 cfu/g (Day-0)	Samples with enumeration > 100 cfu/g (End of shelf-life)
Feta-type	2.44	0.95	-1.49	28/30	0/30
MRSC	7.84	7.89	+0.05*	30/30	30/30
SRSC	6.70	6.64	-0.06*	30/30	30/30
Blue-veined	7.59	7.28	-0.31*	30/30	30/30
SPSHC	4.60	4.81	+0.21*	30/30	30/30

Legend: *, growth potential to consider with caution as initial levels of *L. monocytogenes* were exceptional.

The origin of the contamination was investigated. In this factory, cheeses were made from bovine, caprine and ovine milk. Only batches made from ovine milk were contaminated at this period. Most probable cause of cheese spoilage was thus the use of contaminated milk. The concerned producer was not a dairy farmer and bought milk to a neighboughring farm. Milk samples were analyzed, and enumerated levels of the pathogen were comprised between 3.5 and $5.4 \log_{10}$ cfu/mL. After investigation in the herd, a single ewe was responsible for the excretion of *L. monocytogenes*. When milk of this animal was not pooled

anymore with milk of other ewes, *L. monocytogenes* was not detected anymore in 25 mL of milk. The fact that a single animal can be responsible for the contamination of tank milk is not new, as it was already reported for bovine milk by Hunt *et al*. (2012) and for goat milk by Delhalle *et al*. (2012). Nevertheless, the levels of *L. monocytogenes* were much lower in the latter study, *i.e.* 280 cfu/mL, in comparison to the present situation.

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Chapter 6

Belgian artisanal cheeses resident microbiota

Outline

Challenge studies detailed in Chapter 5 allowed the calculation of δ for *L. monocytogenes* in diverse varieties of Belgian artisanal cheeses. For some cheese types, *i.e.* SRSC, MRSC and SHC, inter-farm differences were observed, in terms of potential risk for food safety associated with spoilage by the pathogen. Furthermore, huge inter-batch variability was observed for some farms. These samples did not differ significantly in terms of pH, a_w and dry matter, and they were produced using a similar production process. A hypothesis to explain these behavioral differences could be the influence of cheese resident microbiota, as some bacterial species or consortia could be able to inhibit the growth of the pathogen. The goal of the present chapter was thus to explore resident microbiota of the cheese varieties for which challenge studies were performed, using NGS. Another objective was to look for potential correlation between the presence of specific bacterial species and calculated δ of *L. monocytogenes* in SRSC and SHC.

Paper: Study of the microbial diversity of a panel of Belgian artisanal cheeses associated with challenge studies for *Listeria monocytogenes*

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Contribution of Amaury Gérard to the paper:

Amaury Gérard performed deoxyribonucleic acid (DNA) extraction on cheese suspensions. He was also responsible for the statistical treatment of DNA sequencing data. Finally, he wrote the paper and drew graphs.

1. Abstract

High throughput sequencing could become a powerful tool in food safety. This study was the first to investigate artisanal cheeses from Belgium (31 batches) using metagenetics, in relation to *L. monocytogenes* growth data acquired during a previous study. Five cheese types were considered, namely UACC, SRSC, MRSC, GSHC and SPSHC. Each batch was analyzed in triplicate the first and the last days of storage at 8°C. Globally, 2,697 operational taxomomic units (OTUs) belonging to 277 genera and to 15 phyla were identified. *Lactococcus* was dominant in all types, but *Streptococcus* was co-dominant in SRSC and SPSHC. The dominant population was not always associated with added starter cultures. Bacterial richness and diversity were significantly higher in both types of soft cheeses than in other categories, including genera like *Prevotella*, *Faecalibacterium* and *Hafnia-Obesumbacterium* in MRSC and *Brevibacterium*, *Brachybacterium*, *Microbacterium*, *Bacteroides*, *Corynebacterium*, *Marinilactibacillus*, *Fusobacterium*, *Halomonas* and *Psychrobacter* in SRSC. A strong correlation was observed between no growth of *L. monocytogenes* in a SRSC and the presence of an unknown *Fusobacterium* (relative abundance around 10%). This *in silico* correlation should be confirmed by further experiments *in vitro* and *in situ*.

Keywords: Metagenetics, Cheese, Bacteria, 16S rRNA gene, Ecology, Challenge studies.

2. Introduction

Cheese is one of the oldest dairy and fermented products, and was already produced 8,000 years ago in the Middle-East (Gobbetti *et al*., 2018b). Nowadays, more than 1,200 cheese varieties could be found worldwide, varying in terms of texture, aspect, aroma and flavor (Barthelemy and Sperat-Czar, 2001; Tilocca *et al*., 2020). Although some cheese varieties from France, Italy and Latin America have been extensively studied and registered as PDO, Belgian cheeses remain relatively unknown. However, cheese production is well established in Belgium, with more than 250 artisanal cheese producers and several famous industrial cheese factories (personal communication). Artisanal cheeses are essentially handmade in farms and using raw milk (Kamimura *et al*., 2020). Raw milk cheeses present more pronounced tastes and flavors than cheeses produced from heat treated milk (Yoon *et al.,* 2016). In addition to sensorial and technological roles, microbiota of raw milk cheeses could play an antagonistic role against foodborne pathogens, including *L. monocytogenes* (Choi *et al*., 2020; Yoon *et al*., 2016). Cheese microbiota originates from two major sources, namely inoculated microorganisms and resident microbiota (Afshari *et al*., 2020). According to Dugat-Bony *et al*. (2016), inoculated microorganisms represent less than 50% of cheese microbiota, but this proportion is influenced by the type of cheese and the type of milk used for manufacture. The remaining part of the population is composed of the resident microbiota. The structure of the latter is influenced by a lot of factors, including raw milk microbiota

(governed itself by farming practices), people working in the workshop, water- and airflows, production tools, surfaces, wooden shelves and natural ripening cellars (Irlinger *et al*., 2015).

Raw milk cheeses have commonly been identified as potential vectors of *L. monocytogenes* (Gérard *et al*., 2018). Consequently, several listeriosis outbreaks associated with contaminated samples occurred worldwide (Martinez-Rios and Dalgaard, 2018). During a previous project, challenge studies were performed to determine δ of *L. monocytogenes* in 32 Belgian artisanal cheeses (Gérard *et al*., 2020a). For some batches of SRSC, MRSC, SPSHC and GSHC, an unexpected decrease in the levels of the pathogen during shelf-life was observed. Physico-chemical characteristics of the samples did not allow to explain this inhibition.

A hypothesis was that resident microbiota of these cheeses acted as an inhibitor on *L. monocytogenes*. For a long time, food microbiota has been exclusively studied using classical culturing methods, missing the presence of all non-culturable microorganisms, and underestimating its exceptional diversity (Afshari *et al*., 2020; Bozoudi *et al*., 2016). The emergence of next generation sequencing (NGS) technologies allowed a huge revolution in deciphering food microbiota, including cheese (Afshari *et al*., 2020). Although NGS technologies were already used to characterize diverse food matrices, their use in food safety remains an emerging trend (Weimer *et al*., 2016). The presence of some particular bacterial species could be a clue to predict the ability of foodborne pathogens, including *L. monocytogenes*, to grow or to be inhibited (Jagadeesan *et al*., 2019).

Recently, various studies on the microbial diversity of multiple cheese varieties have been conducted in diverse parts of the world, including *Bola de Ocosingo* (Mexico), *Cheddar* (USA), *Livanjski* (Czech Republic), *Mozzarella* (Italy), *Rushan* (China) and *Serra da Canastra* (Brazil) (Aldrete-Tapia *et al*., 2018; Choi *et al*., 2020; Kamimura *et al*., 2020; Marino *et al*., 2019; Vladimir *et al*., 2020; Xue *et al*., 2018). To our knowledge, the only Belgian cheese which has already been studied using metagenetics is *Herve* cheese, which is the only Belgian cheese registered as PDO (Delcenserie *et al*., 2014). However, a lot of other products from Belgium deserve more attention.

The main aim of this study was to acquire an in-depth knowledge of the microbiota of cheese varieties previously analyzed by challenge studies by Gérard *et al*. (2020a). For this purpose, the exact same batches as those used during challenge studies were considered. Potential correlations between the presence of bacterial taxa and δ of *L. monocytogenes* evaluated during these challenge studies were also explored, as a first approach.

3. *Material and methods*

1. **Sampling and cheese definition**

Based on previous knowledge acquired on Belgian artisanal cheeses (Gérard *et al*., 2020b), a classification into five major varieties was used during this study (see description in **Table** 6-1), based on manufacturing practices and final characteristics of the products, namely (a) UACC (b) SRSC (c) MRSC, (d) GSHC and (e) SPSHC.

Both types of SHC have MFFB > 54%. Hard cheeses (*i.e.* MFFB < 54%) and blue-veined cheeses were not considered in this study, as these types are not common in Belgium. Cheeses were considered as artisanal when they were transformed by hand directly in farms or in cheese factory directly buying milk to neighbouring farms. Studied batches were distributed as follow: (a) 11 UACC, (b) 4 SRSC, (c) 4 MRSC, (d) 4 GSHC and (e) 8 SPSHC. All batches considered in the present paper are the same as those used in a previous study, published as Gérard *et al.* (2020a). Samples were collected from different farms, directly after production or after ripening, respectively for UACC and ripened cheeses, corresponding to day-0 in the following parts of this article. Each collected batch was composed of at least 12 cheese wheels or pieces.

Table 6-1. Characteristics of the five types of cheese considered during this study.

	UACC	MRSC	SRSC	SPSHC	GSHC
Starters	L. lactis subsp. lactics / L. lactis subsp. cremoris / Leuconostoc spp.	L. lactis subsp. lactics / L. lactis subsp. cremoris / Leuconostoc spp. / P. candidum	L. lactis subsp. lactics / L. lactis subsp. cremoris / Leuconostoc spp. / B. linens	L. lactis subsp. lactics / L. lactis subsp. cremoris / S. thermophilus	L. lactis subsp. lactics / L. lactis subsp. cremoris / S. thermophilus
Curd	Lactic	Enzymatic	Enzymatic	Enzymatic	Enzymatic
Type of milk	P: UACC9 / R: UACC1-8 and UACC10-12	P: MRSC1 / R: MRSC2-4	R	R	P: GSHC1-3 / R: GSHC4
Maximal temperature during manufacture	Room temperature	<40°C	<40°C	<40°C	<40°C
Pressing	No	No	No	Yes	Yes
Specific ripening practices	/	Turning	Turning + / Rind washing	Turning + / Rind washing	Turning
Ripening duration (weeks)	/	2	3	4	8
Rind	No rind	White molds	Red/orange bacteria	Natural crust	Artificial coating
Weight (kg)	~0.25	0.25-0.30	0.30-0.50	1.00-2.50	>10.00
Shelf-life (days)	14	30	30	30	30
pH (Gérard et al., 2020a)	~4.4	~5.8	~5.8	~5.8	~5.8

(Continued)

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Table 6-1. (Continued)

	UACC	MRSC	SRSC	SPSHC	GSHC
a_w (Gérard et al., 2020a)	~0.99	~0.97	~0.97	~0.96	~0.96
Dry matter (Gérard et al., 2020a)	~25%	~50%	~50%	~60%	~60%
Growth of L. monocytogenes (number of batches with growth/total number of batches; Gérard et al., 2020a)	0/11	4/4	3/4	4/8	3/4

Legend: P, pasteurized milk; R, raw milk.

2. Microbial challenge tests for *L. monocytogenes*

Gérard *et al*. (2020a) performed challenge studies for *L. monocytogenes* in cheese, in agreement with available guidelines and recommendations (EURL *Lm*, 2014; FASFC, 2016). This part, as well as parts 2.3 to 2.5 are presented as a reminder of the methodology developed during the previous study of Gérard *et al*. (2020a). Among the 12 cheeses/pieces collected per batch, six were inoculated at a level of 100 cfu/g with a cocktail of three *L. monocytogenes* strains isolated from dairy products (12MOBO53LM, 12MOBO96LM and 12MOBO98LM) and provided by EURL *Lm*. Briefly, cryobeads containing each strain were suspended in 9 mL of BHI and stored at 37°C for 18 h. These cultures were diluted 1:100 in BHI and stored for 7 days at 7°C. Strains were then pooled in equivalent amounts. The six non-inoculated samples were used as control samples. The pathogen was inoculated in cheese cores using a syringe, except for SRSC and MRSC, for which the inoculum was divided between core and rind. For each batch, three controls and three inoculated cheeses were analyzed at day-0 (see section 3.3 and 3.4 of this chapter), while remaining cheeses were stored at 8 ± 1°C until end of shelf-life. At this time point, the same analyses were performed. Shelf-life of 14 and 30 days was considered for UACC and ripened cheeses, respectively.

3. Samples preparation

Samples of 25 g of cheese, comprising both core and rind, were diluted 10-fold in trisodium citrate (81 g of trisodium citrate + 4050 mL of purified water) and homogenized using Stomacher 400 (Seward, Worthing, United Kingdom). Ten mL of this suspension were kept at -80°C until DNA extraction. The remaining volume was used for microbiological enumerations.

4. Microbiological enumerations

L. monocytogenes was enumerated in samples at day-0 and end of shelf-life, using RAPID'*L. mono* method, detailed by Gérard *et al*. (2020a). Total microbiota was enumerated after pour-plate inoculation of 1 mL of cheese suspension with 15 mL of plate count agar (Bio-Rad, Hercules, CA, USA), incubated at 22°C for 72 h, as adapted from ISO 4833-1:2013 method (ISO, 2013). LAB counts were determined by pour-plate inoculation with 15 mL of MRS agar (Tritium Microbiologie, Eindhoven, Netherlands), following the same incubation scheme (ISO, 1998).

5. δ calculation

δ was calculated according to guidelines provided by EURL *Lm* (2014) and as described by Gérard *et al*. (2020a), *i.e.* "as the difference between the median contamination at use-by-date and the median contamination at day-0, expressed as $\log_{10}$ cfu/g".

6. DNA extraction

For each batch, DNA was extracted from three samples at day-0 and three samples at the end of shelf-life, using Fast DNA SPIN Kit with CLS-TC (MP Biomedicals, Santa Ana, CA, USA), from 200 µL of cheese suspension. DNA concentration and quality were checked using Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Extracts were stored at -18°C until use.

7. Libraries preparation and sequencing

Libraries were prepared under accreditation ISO 17025 by amplifying V1-V3 regions of the 16S ribosomal ribonucleic acid (rRNA) bacterial gene. Sequences of forward and reverse primers, with overhand adapters, used during this study were 5'-GAGAGTTTGATYMTGGCTCAG-3' and 5'-ACCGCGGCTGCTGGCAC-3', respectively. Amplicons were purified using Agencourt AMPure XP bead kit (Beckman Coulter, Pasadena, CA, USA), indexed using Nextera XT index primers 1 and 2 (Illumina, San Diego, CA, USA), quantified by Quant-IT PicoGreen (Thermo Fisher Scientific, Waltham, MA, USA), and diluted to a concentration of 10 ng/µL. Each DNA sample was then quantified by qualitative polymerase chain reaction (qPCR) with KAPA SYBR® FAST qPCR Kit (Kapa Biosystems, Wilmington, MA, USA). Finally, samples were normalized, pooled and sequenced using Illumina MiSeq technology with v3 reagents (Illumina, San Diego, CA, USA), using paired end reads, by GIGA Genomics platform (Liège, Belgium). A co-sequencing of a mock community was conducted to assess error rate due to biases introduced during PCR and sequencing steps. Mock community was composed of known proportions of *Carnobacterium maltaromaticum*, *L. lactis* subsp. *cremoris*, *Leuconostoc carnosum*, *Pseudomonas aeruginosa* and *S. thermophilus*. For all sequencing runs, expected proportions of these bacteria were found. Negative controls were also used during DNA extraction and library preparation, and sequenced.

8. Bioinformatics

Sequence reads were processed using respectively Mothur v1.44.3 and VSearch for alignment, clustering and chimera detection (Rognes *et al*., 2016; Schloss *et al*., 2009). Sequences were clustered into operational taxonomic units (OTUs) at 97% of identity. SILVA 138 database of full-length 16S rDNA gene sequences was used for alignments of unique sequences and taxonomical assignations (Quast *et al*., 2013). Finally, cleaned sequences were rarefied to 6,000 reads per sample. All sequence reads are publicly available on National Center for Biotechnology Information (NCBI) website under the Bioproject ID PRJNA672908.

9. Statistics

All statistical analyses were performed at the genus level, as identification at the species level based on short 16S rRNA gene sequences should only be considered carefully. Regarding α-diversity, ecological indicators, namely Goods's coverage, the number of genera, Chao1 estimator of richness, reciprocal Simpson diversity index and Simpson evenness, were calculated using Mothur v1.44 (Schloss *et al*., 2009). For bacterial enumeration and α-diversity indicators, statistical differences between groups were identified by Kruskal-Wallis test, using Minitab 17 (State College, PA, USA). Barplots were built using Microsoft Excel (Redmond, WA, USA), including only genera with relative abundance > 1% in at least one type of cheese at day-0 or end of shelf-life. Structure of the subdominant and minor communities, or β-diversity, was assessed using Yue and Clayton Theta dissimilarity matrices built using Mothur, taking into account proportions of both shared and non-shared genera from the populations, and not comprising the dominant genera, *i.e.*

Lactococcus and *Streptococcus* (Yue and Clayton, 2005). Non-metric multidimensional scaling (NMDS) was performed using Mothur and considered as satisfying when stress value was < 0.20. Finally, plots were built using RStudio and R package ggplot2 (Wickham, 2016; RStudio Team, 2020). Analysis of molecular variance (AMOVA) was performed to reveal eventual significant population structure differences, using Mothur. For SHC and SRSC, in order to look for correlations between δ of *L. monocytogenes*, calculated during challenge studies, and the presence of specific bacterial genera, canonical correspondence analyses were performed, using R package vegan (Oksanen *et al*., 2019). Observations were confirmed by building Spearman correlation matrices with R and false discovery rate (FDR) corrections for multitesting. Permutation tests were performed using R package wPerm (Weiss, 2015).

4. Results

1. Bacterial enumerations

Total microbiota at 22°C and LAB at 22°C were enumerated in all samples. Bacterial counts by type of cheese are summarized in **Table** 6-2 (averages ± standard deviations). In all types of cheese, level of total microbiota was comprised between 7.0 and 8.2 $\log_{10}$ cfu/g, on average, at both day-0 and end of shelf-life. Total and LAB counts were the lowest in GSHC at day-0. Both levels were significantly higher in UACC than in MRSC and GSHC. At the end of shelf-life, levels did not differ significantly between types. A significant difference was observed between the levels of total microbiota in UACC at day-0 and at the end of shelf-life. The majority of total microbiota was thus composed of LAB, with enumerations of at least 6.9 $\log_{10}$ cfu/g. At day-0, *L. monocytogenes* levels were always comprised between 1.48 and 2.71 $\log_{10}$ cfu/g. Globally, at the end of shelf-life, final contamination was comprised between < 1 and > 7 $\log_{10}$ cfu/g. A conclusion of challenge studies was that contamination systematically decreased during storage of UACC at 8 ± 1°C. On the contrary, both types of soft cheeses, *i.e.* SRSC and MRSC, allowed the growth of *L. monocytogenes*, but at different extents. Maximal levels reached in SRSC (around 4 $\log_{10}$ cfu/g) were lower than in MRSC (up to > 7 $\log_{10}$ cfu/g). An exception was observed for batch SRSC1, in which levels of the pathogen decreased during shelf-life. In GSHC and SPSHC, final levels were generally lower than 3 $\log_{10}$ cfu/g, but huge inter-farms, inter-batches and intra-batch variability was observed.

Table 6-2. Total microbiota and LAB counts at day-0 and end of shelf-life (averages ± standard deviations) expressed as $\log_{10}$ cfu/g.

Sampling times	Analyses	UACC	SRSC	MRSC	GSHC	SPSHC
Day-0	Total microbiota	8.216±0.381[a]	8.156±0.187[abc]	7.678±0.776[bc]	7.077±0.928[c]	8.014±0.408[ab]
	LAB	8.091±0.381[a]	7.930±0.266[abc]	7.263±0.804[bc]	7.012±0.682[c]	8.046±0.328[ab]
End of shelf-life	Total microbiota	7.661±0.639[a]	8.221±0.159[a]	7.714±0.702[a]	7.402±0.608[a]	7.926±0.314[a]
	LAB	7.934±0.501[a]	7.417±0.467[a]	7.642±0.793[a]	6.907±0.991[a]	7.823±0.361[a]

Legend: by line, enumeration levels not sharing common superscript letters were significantly different.

2. α-diversity

α-diversity metrics, including number of observed genera, Chao1, reciprocal Simpson index and Simpson evenness, were used to assess community richness and diversity. Results are summarized in **Erreur ! Source du renvoi introuvable.** for each type of cheese. For all samples at day-0 and end of shelf-life, Good's coverage was > 0.99, meaning that although the number of sampled sequence reads (*i.e.* 6,000) was limited, this sampling effort allowed to produce an accurate caption of cheese microbial communities. For all types of cheese, no significant differences in bacterial richness and diversity were observed between samples at day-0. Regarding richness, at the end of shelf-life, the number of genera was significantly higher in soft cheeses (MRSC and SRSC), in comparison with all other types of cheese. Chao1 richness indicator confirmed this observation for SRSC at the end of shelf-life. Regarding diversity, reciprocal Simpson index enlightened the same conclusion. No significant differences were observed at day-0, regarding Simpson evenness but, at the end of shelf-life, significant differences were observed between soft cheeses and other types. Between day-0 and end of shelf-life, significant differences were observed for MRSC and SRSC regarding Simpson evenness.

3. Cheese microbiota

Challenge studies performed in accordance with EURL *Lm* (2014) guidelines require two sampling times, namely day-0 and end of shelf-life. Cheese microbiota was thus studied at these end points, in the exact same batches as in published paper of Gérard *et al*. (2020a). Overall, 1,107,561 reads were obtained after treatment of raw data in cheeses sampled at day-0 and end of shelf-life, and clustered into 2,697 OTUs, belonging to 277 genera and 15 phyla. Ninety-eight genera were common between samples from day-0 and end of shelf-life. One hundred and twenty-four and 55 unique genera were observed at day-0 and end of shelf-life, respectively. Only five phyla represented more than 1% of sequence reads in at least one type of cheese at day-0 or end of shelf-life, namely *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, *Proteobacteria* and *Fusobacteria*. Barplots of the bacterial genera in all types of cheese are presented in **Figure** 6-1. For clarity and readability improvement, only genera with relative abundance $> 1\%$ in one type of cheese at day-0 and/or end of shelf-life were plotted. Supplementary files 6-1 to 6-5 show plots for individual samples.

a) Dominant microbiota

Bacteria from the genus *Lactococcus* were dominant in all types of cheese, at both day-0 and end of shelf-life. Most of these sequences corresponded to *L. lactis*, a major starter culture. A co-dominance of *Lactococcus* with *Streptococcus* (relative abundance $> 25\%$) was observed in SRSC and SPSHC. Most *Streptococcus* sequences were linked to *S. thermophilus*.

Table 6-3. α-diversity metrics by type of cheese.

Types of cheese	Good's coverage (%)		Number of genera			Chao1			Inverse Simpson			Simpson evenness		
	Day-0	End of shelf-life	Day-0	End of shelf-life	Statistical letters	Day-0	End of shelf-life	Statistical letters	Day-0	End of shelf-life	Statistical letters	Day-0	End of shelf-life	Statistical letters
UACC	0.999±0.001	0.999±0.001	15.94±2.49[a]	8.64±8.57[b]	$H_{1,67}$=1.16 p=0.281	21.55±3.50[a]	13.23±16.24[b]	$H_{1,67}$=0.43 p=0.510	1.12±0.17[b]	1.10±0.13[b]	$H_{1,67}$=0.29 p=0.590	0.18±0.13[a]	0.20±0.11[a]	$H_{1,67}$=1.04 p=0.307
MRSC	0.999±0.001	0.999±0.000	26.92±4.46[a]	18.00±6.30[a]	$H_{1,24}$=3.41 p=0.065	34.90±4.30[a]	22.50±8.49[ab]	$H_{1,24}$=1.61 p=0.204	1.54±0.99[ab]	1.51±0.74[b]	$H_{1,24}$=1.92 p=0.166	0.12±0.07[a]	0.08±0.02[b]	$H_{1,24}$=2.08 p=0.149
SRSC	0.998±0.002	0.999±0.000	28.00±3.57[a]	25.00±5.60[a]	$H_{1,24}$=2.10 p=0.148	38.60±4.90[a]	38.92±24.77[a]	$H_{1,24}$=2.10 p=0.148	1.90±1.29[a]	2.08±1.03[a]	$H_{1,24}$=1.85 p=0.173	0.18±0.26[a]	0.08±0.03[b]	$H_{1,24}$=0.71 p=0.401
GSHC	0.999±0.000	0.999±0.001	7.08±4.19[a]	8.92±7.05[b]	$H_{1,24}$=0.16 p=0.686	10.29±7.35[a]	13.53±14.36[b]	$H_{1,24}$=0.04 p=0.840	1.07±0.08[b]	1.20±0.30[b]	$H_{1,24}$=0.12 p=0.729	0.20±0.12[a]	0.19±0.10[a]	$H_{1,24}$=0.21 p=0.64
SPSHC	0.999±0.000	0.999±0.000	10.64±1.58[a]	7.29±3.32[b]	$H_{1,49}$=0.14 p=0.711	17.00±2.60[a]	9.01±5.30[b]	$H_{1,49}$=0.26 p=0.610	1.24±0.26[b]	1.25±0.27[b]	$H_{1,49}$=0.05 p=0.826	0.21±0.14[a]	0.20±0.08[a]	$H_{1,49}$=0.41 p=0.522
Statistical letters			$H_{4,96}$=8.47	$H_{4,92}$=37.03		$H_{4,96}$=6.96	$H_{4,92}$=33.18		$H_{4,96}$=10.47	$H_{4,92}$=27.41		$H_{4,96}$=0.212	$H_{4,92}$=3.81	
p-value			0.076	<0.001		0.138	<0.001		0.033	<0.001		0.212	<0.001	

Legend: By column, values not sharing superscript letters are signifianclty different. Significant p-values, i.e. < 0.05, are written in italic bold; H correspond to H-statistics used for Kruska-Wallis tests.

Regarding UACC, GSHC and SPSHC, no other genera with relative abundance > 1% were observed. For the latter types of cheese, relative abundances of the dominant/co-dominant genera, *i.e. Lactococcus* and *Streptococcus*, were higher at end of shelf-life than at day-0. In SPSHC, cumulative proportion of both genera was $98.0 \pm 3.5\%$ and $99.0 \pm 1.2\%$ at day-0 and end of shelf-life, respectively. Nevertheless, 101 genera were observed in SPSHC at day-0, while only 40 were identified in GSHC (27 in common). At the end of shelf-life, only 38 genera were observed in each type of semi-hard cheese (19 in common). In contrast, relative abundances of *Lactococcus* and *Streptococcus* were lower at end of shelf-life than at day-0 in both types of soft cheeses.

b) NSLAB

Major NSLAB observed during this study included species from genera *Enterococcus*, *Lactobacillus* (and possibly newly described genera *Companilactibacillus*, *Lacticaseibacillus*, *Lactiplantibacillus*, *Lentilactobacillus*, *Levilactobacillus* and *Ligilactobacillus*), *Lactococcus*, *Pediococcus*, *Streptococcus* and *Weissella*. Proportions of these genera were variable between cheese types, but often < 1% of relative abundance.

c) Other genera with relative abundance > 1%

Bifidobacterium, mainly *Bifidobacterium animalis* subsp. *lactis*, were observed at day-0, in all types of cheeses, but were not detected anymore at end of shelf-life.

Although *Lactococcus* and *Streptococcus* were (co-)dominant in SRSC and MRSC, additional genera with a relative abundance > 1% were observed in soft cheeses, including *Prevotella* ($4.0 \pm 13.7\%$; 1 cheese out of 4), *Faecalibacterium* ($3.3 \pm 9.9\%$, 1/4) and *Lachnospiraceae* family ($1.0 \pm 2.6\%$, 1/4) in MRSC, and *Brevibacterium* ($11.3 \pm 26.3\%$, 1/4), *Brachybacterium* ($3.4 \pm 7.7\%$, 2/4), *Microbacterium* ($2.3 \pm 5.8\%$, 2/4), *Bacteroides* ($1.9 \pm 6.3\%$, 2/4) and *Staphylococcus* ($1.7 \pm 5.2\%$, 3/4) in SRSC. In MRSC, *Prevotella*, *Faecalibacterium* and *Lachnospiraceae* were not observed at end of shelf-life samples. On the opposite, relative abundances of the genera *Hafnia-Obesumbacterium* (from $0.0 \pm 0.1\%$ to $15.5 \pm 25.4\%$, 3/4) and *Enterococcus* (from undetected to $2.0 \pm 4.1\%$, 3/4) were increased. In SRSC, *Bacteroides* was not detected anymore at the end of shelf-life, while relative abundance of *Staphylococcus* fell to $0.2 \pm 0.3\%$. *Corynebacterium* (2/4), *Marinilactibacillus* (4/4), *Fusobacterium* (1/4), *Halomonas* (1/4) and *Psychrobacter* (4/4) reached relative abundances > 1% at the end of shelf-life. In addition to that, variability between some triplicates from a given batch was sometimes observed (see Supplementary files 6-1 to 6-6).

d) Foodborne pathogens

Regarding the detection of potential foodborne pathogens, metagenetics allowed to observe *L. monocytogenes*, *E. coli* and *Staphylococcus* spp.

Using metagenetics based on V1-V3 regions of 16S rRNA gene sequencing and sampling effort of 6,000 sequences, *L. monocytogenes* was only detected in seven

MRSC samples, at end of shelf-life. All types of cheese put together, eight OTUs associated to *Staphylococcus* were observed, including *Staphylococcus aureus* (10 reads) and *Staphylococcus equorum* (2,181 reads).

e) Observation of unexpected bacterial genera

More surprising bacteria were also observed during this study. In three SRSC samples from the same factory, a huge proportion of an unknown species from the genus *Fusobacterium* has been observed, *i.e.* 12.18% of all sequence reads. Four OTUs from the genus *Ralstonia* were also observed in all types of cheese at day-0 and end of shelf-life, including *R. pickettii*.

4. β-diversity

Community structure, or β-diversity, was assessed not considering the two dominant bacterial genera, *i.e. Lactococcus* and *Streptococcus*, as their important weight in the analysis would have masked the potential differences between subdominant and minor communities. NMDS and AMOVA revealed an influence of the time of sampling on subdominant community structure in SRSC, MRSC, SPSHC and GSHC (**Figure** 6-2 C-F; all p-values < 0.001). Subdominant community structure of UACC did not significantly vary during shelf-life (p-value = 0.160). Subdominant community structure was also compared between types of cheese. At day-0, few significant differences were observed, namely SPSHC vs. MRSC (p-value = 0.003) and SPSHC vs. UACC (p-value = 0.002). At end of shelf-life, subdominant community structure was more different between types of cheese, with all pairwise tests with p-values < 0.002, excepting for GSHC vs. SPSHC and GHSC vs. UACC, for which no significant differences were observed (**Figure** 6-2 A). Consequently, it appeared that the differentiation in cheese community structure occurred during storage at 8°C.
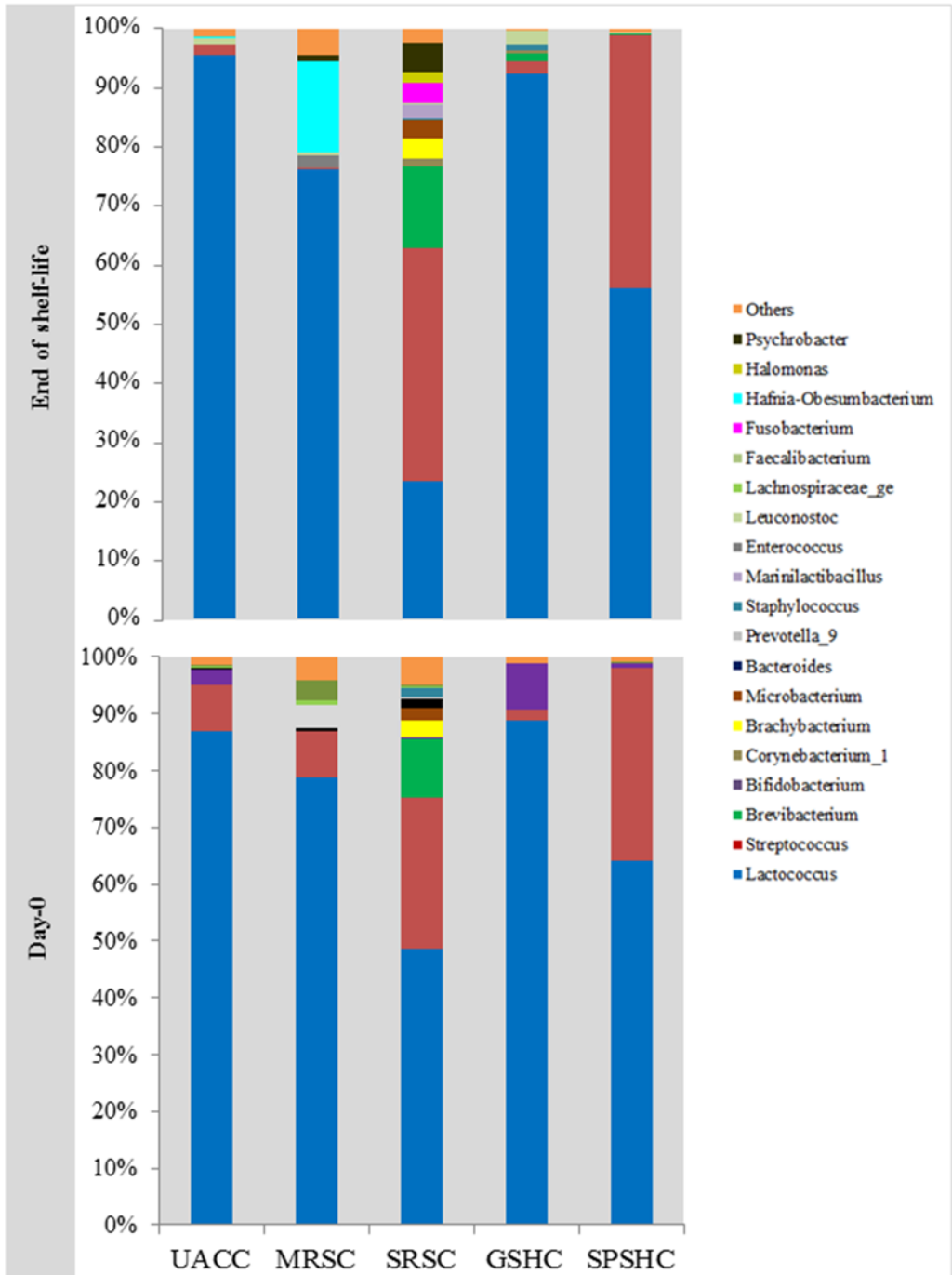
Figure 6-1. Relative abundance of bacterial genera in all types of cheese at day-0 and end of shelf-life. Only genera with relative abundance > 1 % were plotted.
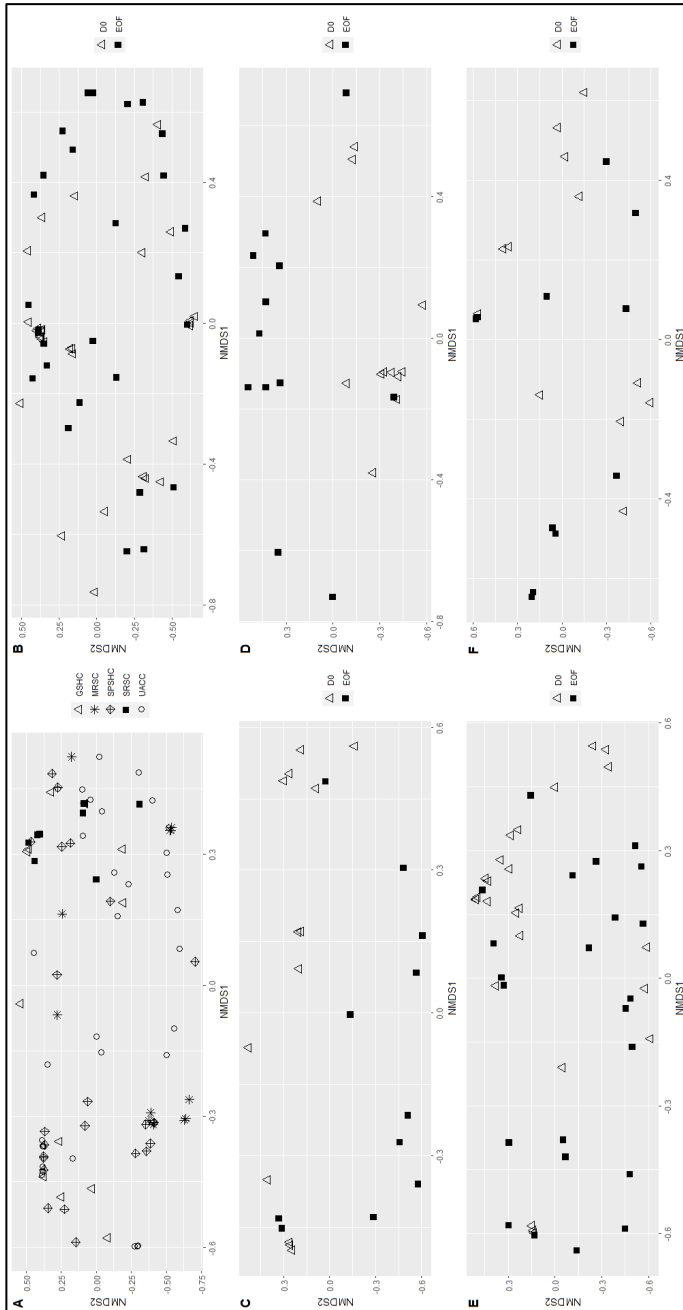
Figure 6-2. NMDS highlighting differences in cheese subdominant community structure (Yue and Clayton theta dissimilarity matrix); A, all types of cheese at end of shelf-life; B, UACC; C, SRSC; D, MRSC; E, SPSHC; F, GSHC; D0, day-0; EOF, end of shelf-life; *, significant differences between groups (p-value < 0.050).

5. Correlation between growth potential of *L. monocytogenes* and resident microbiota

Canonical correspondence analyses were performed to look for correlations between δ of *L. monocytogenes*, calculated from challenge studies (Gérard *et al.*, 2020a), and the presence of specific genera identified using metagenetics. As a reminder, in this previous paper, it was reported that three batches of SRSC from a unique farm did not allow the growth of *L. monocytogenes*, with all δ comprised between -1.05 and -1.68 $\log_{10}$ cfu/g, from an initial contamination of approximatively 2 $\log_{10}$ cfu/g. A high inter-farm variability in δ values was also observed for both types of SHC. Canonical correspondence analysis triplots did not allow the identification of relevant correlations between δ of *L. monocytogenes* in SHC and the presence of particular bacterial genera. Canonical correspondence analysis triplot for SRSC was more interesting (**Figure** 6-3). The three samples in which the pathogen was unable to grow (9-10-11) are clearly separated from other cheeses and located on the left part of the plot. Based on graphical representation, it seems that the inability of *L. monocytogenes* to grow in SRSC could be correlated to the dominance of *Lactococcus*. No growth of *L. monocytogenes* was also associated to the presence of the genera *Alkalibacterium*, *Arcobacter*, *Clostridiisalibacter*, *Fusobacterium*, *Marinilactibacillus*, *Pseudoalteromonas*, *Psychrilyobacter* and *Staphylococcus*. Spearman correlation coefficients calculated with permutation tests confirmed that four of these genera were significantly correlated with the no growth of *L. monocytogenes*, namely *Lactococcus*, *Psychrilyobacter*, *Fusobacterium* and *Alkalibacterium* (**Table** 6-).

5. *Discussion*

1. Bacterial enumerations

Enumeration of total microbiota and LAB reached expected levels. Indeed, comparable values were reported by Delcenserie *et al.* (2014) and Kamimura *et al.* (2020) in *Herve* and *Serra da Canastra*, respectively. In cheese, LAB represent a majority of total microbiota. Most LAB generally come from starter cultures (SLAB), but NSLAB were frequent (Choi *et al.*, 2020). NSLAB are mainly facultative hetero-fermentative bacteria, including *Lacticaseibacillus* spp. (comprising species previously known as *Lactobacillus casei*, *Lactobacillus paracasei* or *Lactobacillus rhamnosus*) and *Lactiplantibacillus* spp., playing important roles in the development of cheese aromas and flavors (Choi *et al.*, 2020; Zheng *et al.*, 2020).

Figure 6-3. Canonical correspondence analysis triplot for SRSC. Green labelled numbers correspond to cheese samples, red labels to bacterial genera and black arrow to positive δ of *L. monocytogenes*. Cheese samples not allowing the growth of *L. monocytogenes*, *i.e.* 9-10-11, are grouped on the right side of the figure.
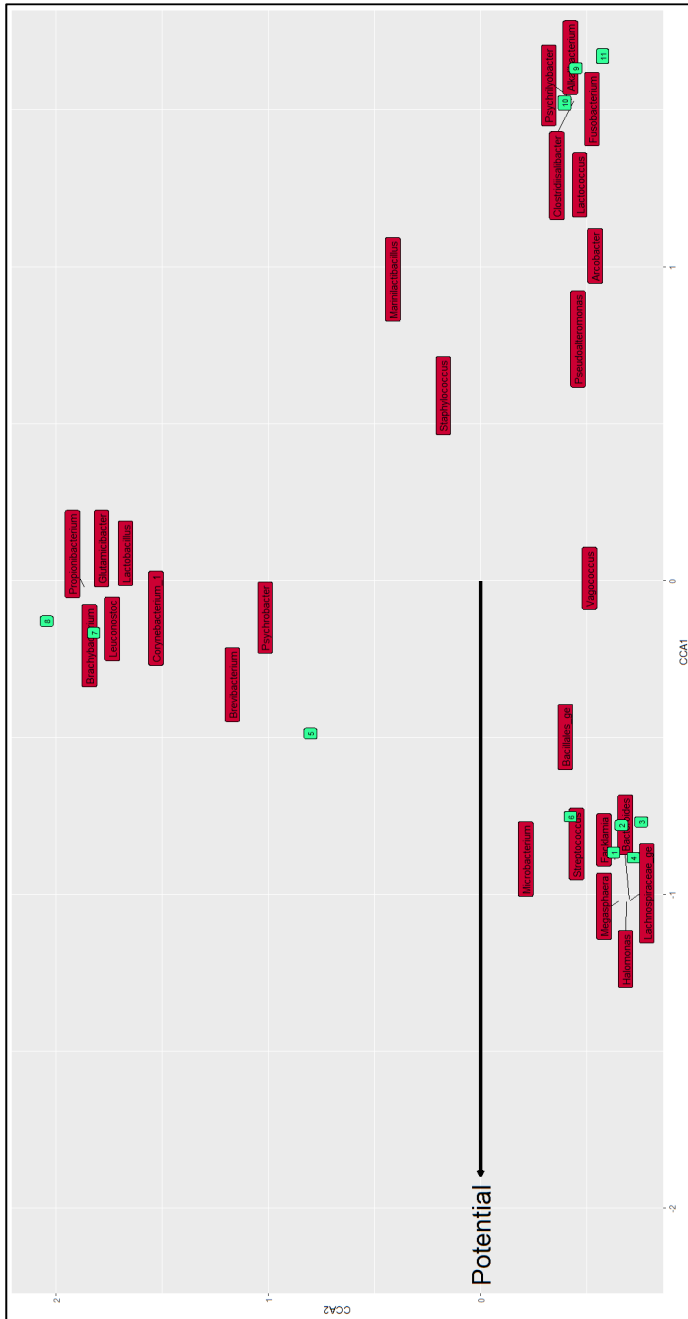
Table 6-4. Spearman correlation coefficient and significativity (p-values corrected for multitesting using FDR method) for the genera suspected to be correlated with no growth of *L. monocytogenes* from canonical correspondence analysis.

Genera	Spearman correlation coefficient	p-values
Lactococcus	-0.620	*0.002*
Psychrilyobacter	-0.511	*0.022*
Fusobacterium	-0.511	*0.024*
Alkalibacterium	-0.511	*0.024*
Clostridiisalibacter	-0.408	0.118
Staphylococcus	0.224	0.306
Pseudoalteromonas	-0.092	0.677
Arcobacter	-0.052	0.814
Marinilactibacillus	0.001	0.995

Legend : corrected p-values in italic bold are significant (*i.e.* < 0.050).

2. Cheese microbiota

a) Dominant microbiota

Lactococcus were dominant in all cheese types, but *Streptococcus* was co-dominant in SPSHC and SRSC. For the latter type of cheese, this observation was quite surprising. From **Table** 6-1, it can be seen that *S. thermophilus* was not used as starter culture during manufacture of SRSC, although it was the case during SPSHC production. From these facts, it should be said that dominant microbiota is not necessarily linked to selected starter cultures. Regarding cheese dominant microbiota reported in the literature, Aldrete-Tapia *et al*. (2018) and Falardeau *et al*. (2019) observed the dominance of *S. thermophilus* in *Bola de Ocosingo* and *Gruyere*, respectively, while a dominance of *L. lactis* in *Brie*, *Cheddar*, cores of *Époisses*, *Herve*, *Jarlsberg* and rinds of *Saint-Marcellin* was also reported (Delcenserie *et al*., 2014; Dugat-Bony *et al*., 2016; Falardeau *et al*., 2019). In *Gouda* cheese, Oh *et al*. (2016) reported only a low relative abundance of the *Streptococcus* genus (< 0.1%). This is not in accordance with the present study, as the genus *Streptococcus* represented $2.0 \pm 3.0\%$ of the reads in GSHC at day-0 and end of shelf-life. Nevertheless, it can be observed that, from identical starter culture in GSHC and SPSHC, different bacterial profiles were obtained. A hypothesis to explain the dominance of *Streptococcus* in some samples could be the inhibitive effect of salt on the growth of *Lactococcus* (Ceugniez *et al*., 2017). Another one could be the influence of the temperature during cheese production, as *S. thermophilus* is a thermophilic LAB. Nevertheless, no (half-) cooked cheeses were included in this study. *Lactococcus* spp. and *Streptococcus* spp. are part of the dominant microbiota of raw milk (1-4 $\log_{10}$ cfu/g) and of the major commercial starters available for cheese production (Aldrete-Tapia *et al*., 2018; Tilocca *et al*., 2020). Kamimura *et al*. (2020) suggested that *Lactococcus* and *Streptococcus* are the

most adapted genera regarding physicochemical conditions met during cheese production, ripening and storage. In *Gruyere* and *Comté*, a co-dominance of *Streptococcus* with *Lactobacillus* was already observed (Wei *et al*., 2016), but *Lactobacillus* was never found in dominant position in our samples. During a study on Rushan cheese, Xue *et al*. (2018) identified *Acetobacter* and *Acinetobacter* as (co-) dominant genera but, in the present study, these genera were either not detected or had a really low relative abundance ($< 0.1\%$), respectively. Another SLAB, *Leuconostoc*, mainly *Leuconostoc pseudomesenteroides*, was observed in all types of cheese at both sampling points, but as a part of the subdominant population. It was also the case in *Gouda* cheese, in which *Leuconostoc* represented around 1% of the sequences (Oh *et al*., 2016). Although *Leuconostoc* is included in most commercial starters as citrate fermenter, it was not used during manufacture of GSHC (Gobbetti *et al*., 2018a).

Regarding semi-hard cheeses, it was observed that bacterial richness was much lower in GSHC (40 genera) than in SPSHC (101 genera). The coating around GSHC prevented the development of surface microbiota, explaining these differences. Both types of semi-hard cheese had a poorly diversified microbiota at the end of shelf-life, with only 38 observed genera in total. In *Edam*, another semi-hard cheese similar to *Gouda*, genera *Acetobacter*, *Alkaliphilus*, *Bacillus*, *Cellulomonas* and *Propionibacterium* were part of the subdominant microbiota (Nalepa *et al*., 2020), but none of these taxa were observed in SPSHC and GSHC from the present study.

b) NSLAB

Many genera of NSLAB were identified during this study. All these genera remained subdominant or minor in our samples, but their presence in cheese was not surprising, as NSLAB are part of natural raw milk microbiota. They have also been isolated from cheese production environment (Choi *et al*., 2020).

c) Other genera with relative abundance > 1%

As detailed in part 4.3., *Bifidobacterium* were observed in all cheese types. Bacteria of the latter genus are known for their probiotic properties (Demers-Mathieu *et al*., 2016). Demers-Mathieu *et al*. (2016) mentioned that some *Bifidobacterium* species, including *B. animalis* subsp*. lactis*, could survive in *Cheddar* up to several months of ageing and storage. Delcenserie *et al*. (2013) discovered two *Bifidobacterium* species able to grow during ripening of French cheeses, namely *B. crudilactis* and *B. mongoliense*, but the latter species were not detected in our samples, and the genus was not identified anymore at end of shelf-life.

In SRSC and MRSC, subdominant microbiota was composed of several additional genera, at both day-0 and end of shelf-life, but differences were observed according to the cheese varieties. This inter-farm diversity is known as the terroir effect and is a major characteristic of artisanal cheeses (Turbes *et al*., 2016). Nevertheless, this concept is questionable, as an opposed idea, observed by Wolfe *et al*. (2014), suggests that reproducible rind microbial communities could be found on cheese

samples collected from various parts of the world. In other words, the impact of fermentation phenomena on cheese microbial composition could be greater than the geographical influence. Differences between cheeses within a given batch highlight the intrinsic variability of an artisanal production process, as well as the variability introduced by the sampling procedure. These variations could also be introduced by the sampling effort of 6,000 sequence reads per sample used in this work.

Most subdominant genera in SRSC and/or MRSC samples were already observed in cheese. *Brevibacterium* had an important relative abundance (> 10% at day-0 and end of shelf-life) in SRSC. Bacteria from these genera are rind colonizers, especially *B. linens*, which is responsible for the red-orange color of SRSC rinds and was used as ripening starter in SRSC manufacture (Fox *et al.*, 2017; Wei *et al.*, 2016). *Staphylococcus* and *Micrococcus* also contribute to this aspect by producing pigments (Ceugniez *et al.*, 2017). As already mentioned, *Staphylococcus* was observed in SRSC samples during this study, but it was not the case of *Micrococcus*. As alkalophiles, the presence of the genera *Corynebacterium* and *Brachybacterium* on the surface of washed rind cheeses is common, provided that this environment is de-acidified due to the metabolic activities of yeasts and moulds (Wei *et al.*, 2016). In this study, relative abundance of *Corynebacterium* was relatively low, especially at day-0 ($0.1 \pm 0.4\%$ in SPSHC and $0.2 \pm 0.4\%$ in SRSC), but was increased in SRSC at the end of shelf-life ($1.2 \pm 2.2\%$). *Brachybacterium* was part of the subdominant population of SRSC, with relative abundance of $3.4 \pm 7.7\%$ and $3.5 \pm 7.5\%$ at day-0 and at the end of shelf-life, respectively. *Marinilactibacillus* (mainly *Marinilactibacillus psychrotolerans*) and *Halomonas* are halotolerant bacteria that were part of the subdominant microbiota of SRSC. They were identified for the first time in seawater, and their presence in cheese can be attributed to cross-contaminations during brining or salting (Yunita *et al.*, 2018). *Halomonas* has often been identified in short ripening cheeses and could play important functions during ripening (Quijada *et al.*, 2018). *M. psychrotolerans* was already observed in *Herve* and *Munster*, two red smear cheeses (Delcenserie *et al.*, 2014; Dugat-Bony *et al.*, 2016. *Psychrobacter* was observed in all SRSC samples at the end of shelf-life. According to Ceugniez *et al.* (2017), *Psychrobacter* is part of the raw milk microbiota, and its growth is promoted in cheese, especially in case of cold ripening and during storage. Some *Psychrobacter* species have also been isolated from seawater and are thus halotolerant. They could possibly be carried by brine and salt (Falardeau *et al.*, 2019). Finally, the presence of *Microbacterium* in various types of cheeses is well documented, originating from raw milk and contributing to cheese flavor (Delcenserie *et al.*, 2014; Irlinger *et al.*, 2015; Tilocca *et al.*, 2020). *Bacteroides* are abundant in dairy farm environment, on teat skin and in raw tank milk. Their presence in cheese has already been observed in multiple varieties (Falardeau *et al.*, 2019, Milani *et al.*, 2019). These bacteria are part of the natural human gut microbiota, and can be used as probiotics (Tan *et al.*, 2019). Regarding MRSC, the presence of *Faecalibacterium* is not a surprise, as this genus is commonly found in raw milk (Savin *et al.*, 2019). These strict anaerobes could find a suitable environment in cheese cores (Fox *et al.*, 2017). Quigley *et al.* (2012)

observed for the first time the presence of *Faecalibacterium* in cores of soft, semi-hard and hard cheese samples. Interestingly, various species from this genus, including *Faecalibacterium prausnitzii*, are known for their probiotic role (Savin *et al*., 2019). *Prevotella*, another genus including strict anaerobes, was frequently observed in cheese since the emergence of NGS. *Prevotella* were primarily identified in cow rumens, but were also observed in mouth, nose and gut of cows (Fox *et al*., 2017). According to Frétin *et al*. (2018), individuals from the family *Lachnospiraceae* are commonly found on the teat skin, as a result of fecal contamination, provided that these bacteria are part of gut microbiota. Bacteria can thus be transferred to raw milk during milking or to washing water during cleaning and be found in cheese. It was for instance the case in *Parmesan* (Milani *et al*., 2019). On the opposite, Falardeau *et al*. (2019) observed *Lachnospiraceae* in dairy farms, milk and cheese plants, but did not detect its presence in the final cheeses, including MRSC. However, as DNA sequencing do not allow to distinguish dead and alive bacteria, it is possible that all these anaerobes were not metabolically active anymore in cheese during ripening and storage. *Hafnia alvei*, a fecal and water contaminant, represented a huge part of the subdominant microbiota in MRSC. This Gram-negative bacterium is sometimes used as starter culture in MRSC and SRSC, as it influences cheese sensorial properties by producing volatile sulfur compounds (Irlinger *et al*., 2015). To our knowledge, *H. alvei* was not intentionally added in samples considered during this work. A hypothesis to explain the peak in relative abundance of *H. alvei* in MRSC during storage at 7°C is that psychrotrophic Gram-negative bacteria are favored by these conditions (Gobbetti *et al*., 2018b).

d) Foodborne pathogens

Two foodborne pathogens were identified using metagenetics, namely *L. moncoytogenes* and *S. aureus*. *L. monocytogenes* was only observed in seven MRSC sample at end of shelf-life. During challenge studies performed by Gérard *et al*. (2020a), levels of the pathogen were the highest in concerned batches at end of shelf-life 6-7 $\log_{10}$ cfu/g), while level in other varieties was generally < 3 $\log_{10}$ cfu/g. Given the random sampling effort used in this study, *i.e.* 6,000 sequences/sample, and cheese total microbiota assessed by plate counts (*i.e.* 7-8 $\log_{10}$ cfu/g), it was expected that the sensitivity of metagenetics was not sufficient to detect *L. monocytogenes* in the latter samples, as it is also the case for many other minor microbial species. Indeed, the probability to randomly select sequences of minor bacteria is limited in contrast to sequences of sub-dominant or dominant microbiota. As expected, metagenetics is not the most adequate tool when looking for pathogens in food.

Regarding *Staphylococcus*, according to Gobbetti *et al*. (2018a), this genus is part of natural raw milk microbiota, but is also transmitted by cheesemakers' hands (Castellanos-Rozo *et al*., 2020). According to Irlinger *et al*. (2015), *Staphylococcus* spp. were identified on the rinds of nearly all cheese varieties, their halotolerance allowing them to find a suitable environment in and on cheese.

e) Observation of unexpected bacterial genera

As a reminder, *Fusobacterium* has been observed in three SRSC samples from a same batch, with relative abundance around 10%. The presence of *Fusobacterium* in cheese has already been reported by Delcenserie *et al.* (2014), but with a much lower relative abundance (2.54% and 4.39% in raw and pasteurized milk SRSC samples, respectively). To our knowledge, no other papers mentioned the presence of this genus in cheese. Interestingly, cheese samples from this farm were the only SRSC in which *L. monocytogenes* levels decreased during challenge studies (Gérard *et al.*, 2020a). The second unexpected genus observed in this study was *Ralstonia*. Species of this genus are known as plant pathogens and can sometimes be found in raw milk (Salazar *et al.*, 2018). However, *Ralstonia* are also known as potential contaminants from DNA extraction kits, reagents for PCR or water (Salter *et al.*, 2014). Further investigations should be performed in order to confirm that these bacteria were metabolically active during cheese ripening and storage.

3. Correlation between growth potential of *L. monocytogenes* and resident microbiota

Canonical correspondence analysis did not identify correlations with the presence of particular genera and δ of *L. monocytogenes* in SPSHC. This variability could be explained by the bias introduced by the differential dispersion of *L. monocytogenes* into cheese following inoculation during challenge studies, as hypothesized by Gérard *et al.* (2020a). Another explanation could be differences in the composition of dominant microbiota at deeper taxonomic levels, *i.e.* species, subspecies or strains.

Canonical correspondence analysis performed for SRSC revealed more interesting results, with the three samples of interest (*i.e.* samples in which *L. monocytogenes* levels decreased during challenge studies performed by Gérard *et al.* (2020a)) clustered clearly apart from other batches. A first significant correlation was found with the presence of *Lactococcus* as only dominant genus. Although *Lactococcus* spp., including *L. lactis*, are known for their production of bacteriocins inhibiting the growth of *L. monocytogenes*, this correlation could be doubtful as such, as Lactococcus were used as main starter during manufacture of all SRSC samples considered in this study. Nevertheless, inhibition of *L. monocytogenes* by *Lactococcus* spp. is often strain-dependent. Although some batches present similar levels of *Lactococcus* spp., the differential dominance of *Lactococcus* strains could be a clue to explain differences observed regarding δ of *L. monocytogenes*.

A strong correlation with the presence of *Fusobacterium* was reported by canonical correspondence analysis and Spearman correlation coefficients. As detailed in part 3.3.5., *Fusobacterium* represented $12.2 \pm 3.0\%$ of the sequences associated with the three samples not allowing the growth of the pathogen, and this genus was not observed in other samples. It seems that this genus represents the most interesting pathway to investigate, as its presence in cheese was only reported once, in 2014, in samples from the same producer, but with much lower relative abundances. Other genera significantly correlated to the negative δ of

L. monocytogenes were *Alkalibacterium* (29 reads), *Clostridiisalibacter* (26 reads) and *Psychrilyobacter* (27 reads). It was already reported that *Alkalibacterium kapii*, an alkalophilic bacteria, finding suitable environment on cheese surfaces, was able to inhibit the growth of *L. innocua* during *Raclette* cheese ripening (Roth *et al.*, 2011). *Clostridiisalibacter* are halophilic bacteria which were already observed in SRSC (Delcenserie *et al.*, 2014), but their ability to inhibit *L. monocytogenes* has never been investigated. *Psychrilyobacter* is a genus from the *Fusobacteria* phyla, which is commonly observed in marine environments. Its presence in cheese was never reported, although it was already observed in cheese production environment (Schön *et al.*, 2016). All the latter genera represent thus interesting perspectives to investigate, to confirm their potential influence on the growth of *L. monocytogenes*.

6. Conclusions

Microbial populations of cheeses, especially subdominant and minor populations, are strongly influenced by many factors. Each paper on this topic identified novelties: new species, taxa observed in cheese for the first time, or at least unexpected relative abundance of known taxa. It was the case for Belgian samples investigated during this study. The major surprise was the identification of a high proportion (> 10%) of *Fusobacterium* in three SRSC samples from the same factory, which did not allow the growth of *L. monocytogenes* during previously performed challenge studies. Otherwise, it was observed that the production technology has a strong influence on cheese subdominant microbiota, and that starter cultures did not always govern cheese microbial community structure. Regarding dominant microbiota, *Lactococcus* and/or *Streptococcus* were dominant in all cheese types, corresponding mainly to *L. lactis* and *S. thermophilus*. Nevertheless, strains could be different between cheese types or batches. A deeper knowledge could be acquired through analysis of oligotypes. Knowing with precisions strains met in each batch could allow to improve understanding of the results of challenge studies with *L. monocytogenes*, as production of bacteriocins or other antimicrobial compounds is strain dependent. Considering separately core and rind could also have been interesting. In addition to that, using NGS to study fungal communities of Belgian cheeses would represent an added value. Correlations analyses were a first approach in order to draw hypotheses in order to explain the unexpected decrease of *L. monocytogenes* levels during storage of three SRSC samples from the same producer. Further studies should be performed to assess the real influence of the identified genera on the growth of the pathogen. It is also important to characterize in detail the *Fusobacterium* sp., as observed species was not listed in databases. At least two species of this genus, *i.e. Fusobacterium nucleatum* and *Fusobacterium necrophorum*, are known as human pathogens. High relative abundance of *Fusobacterium gastrosuis* has also been associated to stomach ulceration in pigs. Food safety aspects associated to the presence of this unknown *Fusobacterium* should be investigated. Finally, it is now important to go beyond diversity studies, and metatranscriptomics could be a powerful tool to understand the role of bacterial taxa during cheese production and storage.

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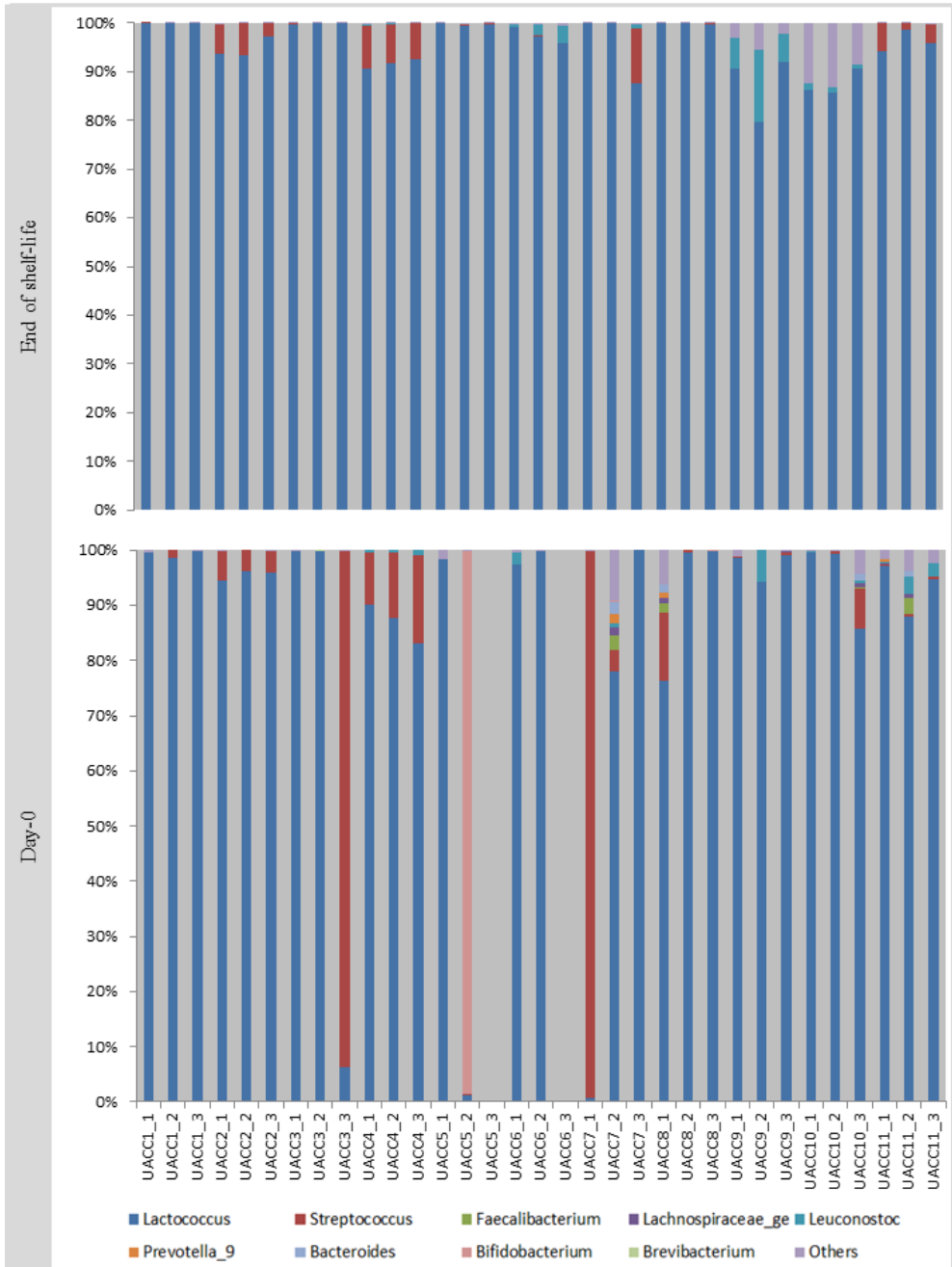
Supplementary material 6-1. Relative abundance of bacterial genera by UACC sample at day-0 and enf of shelf-life. Only genera with relative abundance ≥ 1 were plotted.
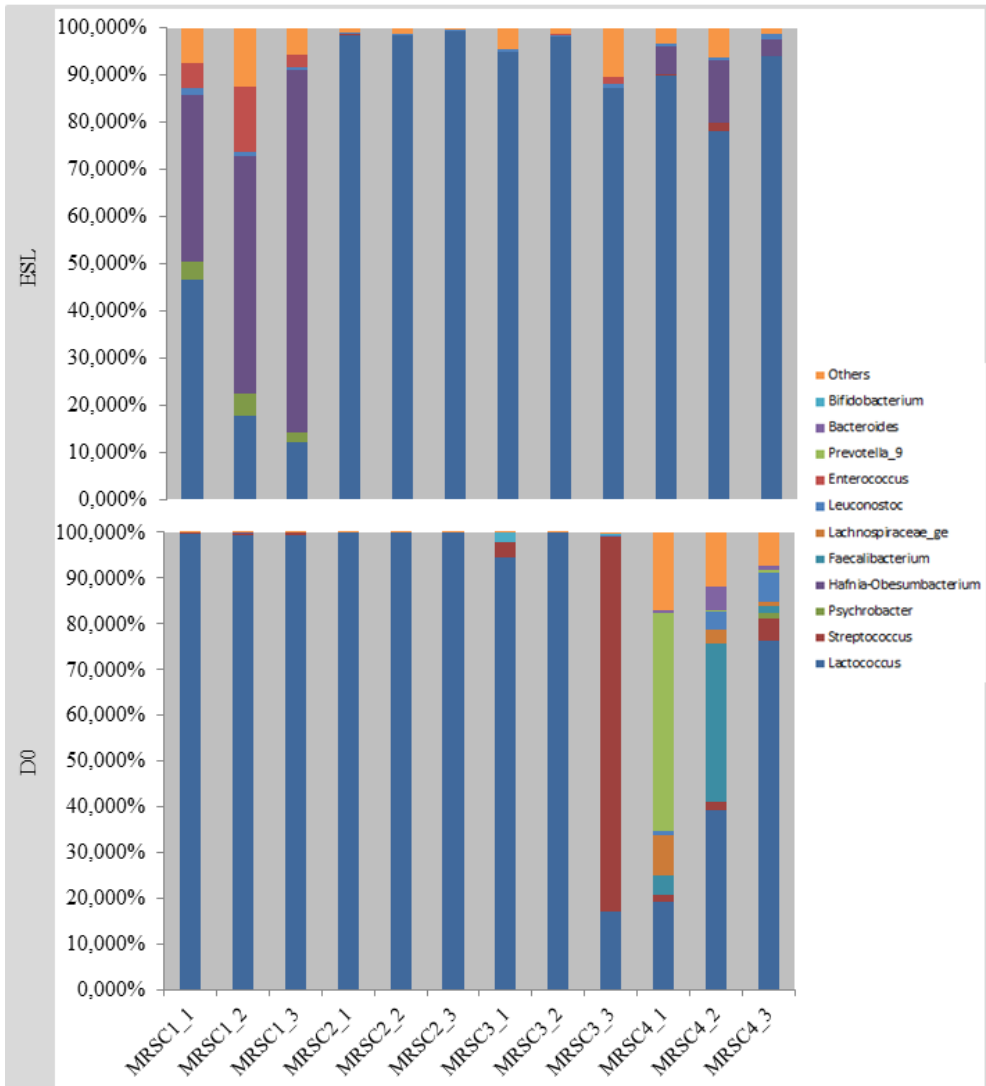
Supplementary material 6-2. Relative abundance of bacterial genera by MRSC sample at day-0 and end of shelf-life. Only genera with relative abundance ≥ 1 % were plotted.
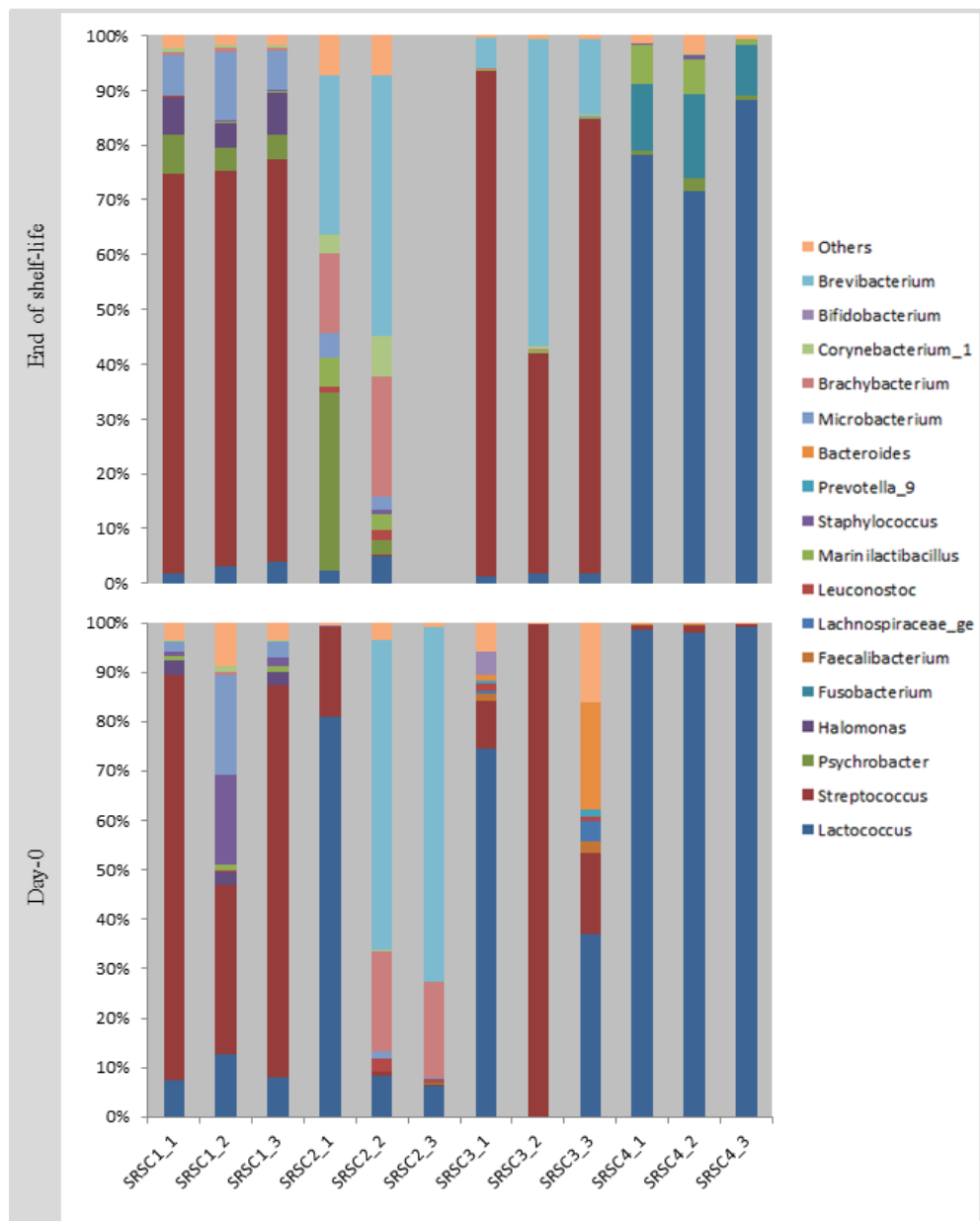
Supplementary material 6-3. Relative abundance of bacterial genera by SRSC sample at day-0 and end of shelf-life. Only genera with relative abundance ≥ 1 % were plotted.
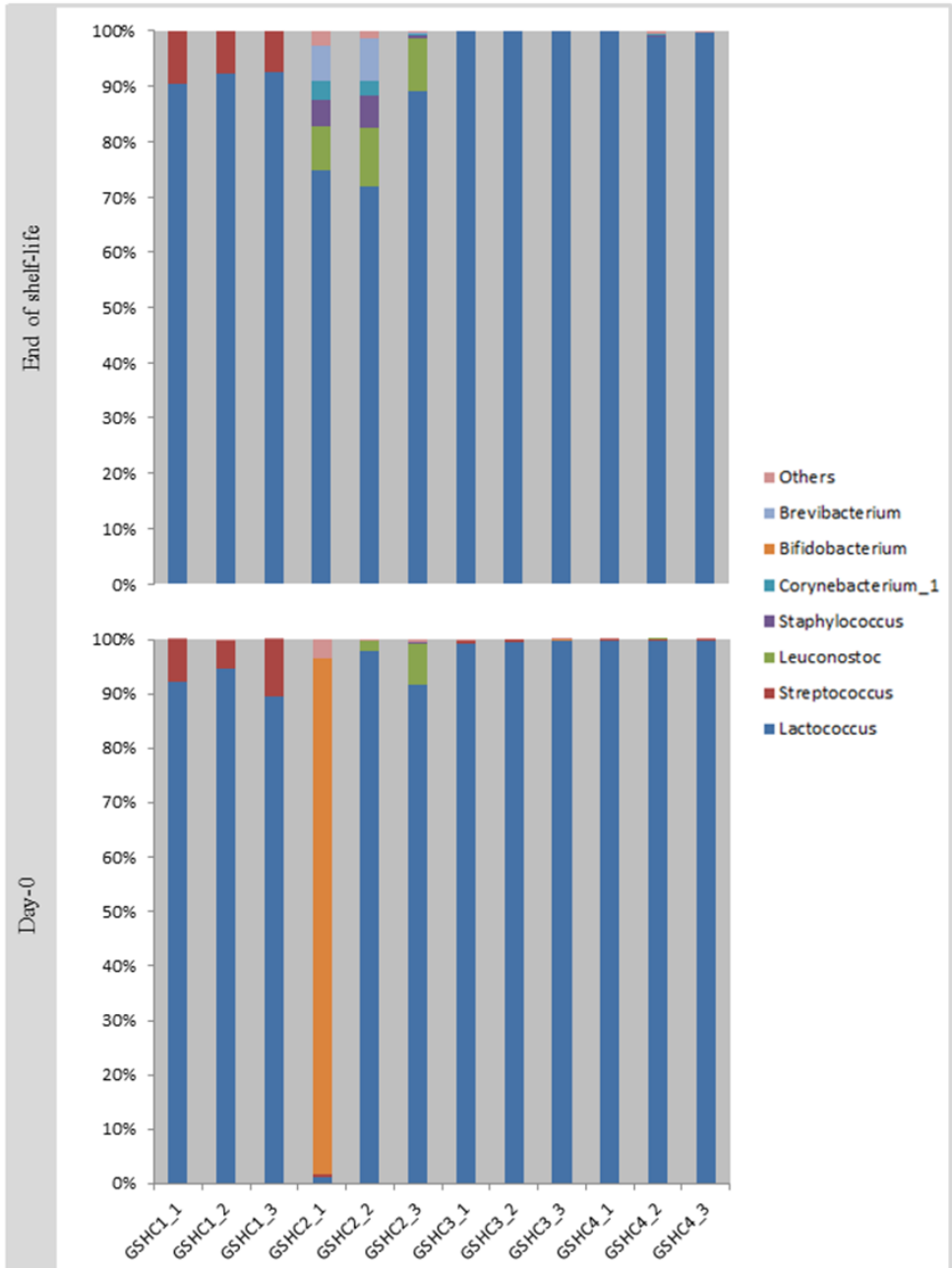
Supplementary material 6-4. Relative abundance of bacterial genera by GSHC sample at day-0 and end of shelf-life. Only genera with relative abundance ≥ 1 % were plotted.
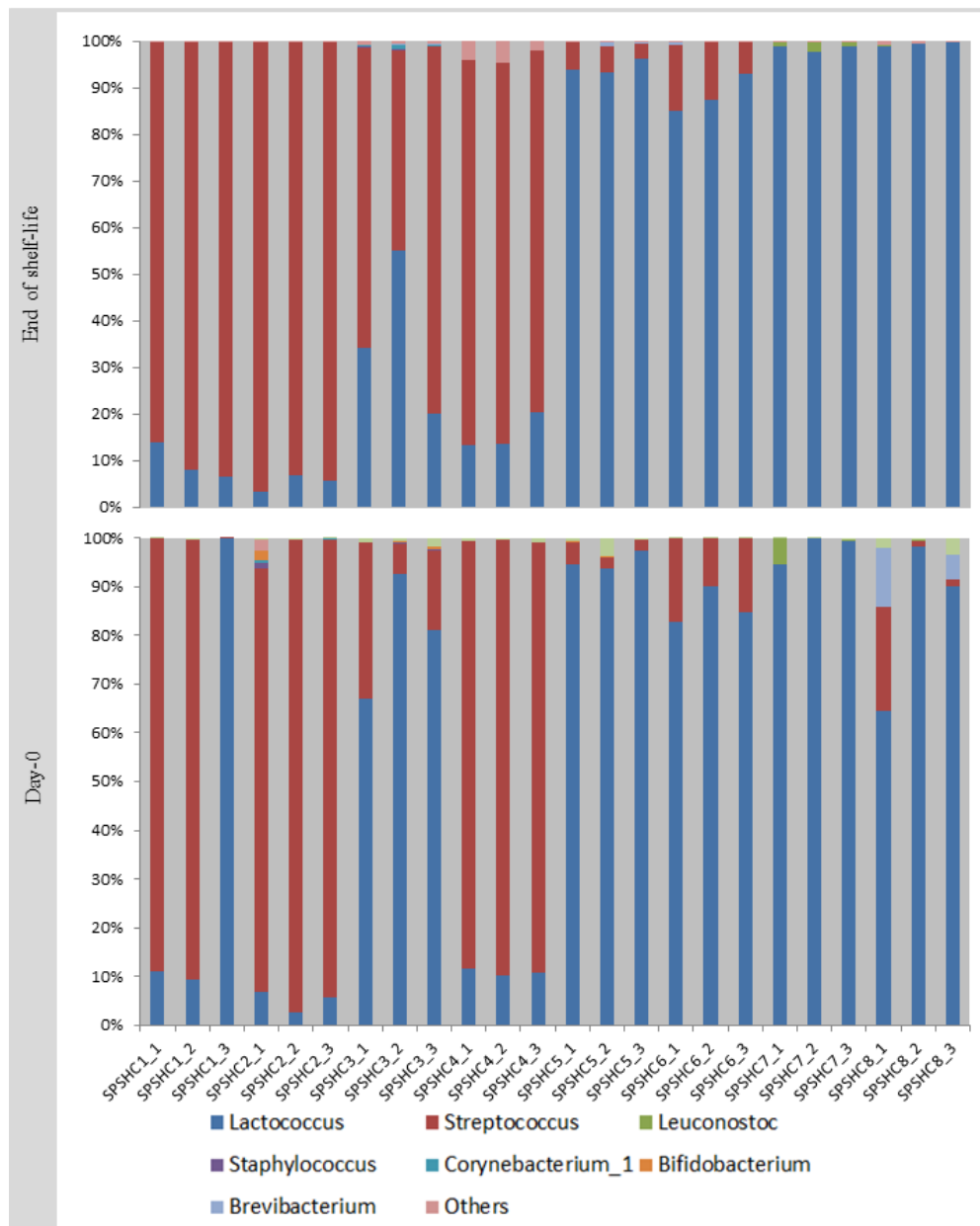
Supplementary material 6-5. Relative abundance of bacterial genera by SPSHC sample at day-0 and end of shelf-life. Only genera with relative abundance ≥ 1 % were plotted.

Supplementary material 6-6. Average (± standard deviation) relative abundance (%) of main bacterial genera considered in this paper. UACC, unripened acid-curd cheeses; MRSC, mold-ripened soft cheeses; SRSC, smear-ripened soft cheeses; GSHC, Gouda-type semi-hard cheeses; SPSHC, Saint-Paulin-type semi-hard cheeses; D0, day-0; ESL, end of shelf-life; /, undetected genus.

Bacterial genera	UACC		MRSC		SRSC		GSHC		SPSHC	
	Day-0	End of shelf-life	Day-0	End of shelf-life	Day-0	End of shelf-life	Day-0	End of shelf-life	Day-0	End of shelf-life
Lactococcus	85.7±28.4	95.5±5.3	78.6±33.3	76.3±32.2	44.3±42.2	23.5±36.0	88.7±27.8	92.4±9.8	62.5±40.0	56.1±41.5
Streptococcus	8.8±23.8	1.9±3.2	7.9±23.4	0.2±0.5	28.7±37.0	39.5±39.7	2.1±3.6	2.0±3.7	35.5±40.4	42.9±41.3
Brevibacterium	/	/	/	/	11.3±26.3	13.8±20.9	/	1.2±2.8	0.1±0.5	0.1±0.2
Bifidobacterium	3.2±17.7	/	0.2±0.6	/	0.4±1.3	/	7.9±27.4	/	0.7±2.7	/
Corynebacterium	/	/	/	/	0.2±0.4	1.2±2.2	/	0.6±1.2	0.1±0.4	0.1±0.3
Brachybacterium	/	/	/	/	3.4±7.7	3.5±7.5	/	/	0.0±0.1	/
Microbacterium	/	/	/	/	2.3±5.8	3.1±4.3	/	/	/	/
Bacteroides	0.2±0.5	/	0.6±1.5	/	1.9±6.3		/	/	/	/
Prevotella	0.1±0.4	/	4.0±13.7	/	0.2±0.4		/	/	/	/
Staphylococcus	/	/	/	/	1.7±5.2	0.2±0.3	/	0.9±2.0	0.1±0.2	/
Marinilactibacillus	/	/	/	/	0.2±0.5	2.2±2.8	/	/	/	/
Enterococcus	0.5±1.2	1.2±2.9	1.0±2.1	2.0±4.1	/	0.3±0.6	0.8±2.3	2.3±4.3	0.2±1.1	0.2±0.5
Leuconostoc	0.1±0.3	/	1.1±2.6	0.6±0.4	0.5±0.8	0.3±0.6	/	/	/	/
Lachnospiraceae	0.3±0.8	/	3.3±9.9	/	0.4±1.2	/	/	/	/	/
Faecalibacterium	/	/	/	/	0.3±0.8		/	/	/	/
Fusobacterium	/	/	/	/	/	3.3±5.8	/	/	/	/
Hafnia-Obseumbacterium	/	/	0.0±0.1	15.5±25.4	0.7±1.2	1.8±3.1	/	/	/	/
Halomonas	/	/	/	/	/	1.8±3.1	/	/	/	/
Psychrobacter	/	/	0.1±0.3	0.9±1.7	/	5.0±9.4	/	/	/	/

Chapter 7

Attemps to isolate *Fusobacterium* sp. from *Herve* cheese, "whole genome sequencing" and comparative genomics

Outline

Surprising results were observed during Chapters 5 and 6 regarding an artisanal raw milk *Herve* cheese. Indeed, during challenge studies with artificially inoculated *L. monocytogenes*, a negative δ was calculated for all batches from the concerned factory. This variety did not significantly differ from other SRSC sudied in terms of manufacturing process, pH, a_w and salt content. Metagenetics on one batch revealed that a significant part of cheese microbiota (*i.e.* around 10.0%) was composed of an unkown species of the genus *Fusobacterium*. These singularities motivated us to have a closer look at this cheese variety. The following chapter will describe activities aiming to isolate and characterize this new species. Nevertheless, it was not possible to make *Fusobacterium* sp. grow on solid media. Metagenomics was thus used to gather its whole genome from cheese DNA sample. A first insight in comparative genomics for *Fusobacterium* spp. will also be proposed in this chapter.

Cheese microbiota of two extra *Herve* batches

In Chapter 6, metagenetics was only performed on one batch of *Herve* cheese. A first step was to check the presence of *Fusobacterium* in all available batches, which were used to assess δ of *L. monocytogenes* during Chapter 5. Simultaneously, these analyses allowed the investigation on the stability of *Herve* microbiota along time.

1. Material and methods

Metagenetics was performed using the protocol detailed in Chapter 6. Cheese suspensions in trisodium citrate used during challenge studies were stored at -80°C until use. Briefly, bacterial DNA was extracted using Fast DNA SPIN Kit with CLS-TC (MP Biomedicals, Santa Ana, CA, USA) and its quality and concentration were checked using NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Libraries were prepared using qPCR primers and purification kit previously described. Sequencing was performed using Illumina MiSeq technology (Illumina, San Diego, CA, USA). The same approach as detailed in Chapter 6 was used for bioinformatics and statistics, combining the use of Mothur v1.44.3, SILVA 138 database and RStudio (Schloss *et al.*, 2009; Quast *et al.*, 2013; RStudio Team; 2020).

2. Herve cheese characteristics

2.1. Physico-chemistry and classical microbiology

Individual physico-chemical and microbiological characteristics of each batch of *Herve* cheese are summarized in **Table** 7-1 (extracted from data acquired during Chapter 5). Highest level of *L. monocytogenes* at end of shelf-life and calculated δ (using EURL *Lm* approach) were also included. For all parameters, no significant differences were observed, except for molds populations. Total microbiota and LAB levels were comparable to data previously reported for artisanal raw milk *Herve* cheese (Delcenserie *et al.*, 2014).

Table 7-1. Physico-chemical characteristics of the three batches of *Herve* cheese.

Batch	Time	a_w	pH	Salt (%)	Dry matter (%)	Total aerobic microbiota (22°C)	LAB (22 °C)	Yeasts	Molds	δ ($\log_{10}$ cfu/g)	Highest level of *L. monocytogenes* at end of shelf-life (cfu/g)
1	D0	0,96-0,97	6,9-7,1	2,7	49,3	$8,1 \times 10^7$	$7,8 \times 10^7$	$9,8 \times 10^5$	<10	-0,68	50
	ESL	0,97	7,2		47,9	$9,6 \times 10^7$	$6,9 \times 10^7$	$6,0 \times 10^6$	$7,0 \times 10^3$		
2	D0	0,97	6,2-6,7	2,0	49,2	$1,0 \times 10^8$	$9,4 \times 10^7$	$>9,3 \times 10^5$	<10	-0,99	90
	ESL	0,97	6,2		48,6	$1,0 \times 10^8$	$8,5 \times 10^7$	$>9,3 \times 10^5$	<10		
3	D0	0,96-0,97	6,9-7,2	2,4	50,2	$1,3 \times 10^8$	$4,0 \times 10^7$	$>9,3 \times 10^5$	$>9,3 \times 10^5$	-1,10	<10
	ESL	0,95-0,97	6,8-7,4		46,3	$>2,0 \times 10^8$	$3,1 \times 10^7$	$>9,3 \times 10^5$	$>9,3 \times 10^5$		

Legend: D0, day-0; ESL, end of shelf-life.

2.2. Microbiota and ecology

Microbiota and ecology were compared between batches using histograms, α-diversity parameters and β-diversity through NMDS.

a) Histograms

Cheese microbiota was characterized at the genus level. **Figure** 7-1 shows relative abundance of genera representing at least 1% of sequence reads in minimum one batch. As observed, 14 genera were concerned but, for all samples at both sampling time, microbiota was dominated by LAB genus *Lactococcus*. Most sequence reads were associated to *L. lactis* subsp. *cremoris*. *Streptococcus* was minor in this cheese variety. *Pseudoalteromonas* was part of the subdominant population at day-0 in batches 2 and 3 and was not identified in batch 1. At end of shelf-life, relative abundance of this genus was lower than 1%. *Pseudoalteromonas* are halophilic bacteria associated with marine environment and possessing enzymes adapted for survival under cold conditions. It was assumed that transmission route for this genus was salting process or washing procedure (Ogier *et al*., 2004; Wolfe *et al*., 2014; O'Sullivan *et al*., 2015).

Another important observation was that *Fusobacterium* genus was observed in all samples at end of shelf-life, with relative abundance between 1 and 15%. It was also identified in batch 2 at day-0 (relative abundance around 2%).

Bacterial profile of batches 2 and 3 looked different from that of batch 1. Interestingly, cumulative relative abundance of dominant microbiota was < 80% in all samples from batch 2. Subdominant microbiota was composed of *Fusobacterium*, *Psychrobacter*, *Psychrilyobacter*, *Marinilactibacillus*, *Marinobacter*, *Vibrio*, *Arcobacter* and *Glutamicibacter*. In batch 1 at end of shelf-life, *Marinilactibacillus* had a relative abundance of 8%. All these genera were also observed by Delcenserie *et al*. (2014). It can be surprising that *Brevibacterium* and *Corynebacterium* did not have a relative abundance > 1%, as both genera are typically found in rinds of SRSC. As an example, during a previous study on raw milk *Herve* cheese rinds, *Brevibacterium* and *Corynebacterium* had relative abundance around 1 and 50%, respectively. Both genera were not detected from *Herve* cores. In this previous study, *Fusobacterium* accounted for 2.5% of sequence reads obtained from raw milk cheese surfaces (Delcenserie *et al*., 2014).

Globally, as a first approach, bacterial profile was variable between batches, although these were manufactured in the same factory, using the same process, and were stored under the same conditions. The most interesting information was that the unknown *Fusobacterium* sp. was observed in all batches. In total, 5,299 sequence reads (out of 178,981, *i.e.* 3.0%) were associated to this OTU.
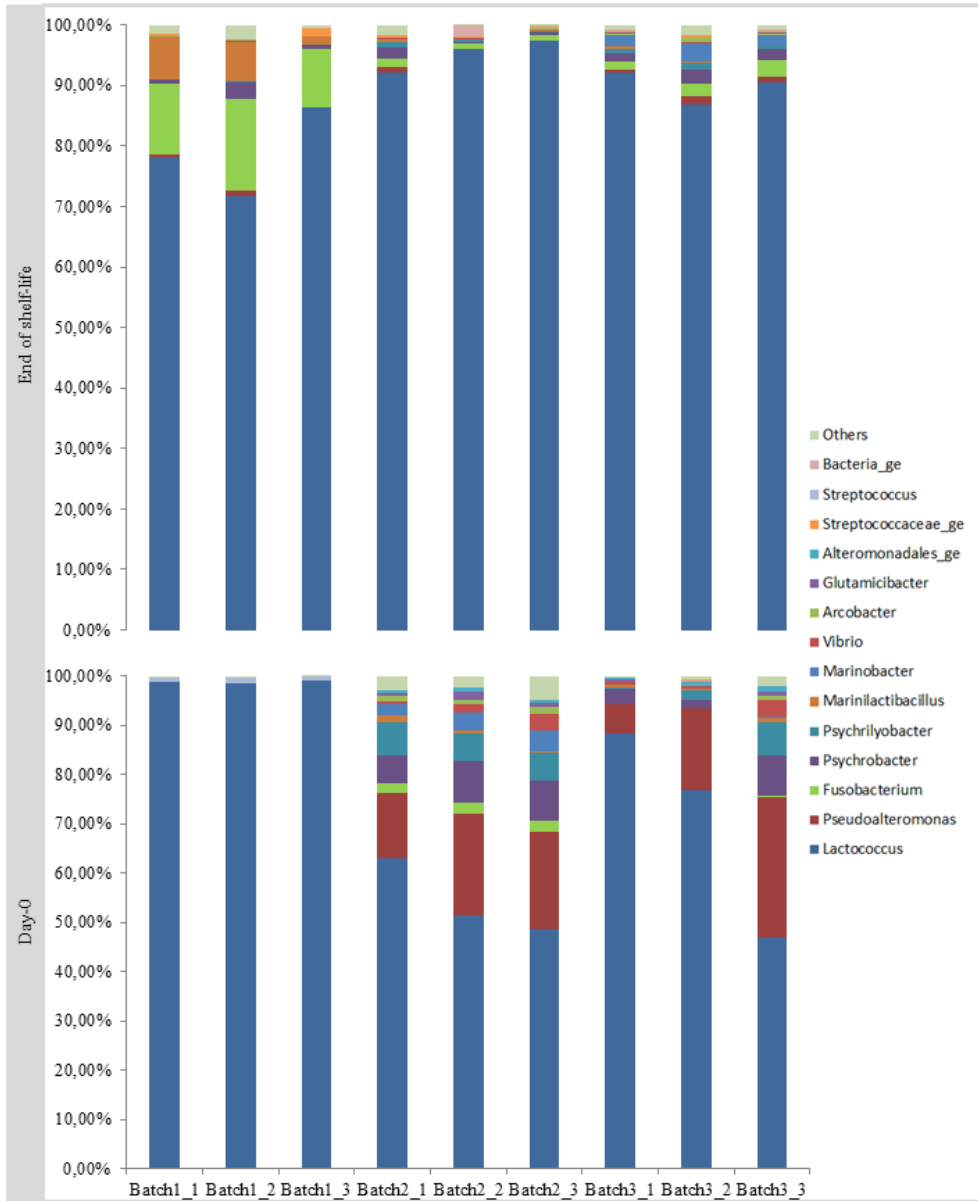
Figure 7-1. Microbiota of three batches of raw milk Herve cheese at day-0 and end of shelf-life at the genus level (in triplicates, from distinct suspensions of 25 g of cheeses). Only genera with relative abundance > 1% in at least one batch at day-0 or end of shelf-life were considered.

b) Ecological indicators

Coverage was > 0.99 for all batches. No significant differences in richness (number of genera and Chao1 estimator) were identified. Similarly, Simpson's

evenness was not significantly different between samples. Inverse Simpson index, characterizing diversity, was significantly higher in batch 2 at day-0.

Tridimensional NMDS was build, based on Bray-Curtis dissimilarity matrix (**Figure** 7-2). It was observed that batches 2 and 3 at day-0 were clustered apart from other samples. Similarly, samples of batch 1 at end of shelf-life were located at the bottom of the graph. Nevertheless, AMOVA and homogeneity of molculare variance (HOMOVA) did not reveal significant differences, meaning that *Herve* community structure did not significantly vary during shelf-life and between batches. However, after correction of p-values obtained from ANOVA using FDR method, significant differences were observed for specific genera. For all batches, relative abundance of *Lactococcus* was significantly different between day-0 and end of shelf-life, but also between all batches at both sampling times. As observed intuitively, relative abundance of *Fusobacterium* was significantly higher in batch 1 at end of shelf-life but, on the opposite, that of *Psychrilyobacter* was lower.
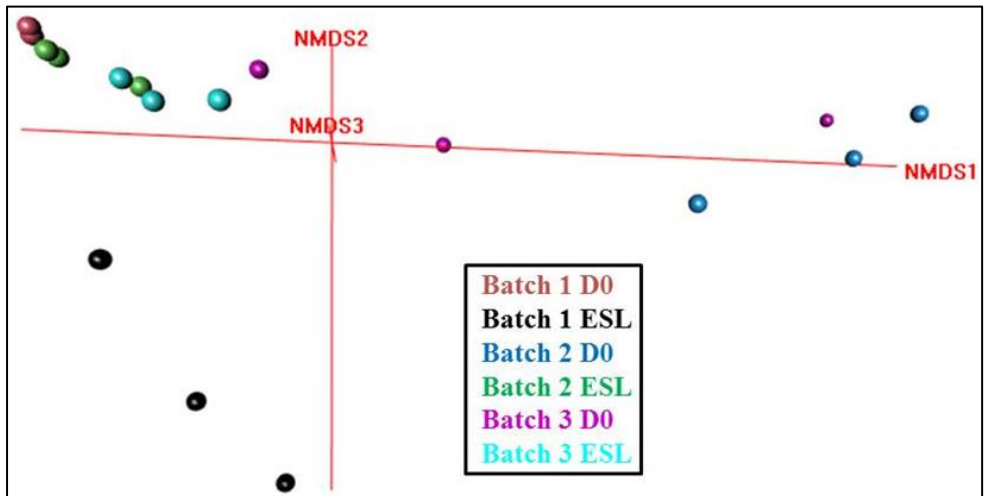
Figure 7-2. NMDS characterizing diversity of the three batches of Herve cheese at day-0 and end of shelf-life, built from Bray-Curtis dissimilarity matrix.

The unknown *Fusobacterium* sp. from *Herve* cheese

Fusobacterium is a genus of Gram-negative bacteria belonging to *Fusobacteria* phylum. These strict fastidious anaerobes are catalase-negative non spore-forming bacilli, producing butyric acid, and having GC content comprised between 26 and 34%. Their optimal growth generally occurs at 35-37°C and at pH 7. *Fusobacterium* are normal inhabitant of animal and human genital and gastrointestinal tracts (De Witte *et al.,* 2017; Garcia-Carretero *et al*., 2017; Rachana *et al*., 2019). The genus currently includes 19 species, according to NCBI Taxonomy, among which some are known pathogenic bacteria, *e.g. F. mortiferum*, *F. necrophorum*, *F. nucleatum* and *Fusobacterium varium* (Schoch *et al*., 2020). These pathogens are for instance

responsible for abscess formation and septicemia (Garcia-Carretero *et al.*, 2017). Nevertheless, some species, including *F. gastrosuis*, have no known pathogenic effects (De Witte *et al.*, 2017). According to literature review, the presence of *Fusobacterium* in food is uncommon.

Full sequence of V1-V3 region of 16S rRNA gene of this unknown *Fusobacterium* sp., assigned using SILVA 138 reference database, was:

5'-TTCTTTGGAGAGTTTATTCTGGCTCAGGATGAACGCTGACAGAATGCTTA ACACATGCAAGTCTACTTGAATTCACTTCGGTGATAGTAAGGTGGCGGACGGG TGAGTAACACGTAAAGAACTTGCCTTACAGTTTGGGACAACTATTGGAAACGA TAGCTAATACCGGATATTATGAATTTTCCGCATGGAAGATTTATGAAAGCTATA TGCGCTGTAAGAGAGCTTTGCGCCCCATTAGCTAGTTGGTGAGGTAACAGCT CACCAAGGCAACGATGGGTAGCCGGCCTGAGAGGGTGAACGGCCACAAGGG GACTGAGACACGGCCCTTACTCCTACGGGAGGCAGCAGTGGGGAATATTGGA CAATGGACCAAAAGTCTGATCCAGCAATTCTGTGTGCACGATGACGGTCTTCG GATTGTAAAGTGCTTTCAGTTGGGAAGAAAGAAATGACGGTACCAACAGAAGA AGCGACGGCTAAATACGTGCCAGCAGCCGCGGT-3'.

This sequence was aligned with known sequences of *Fusobacterium* spp. using Basic Local Alignment Search Tool (BLAST; **Table** 7-2; Johnson *et al.*, 2008). All sequences with identity > 97% corresponded to uncultured bacteria, or at least to uncultured *Fusobacteriaceae* or *Fusobacterium* spp. Most sequences corresponded to bacteria identified in marine environments or organisms. The most closely related known species was *Fusobacterum perfoetens* (in bold in **Table** 7-2). This bacterium was isolated from pig gut microbiome. Sequence identity was 96% only, while clustering into distinct OTUs is generally performed when sequence identity is < 97%.

Alignment with reference 16S rRNA gene sequences of known *Fusobacterium* species is shown in **Table** 7-3. The most closely related species was *F. perfoetens*, with a sequence identity of only 93.6%. Identity with all other *Fusobacterium* species was < 92.0%. **Figure** 7-3 shows phylogenetic tree based on all available sequences of V1-V3 regions of 16S rRNA gene of known species of *Fusobacterium*, built using Molecular Evolutionary Genetics Analysis (MEGA; Kumar *et al.*, 2018) and plotted using interactive tree of life (iTOL; Letunic and Bork, 2016). The unknown OTU was clustered apart from other *Fusobacterium* species, at the root of the tree. The closest species was *F. perfoetens*, as concluded from alignments. On the tree, *Fusobacterium necrogenes* was also close to the unknown *Fusobacteruim* sp. but, in practice, the sequence of this species was totally different, as alignments did not find any significant similarity.

Although these observations were based on relatively small DNA fragments, isolating and characterizing this species was worth the candle. Next parts of this chapter will deal with experiments performed for this purpose.
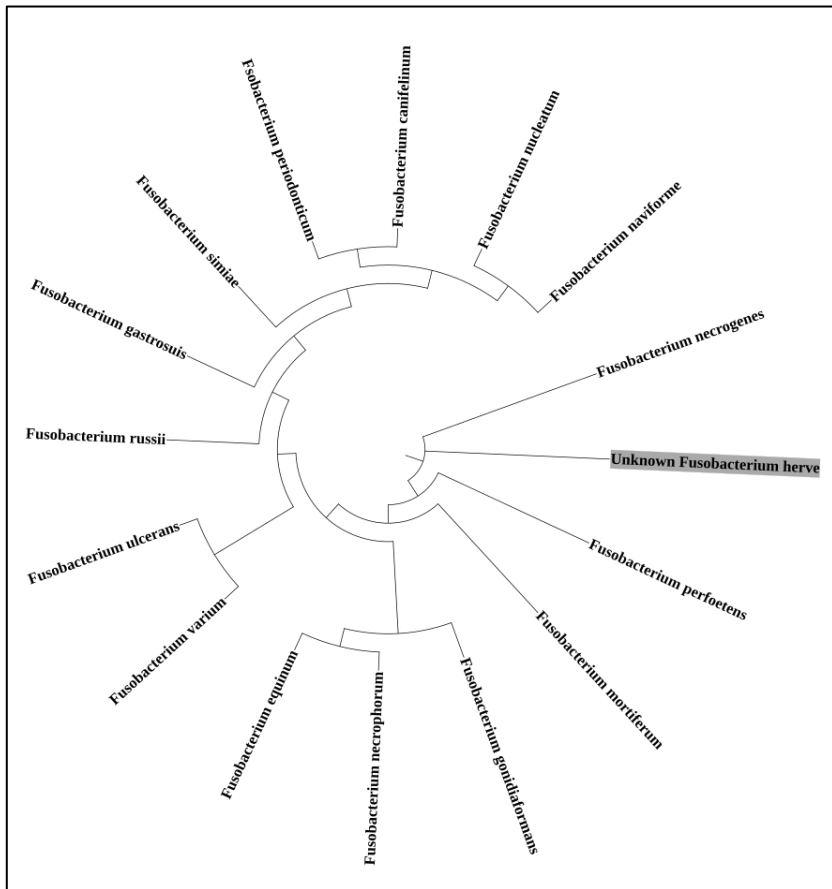
Figure 7-3. Phylogenetic tree built using neighbour-joining algorithm and based on V1-V3 regions of 16S rRNA gene of *Fusobacterium* species. OTU observed in *Herve* cheese is highlighted in grey.

Table 7-2. Most closely related sequences with V1-V3 regions of 16S rRNA gene of the unknown OTU of *Fusobacterium* identified in raw milk *Herve* cheese (adatpted from NCBI BLAST).

Name	Accession	Origin	Reference	Sequence coverage (%)	Identity (%)
Uncultured bacterium	DQ325482.1	Wastewater	Zhao et al., 2008	98.0	99.4
Uncultured bacterium	JF827563.1	Oysters	Fernandez-Piquer et al., 2012	98.0	98.8
Unc. Fusobacteriaceae	KF505953.1	Cutaneous microbiome	Chehoud et al., 2013	95	99.2
Fusobacterium sp.	MH746937.1	Marine environment	Unpublished	98.0	98.0
Uncultured bacterium	KT819906.1	Anaerobic sludge	Unpublished	94.0	99.1
Uncultured bacterium	KT819862.1	Anaerobic sludge	Unpublished	94.0	99.1
Uncultured *Fusobacterium*	KF508201.1	Cutaneous microbiome	Chehoud et al., 2013	95.0	98.8
Uncultured bacterium	KF571755.1	Yellow croaker	Unpublished	98.0	97.6
Uncultured bacterium	KF571752.1	Yellow croaker	Unpublished	98.0	97.6
Uncultured bacterium	JF827482.1	Oysters	Fernandez-Piquer et al., 2012	98.0	97.6
Fusobacteriaceae sp.	KT799837.1	Mariculture fish waste	Unpublished	98.0	97.4
Fusobacterium sp.	MN630848.2	Marine environment	Unpublished	90.0	99.6
Uncultured *Fusobacterium*	EF419218.1	Estuar	Chauhan & Williams, 2007	89.0	99.1
Uncultured bacterium	KT952719.1	Surgeonfish	Miyake et al., 2016	98.0	95.8
Uncultured bacterium	KJ507667.1	Oil reservoir	Unpublished	89.0	98.9
Uncultured bacterium	HG971024.1	Sea lettuce	Unpublished	98.0	95.8
Uncultured bacterium	HG971023.1	Sea lettuce	Unpublished	98.0	95.9
Fusobacteriaceae	HG326493.1	Sea lettuce	Unpublished	98.0	95.6
Uncultured bacterium	LC549969.1	Plankton	Unpublished	98.0	95.6
F. perfoetens	MN537503.1	**Pig gut microbiome**	**Unpublished**	**95.0**	**96.0**

Table 7-3. Alignment of sequence of putative *Fusobacterium* sp. from *Herve* cheese with 16S rRNA gene sequences of all known *Fusobacterium* species (adapted from NCBI BLAST).

Species	Accession	Origin	Reference	Covery	Identity
F. perfoetens	MN537503.1	Pig gut microbiome	Unpublished	99.0	93.6
F. gastrosuis	NR_146837.2	Pig stomach	De Witte *et al.*, 2017	98.0	91.8
F. varium	NR_113384.1	Unknown	Unpublished	98.0	91.6
F. mortiferum	NR_117734.1	Unknown	Unpublished	96.0	91.8
F. russii	NR_044687.2	Unknown	Unpublished	98.0	90.4
F. equinum	NR_028933.1	Horse oral cavity	Dorsch *et al.*, 2001	98.0	90.4
F. canifelinum	NR_042755.1	Unknown	Unpublished	96.0	90.4
F. necrophorum	MN537550.1	Intestine	Unpublished	97.0	89.5
F. simiae	NR_044689.2	Unknown	Unpublished	98.0	88.9
F. naviforme	AF342840.1	Unknown	Conrads *et al.*, 2002	93.0	89.1
F. gonidiaformans	NR_027588.1	Unknown	Lawson *et al.*, 1991	82.0	91.1
F. periodonticum	NR_026085.1	Unknown	Lawson *et al.*, 1991	82.0	90.4
F. ulcerans	NR_044820.1	Unknown	Lawson *et al.*, 1991	82.0	89.6
F. nucleatum	NR_026083.1	Unknown	Lawson *et al.*, 1991	82.0	89.1

Evaluation of a qPCR assay specific to the genus *Fusobacterium*

To facilitate decisions on the selection of candidate clones and to limit costs, ability of PCR primers developed by Nagano *et al.* (2007) to detect the unknown OTU of *Fusobacterium* was assessed.

1. Material and methods

Sequences of forward (FUSO1) and reverse (FUSO2) primers were 5'-GAGAGAGCTTTGCGTCC-3' and 5'-TGGGCGCTGAGGTTCGAC-3', respectively (Eurogentec, Liège, Belgium). Although the original protocol was designed for PCR, it was used in qPCR in the present work. **Table** 7- presents constituents of each qPCR reaction. Master mix used was Takyon^{TM} ROX SYBR 2x MasterMix dTTP blue (Eurogentec, Liège, Belgium). Detection of amplification and fluorescence was thus based on SYBR green. qPCR was performed on CFX96 Touch Real-Time PCR thermocycler (Bio-Rad, Hercules, CA, USA) with the following thermal cycling parameters: 5 minutes at 94°C, followed by 30 PCR cycles composed of 30 seconds at 94°C, 30 secondes at 60°C and 30 seconds at 72°C. A final extension occurred at 72°C. Melting curves were produced using a gradient of 0.5°C/min from 60 to 95°C.

Table 7-4. Reagents involved in each PCR well.

Reagent	Volume (µL)
Takyon^{TM} ROX SYBR 2X MasterMix dTTP blue	10.0
Forward primer (10 µM)	1.0
Reverse primer (10 µM)	1.0
Water	5.5
DNA template	2.5

As positive control, the strain *F. perfoetens* DSM 105865 (Clavel, TU Munich, Freising-Weihenstephan, Germany) was purchased (DSMZ GmbH, Braunschweig, Germany). Lyophilized strain was reactivated anaerobically following instructions from the expeditor, and grown on Columbia agar (Thermo Fisher Scientific, Waltham, MA, USA) for 72 h at 37°C.

Using platinum handle, a single colony was collected and put in 150 µL of Chelex 100 Resin 10% solution (Bio-Rad, Hercules, CA, USA). Tube was incubated at 95°C for 15 minutes and under agitation of 900 rpm, using Thermomixer R (Eppendorf, Hamburg, Germany). Resin was peletted by centrifugation at 10.000 rpm for 3 minutes using MiniSpin plus (Eppendorf, Hamburg, Germany). Supernatant was transferred to new tubes and stored until use.

Three new raw milk *Herve* cheeses from the same factory were also purchased. Samples were refrigerated until use-by-date. Cores (ID *Herve* 1C, 2C and 3C) and surfaces (ID *Herve* 1S, 2S and 3S) were considered separately. Suspensions were prepared by diluting 25 g of respective cheese parts in BPW, and homogenized

using Stomacher BagMixer Lab Blender (Interscience, Ottignies-Louvain-la-Neuve, Belgium). DNA was extracted from 20 µL of this suspension using DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany).

PCR primers were tested on some DNA extracts obtained during Chapter 6, from new *Herve* samples, and on DNA of *F. perfoetens*.

2. *Results and discussion*

PCR results are summarized in **Table** 7-. Sample IDs used in this table correspond to ID's from Chapters 5 and 6. The batch of raw milk *Herve* cheese was SRSC4. Surprisingly, qPCR was positive for samples collected at day-0, in which the unknown OTU was not identified using metagenetics. All new cheeses tested positive for the presence of *Fusobacterium* for both core and surface samples. Cycle threshold (Ct) was lower for surface than for core samples (by 3 PCR cycles, on average). It meaned that unkown *Fusobacterium* sp. could be predominantly located on cheese rinds. This would be in accordance with results reported by Delcenserie *et al*. (2014), who observed *Fusobacterium* in rinds of raw and pasteurized milk *Herve* cheese. Cheese surface are exposed to oxygen. It could thus be surprising to observe fastidious anaerobes there. Nevertheless, it is known that some *Fusobacterium* spp. can contribute to formation of biofilms with anaerobic properties (Horiuchi *et al*., 2020).

Table 7-5. Results of qPCR tests for the detection of *Fusobacterium* in cheese.

Sample	*Fusobacterium* in metagenetics (+/-)	*Fusobacterium* in qPCR (+/-)	Ct
SRSC1 day-0	-	-	/
SRSC1 end of shelf life	-	-	/
SRSC4 day-0	-	+	23.3 ± 0.9
SRSC4 end of shelf life	+	+	24.2 ± 0.0
UACC3 day-0	-	-	/
SPSHC4 day-0	-	-	/
MRSC4 day-0	-	-	/
Herve 1C	/	+	25.5 ± 0.8
Herve 1S	/	+	20.5 ±0 .1
Herve 2C	/	+	23.0±0.1
Herve 2S	/	+	20.1 ± 0.1
Herve 3C	/	+	23.7 ± 0.3
Herve 3S	/	+	20.4 ± 0.2
F. perfoetens (T+)	/	+	8.5 ± 0.6
T-	/	-	/

Legend: +, detected using concerned technique; -, not detected using concerned technique; T+, positive control; T-, negative control including 2.5 µL of DNA-free water instead of template DNA.

Selective isolation of the unknown *Fusobacterium*

Several approaches were tested to isolate the unknown *Fusobacterium* sp. from *Herve* cheese samples (**Figure** 7-4). Nevertheless, none of these methods allowed to reach the objective. First paragraph presented hereafter describes state of the art on media and protocols used to identify and to isolate known species of *Fusobacterium*. After that, a detailed description of what was performed will be proposed.

1
- 3 samples from challenge studies and 3 new *Herve* cheeses at end of shelf-life
- Original suspensions + dilutions 10^{-5} and 10^{-6}
- Medium A + Medium B
- Incubation in anaerobic jars for 72 h at 30 and 37°C
- Streaking of colonies on Medium A and incubation 72 h at 30 or 37°C
- Sequencing of V1-V3 regions of 16S rRNA gene for selected colonies

2
- 3 samples from challenge studies and 3 new *Herve* cheeses, distinguishing cores and rinds
- Dilutions 10^{-2} and 10^{-3}
- Medium A + Medium B
- Incubation in anaerobic jars at 30°C and in anaerobic chamber at 37°C
- Streaking of colonies on Medium A and incubation 72 h at 30 or 37°C
- Scraping of original plates, DNA extraction and qPCR assays

3
- Samples from Approach n°2
- Liquid culture in BHI + neomycin, BHI + erythromycin and BHI + neomycin + erythromycin
- Incubation for 72 h at 37°C in anaerobic chamber
- Collection of 100 µL from each tube for DNA extraction and qPCR assays

4
- Samples from Approch n°2 + BHI tubes positive for *Fusobacterium* sp. in qPCR
- Medium A + Medium B
- Incubation in anaerobic jars for 1 week at room temperature
- Streaking of colonies on Medium A and incubation 72 h in anaerobic jars
- Scraping of original plates, DNA extraction and qPCR assays
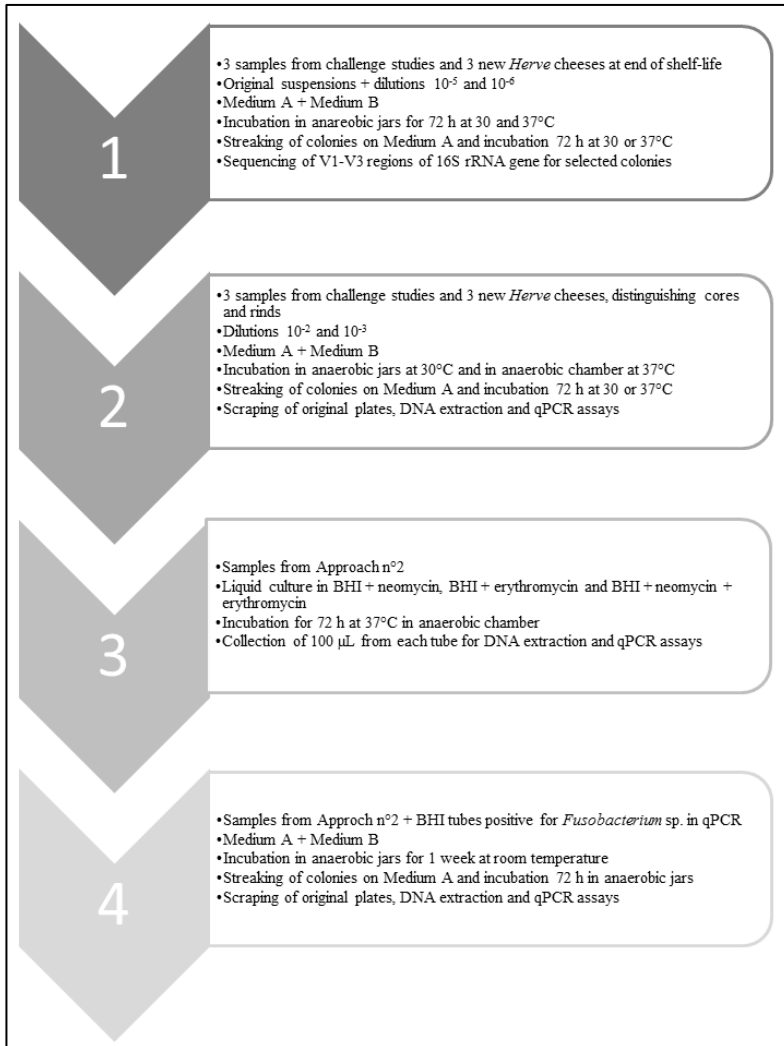
Figure 7-4. Approaches tested to isolate the unknown *Fusobacterium* sp. observed in *Herve* cheese; Medium A, Columbia agar + 5% defibrinated sheep blod; Medium B, Columbia agar + 5% defibrinated sheep blood + 100 mg/L neomycin + 5 mg/L vancomycin + 1 mg/L erythromycin.

1. State of the art on isolation of *Fusobacterium* spp.

Studies detailing procedures for isolation of *Fusobacterium* spp. are not numerous in the literature. **Table** 7-6 gathered all these references, as well as isolation media and incubation times and temperatures. Incubation temperature was always 37°C, with an incubation time of at least 48 h. Media generally contained blood and various antibiotics.

2. Approach n°1

1. Samples

For this first test, six samples were used, namely three suspensions stored at -80°C since challenge studies, related to batch 1 at end of shelf-life, and three new commercial samples of *Herve* cheese considered at end of shelf-life.

2. Method

Suspensions were prepared from 25 g of each new *Herve* cheese samples, including cores and crusts, diluted 10-fold in BPW and homogenized using Stomacher BagMixer Lab Blender (Interscience, Ottignies-Louvain-la-Neuve, Belgium). Initial suspensions and dilutions 10^{-5} and 10^{-6} were spread on Columbia Agar (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 5% defibrinated sheep blood (Medium A; Thermo Fisher Scientific, Waltham, MA, USA), and on the same medium with extra antibiotics, namely 100 mg/L of neomycin, 5 mg/L of vancomycin and 1 mg/L of erythromycin (Medium B; from De Witte *et al*. (2017); all antibiotics were purchased from Sigma-Aldrich, Saint-Louis, MO, USA). Petri dishes were placed in anaerobic jars (Merck, Darmstadt, Germany). Deoxygenation was obtained using Oxoid AnaeroGen 2.5 L patches (Thermo Fisher Scientific, Waltham, MA, USA). Jars were stored at 30 and 37°C for 72 h. For each plate on which microbial growth occurred, six colonies were streaked on Medium A and incubated at respective temperature for 72 h. Isolated colonies were colored using crystal violet and visualized in immersion microscopy. Based on phenotype, 15 colonies were selected and sent to Genalyse Partner s.a. (Sart-Tilman, Belgium) for PCR amplification of V1-V3 regions of 16S rRNA gene, library prepation and Illumina sequencing.

3. Results

No match with the sequence of the unknown OTU of *Fusobacterium* identified in *Herve* cheese were observed, meaning that targeted *Fusobacterium* sp. was not isolated. A first hypothesis was that jars did not guarantee sufficient deoxygenation. Another possibility was that laboratory handlings performed under aerobic conditions, including suspension preparation and plating, could be sufficient to decrease vitality of this fastidious anaerobe.

Table 7-6. Media used for isolation of *Fusobacterium* spp.

Species	Matrix	Medium	Incubation temperature (°C)	Incubation time (h)	Reference
F. canifelinum	Dogs and cats oral cavity	*Brucella* agar	37	NA	Conrads *et al.*, 2004
F. gastrosuis	Pig stomach	Columbia agar + 5% defibrinated sheep blood + N (100 mg/L) + V (5 mg/L) + E (1 mg/L)	37	72	De Witte *et al.*, 2017
F. massiliense	Human duodenum	Columbia agar + 5% defibrinated sheep blood	37	72	Mailhe *et al.*, 2017
F. necrophorum	Humans with sore throat	Blood agar + N (100 mg/L) + V (disc)	37	48	Amess *et al.*, 2007
F. necrophorum	Throat swabs	GN anaerobe medium	37	24-48-120	Batty and Wren, 2005
F. nucleatum	Human oral cavity	Columbia Agar + 5% defibrinated sheep blood + 0.15% crystal violet	37	NA	Abed *et al.*, 2020
F. ulcerans	Ulcers	Blood agar	37	36-48	Adriaans and Shah, 1988
Fusobacterium spp.	NA	Fastidious anaerobe agar + 5% defibrinated horse blood + J (3 mg/L) + V (1 mg/l) + No (1 mg/L)	37	48	Brazier *et al.*, 1991

Legend: NA, not available; N, neomycin; E, erythromycin; V, vancomycin; J, josamycin; No, norfloxacin.

3. Approach n•2

1. Samples

This time, the same three suspensions stored since challenge studies were used, with extra samples 1C, 2C and 3C and 1S, 2S and 3S, corresponding to cores and surfaces of three new *Herve* cheese. The presence of the *Fusobacterium* sp. in all samples had previously been confirmed by qPCR.

2. Method

Medium A and Medium B were used, but with salt content increased to 2.5% (salt content of *Herve* cheese). Laboratory handlings were performed into Anaerobic Workstation – Concept plus (Baker Ruskinn, Sanford, ME, USA). Dilutions 10^{-2} and 10^{-3} were used. Half of the Petri dishes were incubated at 37°C for 72 h into anaerobic incubator, the other half being stored at 30°C in anaerobic jars. Again, colonies able to grow were streaked on Medium A. After that, using a platinum handle, the whole surface of plates was scraped and put in 150 µL of Chelex 100 resin 10% solution for DNA extraction. After quality and concentration control, qPCR was performed using primers FUSO1 and FUSO2.

3. Results

In this case, qPCR test was implemented to avoid huge costs associated to library preparation and DNA sequencing for selected candidate colonies. By performing qPCR on all colonies scraped from Petri dishes, it would have been possible to consider and sequence clones sampled from positive plates only. Nevertheless, none of the plates was positive for *Fusobacterium* genus in qPCR. Positive controls, *i.e.* DNA of *F. perfoetens*, produced signal at expected Ct. Consequently, working in anaerobic station did not allow to solve issues and to isolate the unknown *Fusobacterium* sp.

4. Approach n•3

1. Samples

Samples were the same as those used during approach n°2.

2. Method

Various liquid media were tested, namely (a) BHI with 100 mg/L of neomycin, (b) BHI with 1 mg/L of erythromycin and (c) BHI with 100 mg/L of neomycin and 1 mg/L of erythromycin. Tubes were heated at 95°C to eliminate oxygen before antibiotic addition. After that, tubes were directly placed into anaerobic workstation for cooling, and appropriate concentrations of antibiotics were added. Once cold, 1 mL of cheese original suspension was added to 9 mL of respective growth medium. Tubes were incubated at 37°C for 72 h in anaerobic chamber. After this time, 100 µL of each tube were transferred into 2 mL tubes containing 150 µg of Chelex 100 Resin 10% for DNA extraction and qPCR using FUSO1 and FUSO2 primers.

3. Results

Results were surprising. *Fusobacterium* was detected into three tubes using qPCR, corresponding to three samples and to three growth media. The bacterium was able to grow in one sample from challenge study added to BHI with 1 mg/L of erythromycin. A sample collected from *Herve* surface diluted in BHI with 100 mg/L of neomycin also allowed its growth. Finally, the third positive tube corresponded to a sample of *Herve* core grown in BHI including both antibiotics. All put together, it was not possible to find a logical conclusion to this approach, due to the variability in the results.

5. *Approach n°4*

1. Samples

For this last approach, samples included:

- Tubes from approach n°3 which tested positive for *Fusobacterium* spp. in qPCR;
- *Herve* cheese suspensions (1S, 2S, 3S and 1C, 2C, 3C).

2. Method

In anaerobic workstation, 100 µL of respective cheese suspensions were spread on Medium A and Medium B, with salt content increased to 2.5%. All dishes were incubated in anaerobic jars for one week at room temperature. As the unknown OTU of *Fusobacterium* was able to grow during cheese ripening and storage, this bacterium could be psychrotrophic. It was thus interesting to try to isolate it at a lower temperature than those commonly recommended for other *Fusobacterium* spp. Colonies able to grow were streaked on Medium A. Again, the whole surface of initial Petri dishes was scraped using platinum handle, and bacteria were added to 100 µL of Chelex 100 10% for DNA extraction and qPCR with primers FUSO1 and FUSO2.

3. Results

During this approach, more colonies were able to grow on Medium A. Nevertheless, no colonies were observed on Medium B. Using qPCR, no DNA of *Fusobacterium* has been identified from plates considered in this approach.

6. *Conclusions*

Globally, using all these approaches, it was not possible to isolate the unknown *Fusobacterium* from *Herve* cheese samples. Experiments included three types of samples, various liquid and solid media, two systems allowing incubation under anaerobic conditions, as well as three storage temperatures. Some of them brought interesting information on the bacterium. It is now known that it could be mainly localized on cheese surfaces. A hypothesis to explain the inability to isolate the *Fusobacterium* from *Herve* cheese was that storage of suspensions from challenge studies at -80°C partly degraded bacterial membranes and that damaged cells were not able to grow anymore. That is why it was decided to include new commercial samples in the study. Using qPCR, it was confimed that all these samples contained the targeted bacterium. Uncertainty remained regarding the actual physiological

state of these *Fusobacterium*. Indeed, qPCR do not allow to discriminate between dead, degraded and alive bacterial cells, as DNA of dead cells could still be present in cheese matrix. Nevertheless, approach n°3, with liquid BHI media, demonstrated that *Fusobacterium* sp. was able to grow, suggesting that these bacteria were well alive, and survived to cheese ripening and storage. The question of the inability to isolate and to cultivate this bacterium remains, as Medium A is a particularly rich medium. A likely hypothesis to explain unability to isolate the unkown bacteria could be that it is unculturable, being included in the viable but non-culturable (VBNC) community of cheese. VBNC is a physiological state different from dormance state and allowing survival for long periods following exposure to unfavorable conditions or to cell damages (Ramamurthy *et al*., 2014). In theory, VBNC cells can be reactivated when adequate environmental conditions are provided, meaning that bacteria can become culturable on classical rich growth media (Fakruddin *et al*., 2013; Ayrapetyan and Oliver, 2016). In theory, during experiments, all basic requirements were provided to the targeted *Fusobacterium* sp., including absence of oxygen, various temperatures, and a non-selective growth medium. Another phenomenom which can occur is auxotrophy, *i.e.* the inhability of an organism to synthesize an essential component for its growth. Such bacteria require the inclusion of this given component to the growth medium, or the presence in their environment of other species able to provide this molecule in a sufficient concentration.

In silico reconstruction of the genome of the unknown *Fusobacterium* and comparative genomics

As we failed in the isolation of *Fusobacterium* sp. on growth plates under anaerobic conditions, it was decided to acquire the metagenome of a *Herve* cheese DNA sample. As *Fusobacterium* sp. represented around 10% of the microbiota in this variety, it was thought that it could be possible to reunite its whole genome using NGS. After assembly and annotation, the objectives were, as a first approach, to determine if the targeted *Fusobacterium* sp. could belong to a novel species, and to explore potential differences in metabolic activities between them.

1. *Material and methods*

1. Samples

DNA extracted from sample SRSC4_2 from Chapter 6 was used for this experiment. Indeed, this sample from the first batch of *Herve* cheese which was used for assessment of growth potential showed the highest relative abundance of *Fusobacterium* sp. using metagenetics (see Supplementary material 6-3).

2. DNA sequencing

Library was prepared and sequenced by GIGA Genomics platform (Liège, Belgium). Briefly, library was prepared using Illumina DNA PCR-free Prep kit (San Diego, CA, USA). Library was sequenced using Illumina NovaSeq 6000 sequencer with 300 cycles (San Diego, CA, USA). This sequencing technology provides short

reads (*i.e.* 50-200 nucleotides) with low error rates (*i.e.* 0.5-2.0%). Demultiplexing of reads as well as quality controls were also performed by GIGA Genomics platform.

3. Bioinformatics and data analysis

Genome assembly and annotation were performed using online server PATRIC (Gillespie *et al.*, 2011). Assembler used by PATRIC was SPAdes (Bankevich *et al.*, 2012). PATRIC provided a binning report as output. For each bin, assembled contigs were provided and used for further analyses. Regarding genome annotation, PATRIC used Rapid Annotation using Subsystem Technology (RAST) tool kit (Brettin *et al.*, 2015). JSpecies was used for pairwise comparison between genomes of *Fusobacterium* sp. from *Herve* cheese and of other species of the genus (collected from NCBI), allowing to assess percentage of identity, using average nucleotide identity based on MUMmer (ANIm; Kurtz *et al.*, 2004; Richter *et al.*, 2016).

After gathering 11 genomes of known *Fusobacterium* species, a phylogenomic tree was built, based on the sequence of 100 genes. An unparented species, namely a *Leptotrichia* sp. isolated from oral cavities, was used as tree root. Tree building was based on randomized axelerated maximum likelihood (RAxML) method.

Comparative genomics for *Fusobacterium* spp. was performed using proteome comparison tool available on PATRIC. The figure allowed a visual distinction between shared and unshared genes accross genomes thanks to a color code. Comparison was based on proteins similarity, using protein BLAST (BLASTP). When similarity was found, it was possible for user to know if the relation was uni- or bidirectional.

In addition to that, the tool Genome Group View of PATRIC allowed a comparison of pathways and subsystems between *Fusobacterium* spp. This viewer is associated to Kyoto Encyclopedia of Genes and Genomes (KEGG) maps (Okuda *et al.*, 2008), providing a picture of enzymes potentially produced by concerned genomes and on their role in associated metabolic pathways. Heatmaps were also automatically built, allowing to easily identify shared and unshared enzymes.

2. *Results*

1. General information on acquired data

Assembly algorithm attributed reads to five major bins (**Table** 7-7, derived from binning report). Data acquired for *Fusobacterium* sp. reported genome completeness of 100%. This bin was the only one to meet quality criteria defined by PATRIC, namely completeness ($\geq 80\%$), consistency ($\geq 78\%$) and contamination ($\leq 10\%$), corresponding to the targeted uncultured *Fusobacterium* sp. population. Other bins were attributed to bacterial species commonly found in cheese. *L. lactis* was the dominant population identified during previous steps of this thesis, while *Psychrobacter* and *Marinilactibacillus* were part of subdominant population of *Herve* cheese, observed using metagenetics (Gérard *et al.*, 2021).

Genome of *Fusobacterium* sp. is presented in **Figure** 7-5. Genome size was around 2 Mb. Number of contigs was 213 and GC content was 28.3%. PATRIC identified 2,101 coding sequences (CDS) across the genome. Genes coded for 668

hypothetical proteins, and for 1,433 proteines with functional assignments, among which 1,368 were attributed to cross-genus protein families (PGfam). PATRIC allowed the identification of subsystem superclasses associated with these genes/proteins (**Figure** 7-6). According to Overbeek *et al*. (2005), a subsystem is "a set of functional roles that together implement a specific biological process or structural complex". Around one third were associated to metabolism. An important part of coding sequences was associated to DNA, RNA and protein processing, as well as to stress response and energy production.
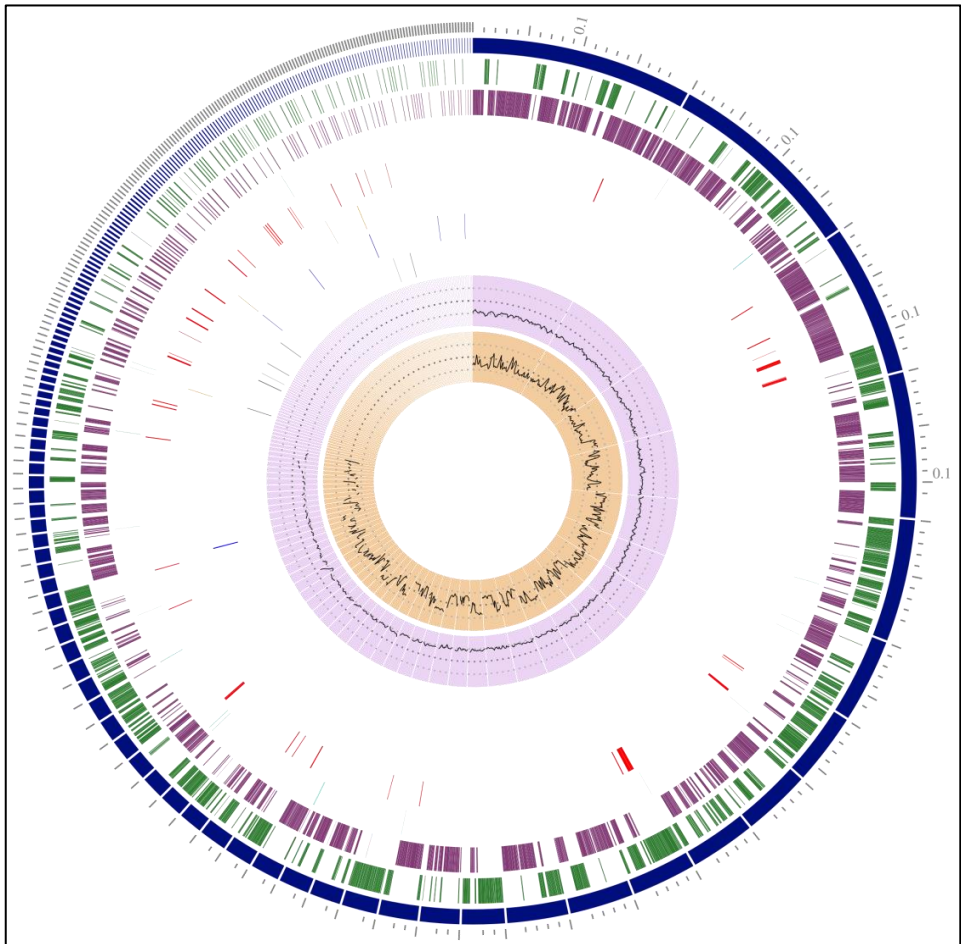
Figure 7-5. Representation of the genome of uncultured *Fusobacterium* sp. from *Herve* cheese. From outermost circle to center: Circle 1, scale in Mb; Circle 2, contigs; Circle 3, forward CDS; Circle 4, reverse CDS; Circle 5, non-CDS features; Circle 6, antimicrobial resistance genes; Circle 7, virulence genes; Circle 8, transporters; Circle 9, drug targets; Circle 10, GC content; Circle 11, GC skew (*i.e* ration (G-C)/(G+C)).
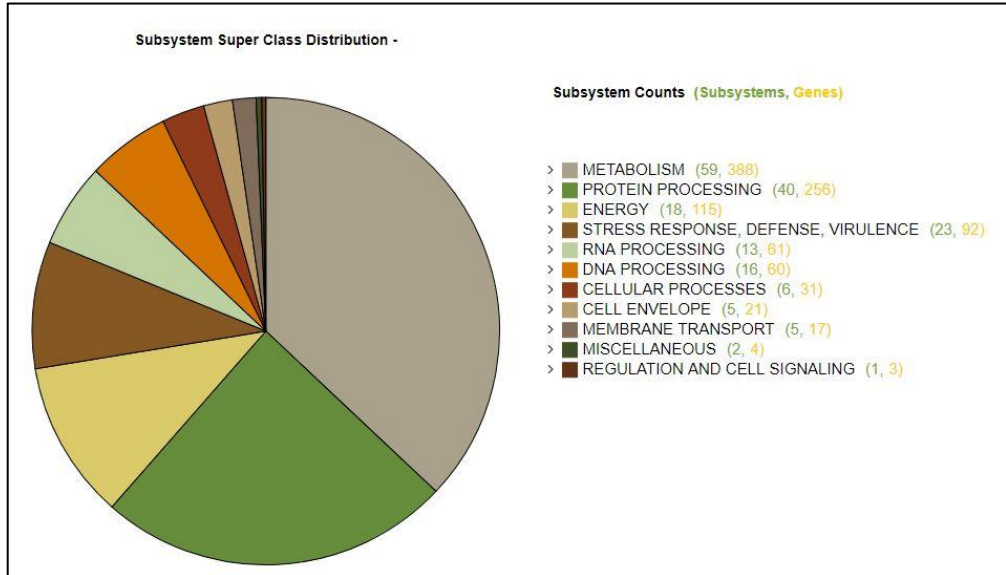
Figure 7-6. Distribution of genes from *Fusobacterium* sp. from Herve cheese between subsystem super classes.

2. Phylogenomic tree and basic comparison of genomes

Pairwise comparisons of genomes performed using JSpecies are presented in **Table** 7-8. Globally, the closest known *Fusobacterium* species could be *F. perfoetens*, but with ANIm of 82.89% only. As a reminder, based on V1-V3 regions of 16S rRNA gene, this species was already considered as the most closely related to the uncultured *Fusobacterium sp*. from *Herve* cheese. ANIm related to pairwise comparisons between other known *Fusobacterium* spp. were comparable, except between *F. periodonticum* and *F. pseudoperiodonticum* (95.38) and between *F. nucleatum* and *F. wasookii* (99.66). Our uncultured *Fusobacterium* could thus correspond to a novel species of the genus.

Phylogenomic tree provided an additional clue (**Figure** 7-7). Taxon most closely related to *Fusobacterium* sp. identified in *Herve* cheese (uncultured *Fusobacterium* clonal population 159267.45 on the figure) was again *F. perfoetens*. Both were clustered together at the root of the tree. As suggested by ANIm, *F. hwasookii* and *F. nucleatum* were closely related. *Leptotrichia* sp. was clustered apart from *Fusobacterium* spp. and allowed the definition of tree root.

Based on these results, it was now important to try look for elements to explain what makes *Fusobacterium* sp. from *Herve* cheese different, in terms of potential metabolic activities and, if possible, to understand how it was able to become a subdominant population in this dairy product.

Table 7-7. Bins obtained after genome assembly using PATRIC.

Genome name	Consistency (%)	Completeness (%)	Contamination (%)	Number of contigs	DNA size (bp)	Good Quality? (Yes/no)
Fusobacterium sp.	90.7	100	4	213	2,063,964	Yes
Psychrobacter pacificiensis	79.8	20.8	0	780	1,311,690	No
Lactococcus lactis	90.9	100	21.4	351	3,177,740	No
Psychrobacter fozii	74.6	97.5	41.6	1,449	4,418,947	No
Marinilactibacillus psychrotolerans	82.0	90.1	42.2	1,222	3,627,764	No

Table 7-8. ANIm between *Fusobacterium* whole genomes.

	F. ulcerans	F. nucleatum	F. necrophorum	F. wasookii	F. varium	F. gonidiaformans	F. perfoetens	F. pseudoperiodonticum	F. periodonticum	F. mortiferum
Fusobacterium sp.	82.48	81.26	82.57	81.26	81.96	81.61	**82.89**	81.83	81.23	81.26
F. ulcerans	/	83.28	83.05	83.28	89.04	83.05	83.10	82.56	82.27	83.28
F. nucleatum	/	/	86.08	99.66	83.35	84.26	83.06	87.45	87.35	83.00
F. necrophorum	/	/	/	86.07	83.27	84.95	83.80	84.62	84.43	83.71
F. wasookii	/	/	/	/	83.35	84.26	83.06	87.45	87.35	83.00
F. varium	/	/	/	/	/	84.14	82.88	83.07	82.97	83.28
F. gonidiaformans	/	/	/	/	/	/	83.35	83.71	83.38	83.4
F. perfoetens	/	/	/	/	/	/	/	82.28	82.33	84.17
F. pseudoperiodonticum	/	/	/	/	/	/	/	/	95.38	82.85
F. periodonticum	/	/	/	/	/	/	/	/	/	82.99

Legend: Highest ANIm between the uncultured *Fusobacterium* sp. from *Herve* cheese and other species is written in bold.
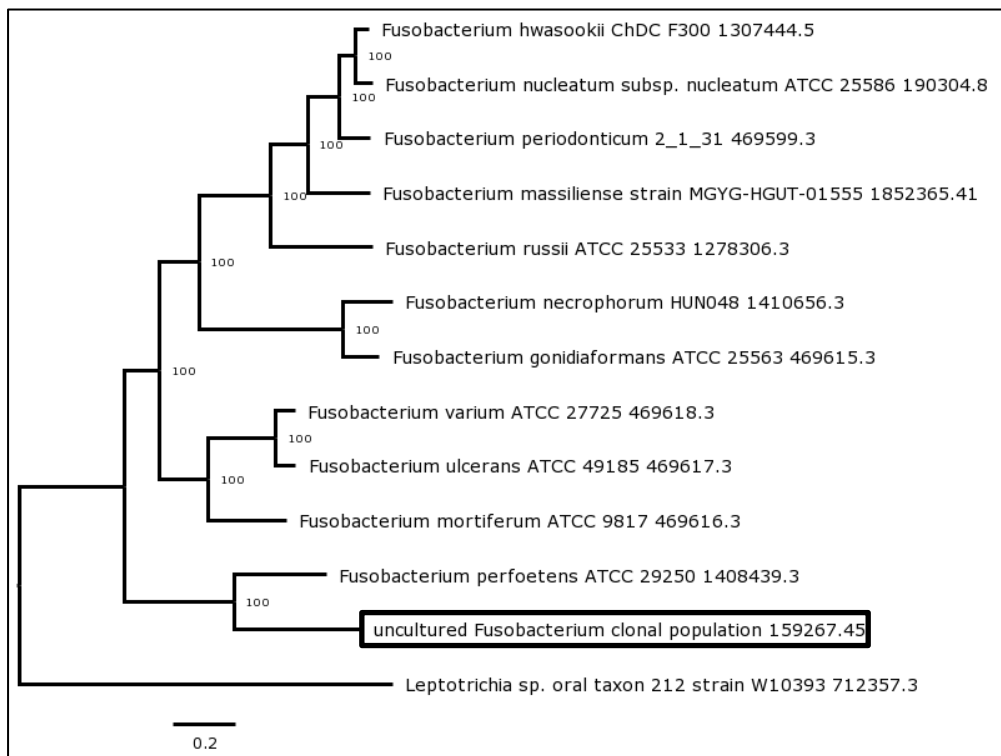
Figure 7-7. Phylogenomic tree comparing reference genomes of *Fusobacterium* spp. with uncultured *Fusobacterium* sp. from *Herve* cheese, based on 100 genes.

3. Comparative genomics for *Fusobacterium* genus

Genome of *Fusobacterium* sp. from *Herve* cheese was compared with genomes of nine other *Fusobacterium* species using PATRIC. **Table** 7- displays basic parameters, including genome length, number of CDS and GC-content for each genome. Excepting *F. necrophorum* and *F. gonidiaformans*, other species presented similar and low GC content, *i.e.* around 25-30%. Genome size was comprised between 1.81 and 3.50 Mb. Uncultured *Fusobacterium* sp. was one of the species presenting the most coding sequences.

From **Figure** 7-8, it was observed that protein identity was generally comprised between 20 and 80% between uncultured *Fusobacterium* sp. and other species, characterized by red to yellow colours. Gaps were also visible, signifying that no protein sequence identity was found in these zones.

Figure 7-9 displays distribution of genes across subsystem classes among *Fusobacterium* spp. Genes were attributed to 24 subsystem classes. The most important in terms of number of dedicated genes were amino acid and derivatives synthesis, protein synthesis, cofactors, vitamins and prosthetic groups synthesis and energy and precursor metabolites generation. Stress response was also an important subsystem class. Differences in relative distribution of genes between pathways

were observed among *Fusobacterium* spp. For instance, uncultured *Fusobacterium* sp. from *Herve* cheese had less genes associated to cofactors, vitamins and prosthetic groups synthesis, in comparison with all other *Fusobacterium* spp. On the opposite, 11% of its genes were associated to amino acids and derivatives synthesis. Interestingly, at the current state, uncultured *Fusobacterium* did not possess the subsystem linked to iron acquisition and metabolism, while it was the case for all other species. Similarly, no prophages, transposable elements and plasmids were identified by PATRIC on its genome. Our unknown *Fusobacterium* sp. could possess a gene involved in sulfur metabolism, only shared with *F. varium* and *F. ulcerans*, and responsible for the production of an enzyme repertoried as Enzyme Commission number EC 1.3.1.84, *i.e.* acryloyl-CoA reductase.

As mentioned by Tambong (2017), ability to answer stress exposure is essential for the survival of bacteria. Prokaryotes can face several types of stress, including osmotic stress, cold or heat shocks, oxidative stress, or presence of antibiotics or toxic compounds in their environment. Among the ten compared genomes, a total of 588 genes involved in stress response, defense and virulence were identified. Regarding osmotic stress, genes producing proteins involved in potassium uptake during hyperosmotic stress were associated with all *Fusobacterium* spp., except our uncultured *Fusobacterium* from *Herve* cheese. Nevertheless, the latter was the only one, with *F. massiliense*, to potentially possess ABC transporters for choline uptake. All genomes theoretically had the required material to produce cold shock protein of the CSP family. These proteins are generally small (*i.e.* 65-75 amino acids) and act as DNA and RNA chaperones in order to ensure effective transcription and translation (Keto-Timonen *et al.*, 2016). Similarly, all species possessed DnaK operon, involved in thermotolerance.

Table 7-9. Basic comparison between *Fusobacterium* genomes.

Species	Size (Mb)	GC content (%)	CDS	Hypothetical proteins	Proteins with functional assigment	PGfams
Uncultured *Fusobacterium* sp.	2.06	28.29	2,101	668	1,433	1,368
F. massiliense	1.81	27.33	1,663	380	1,283	1,650
F. nucleatum	2.17	27.20	2,067	464	1,590	2,054
F. perfoetens	2.10	25.90	1,944	706	1,238	1,942
F. gonidiaformans	1.68	32.69	1,589	391	1,243	1,596
F. varium	3.35	29.35	3,144	951	2,193	3,090
F. necrophorum	2.03	35.10	1,963	1,240	489	1,692
F. russii	1.93	28.60	1,742	547	1,195	1,736
F. mortiferum	2.71	29.29	2,664	805	1,859	2,591
F. ulcerans	3.50	30.30	3,209	1,134	2,075	3,208
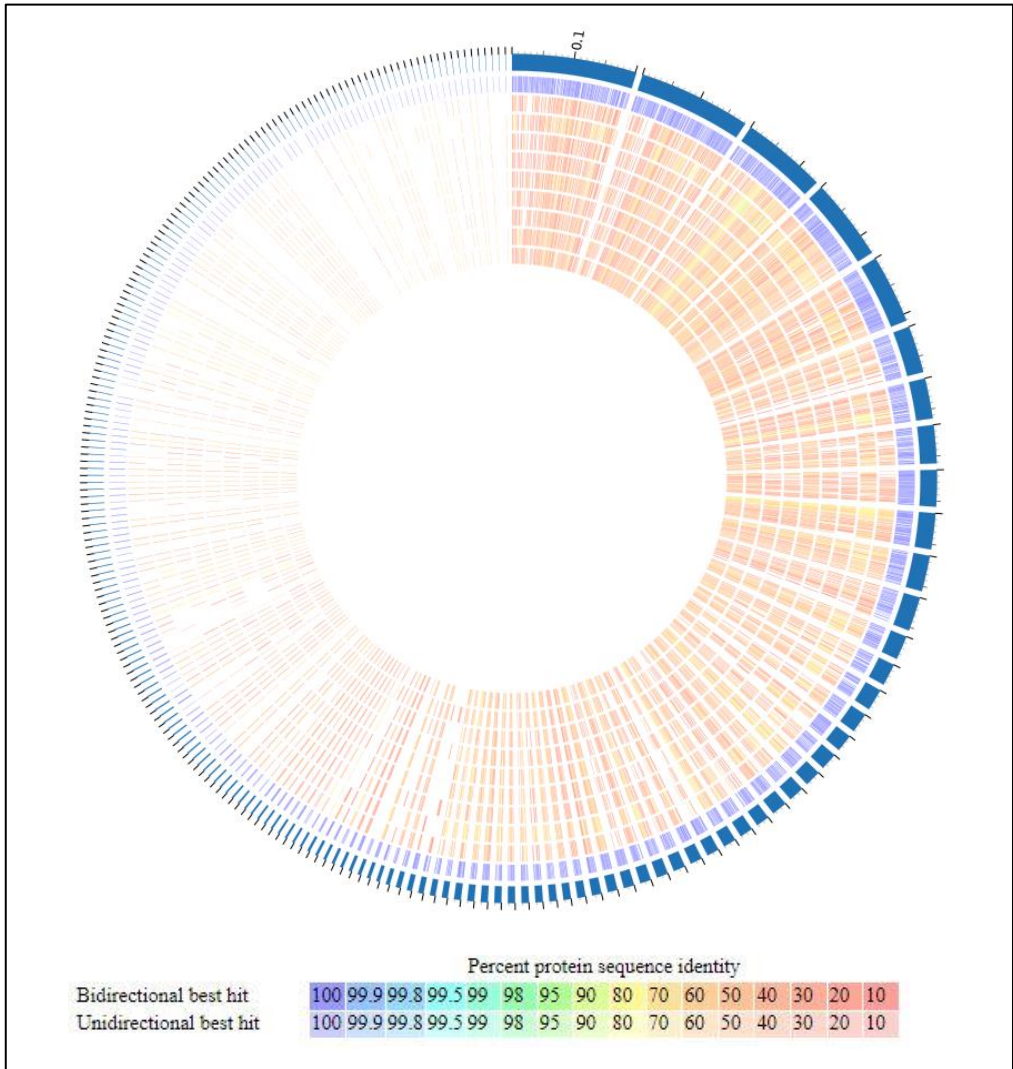
Figure 7-8. Proteome comparison between *Fusobacterium* sp. and reference genomes of other *Fusobacterium* spp. From outermost to centre circles: Circle 1, contigs acquired during assembly of the genome of uncultured *Fusobacterium* sp. from *Herve* cheese with a ladder in Mb; Circle 2, Protein sequences of uncultured *Fusobacterium* sp. as reference; Circle 3, Protein sequences of *F. nucleatum*; Circle 4, Protein sequences of *F. perfoetens*; Circle 5, Protein sequences of *F. massiliense*; Circle 6, Protein sequences of *F. gonidiaformans*; Circle 7, Protein sequences of *F. varium*; Circle 8, Protein sequences of *F. necrophorum*; Circle 9, Protein sequences of *F. russii*; Circle 10, Protein sequences of *F. mortiferum*; Circle 11, Protein sequences of *F. ulcerans*.
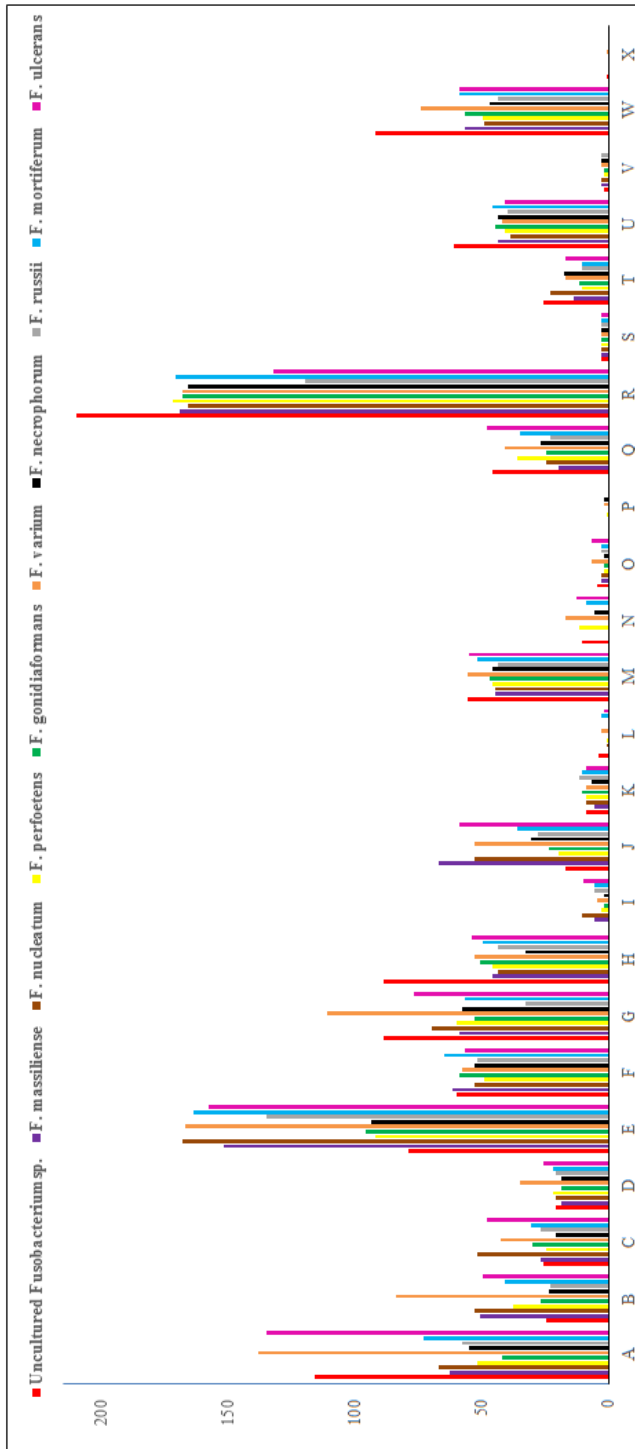
Figure 7-9. Comparison of number of genes belonging to subsystem classes between uncultured *Fusobacterium* sp. from *Herve* cheese and nine other *Fusobacterium* spp. X-axis corresponds to subsystem classes, namely A, Amino acids and derivatives; B, Carbohydrates; C, Cell cycle, cell division and death; D, Cell envelope, capsule and slime layer; E, Cofactors, vitamins and prosthetic groups; F, DNA processing; G, Energy and precursor of metabolites generation; H, Fatty acids, lipids and isoprenoids; I, Iron acquisition and metabolism; J, Membrane transport; K, Metabolite damage and its repair or mitigation; L, Miscellaneous; M, Nucleosides and nucleotides; N, Phosphate metabolism; O, Prokaryotic cell type differentiation; P, Prophages, transposable elements and plasmids; Q, Protein fate; R, Protein synthesis; S, Regulation and cell signaling; T, Respiration; U, RNA processing; V, Secondary metabolism; W, Stress response, defense and virulence; X, Sulfur metabolism.

Regarding oxidative stress, seven genes forming aerotolerance operon were observed only on the genome of *F. varium*. Uncultured *Fusobacterium* sp. from *Herve* could be able to biosynthesise glutathione, while it should not the case of other species based on annotated genomes. Nevertheless, all could be able to interact with glutathione, through the production of glutaredoxin and glutathione peroxidase (EC 1.11.1.9). In addition to its antioxidative action, glutathione also plays a role in resistance to osmotic stress (Smirnova *et al.*, 2001; Zhang *et al.*, 2010).

Finally, a total of 302 genes potentially involved in resistance to antibiotics and toxic compound were observed on the ten genomes. Antibiotics have several mechanisms of action, and *Fusobacterium* spp. appeared well equipped to counteract their effect. All possessed DNA gyrase (EC 5.99.1.3) and RNA polymerases (EC 2.7.7.6), possibly mutated, resulting in a resistance to antibiotics targeting DNA processing and transcription, respectively. Regarding antibiotics targeting cell wall biosynthesis, all species had genes producing homologous proteins, namely EC 5.1.1.1, EC 6.3.2.4 and EC 2.5.1.7. Antbiotics can also target protein synthesis as well as important metabolic pathways. All *Fusobacterium* spp. possessed weapons to counteract both modes of action, but proteins which were not identified among other genomes could be produced by *F. massieliense* and our unknown speices, namely LSU protein L6p, met in large ribosomal subunit and involved in protein synthesis, and enzyme EC 2.3.1.179 (3-oxoacyl synthase). All species could theoretically resist to daptomycin and triclosan. *F. massiliense* and species from cheese had genes encoding for a protein identified as enzyme EC 6.1.1.5 allowing mupirocin resistance, and shared proteins involved in fusidic acid resistance. In total, *Fusobacterium* from *Herve* had 45 genes potentially involved in antibiotics resistance. Sadly, the function of PATRIC allowing to predict antimicrobial resistance, detailed by Antonopoulos *et al.* (2019), was not available for the genus *Fusobacterium*.

Globally, focusing on subsystems associated to stress resistance did not allow to identify which biological traits could be different between the potential novel species and the nine other reference *Fusobacterium* spp. Other subsystem classes were thus investigated. **Table** 7-10 gathers subsystems possessed by uncultured *Fusobacterium* sp. but unshared with other species. Notably, by homology with already annotated genomes, it could possess the necessary material to synthesize histidine, arginine and cysteine, as well as particular enzymes for fatty acids synthesis.

All put together, 545 PGfams could be shared between all studied organisms. Fifty-one PGfams were shared by all other species of *Fusobacterium*, but not by uncultured *Fusobacterium* sp. from *Herve* cheese. On the opposite, analysis based on proteic homology identified 135 PGfams uniquely on unknown *Fusobacterium* sp. genome.

A few differences in subsystems were identified through these comparisons. Identified subsystems did not allow to improve understanding on how *Fusobacterium* sp. was able to become a subdominant taxon in *Herve* cheese. Alternative way to look for differences was to focus on pathways. It was observed

that this bacterium could be the only *Fusobacterium* able to produce a range of enzymes including aldehyde dehydrogenase (EC 1.2.1.3), involved in several pathways (*e.g.* ascorbate and aldarate metabolism, arginine and proline metabolism or glycolysis), alpha-glucosidase (EC 3.2.1.20), three enzymes involved in inositol phosphate pathway (EC 2.7.1.92, EC 4.1.2.29, EC 4.2.1.44), tryptophan synthase (EC 4.2.1.20), and aminopyrimidine aminohydrolase (EC 3.5.99.2). All *Fusobacterium* spp. were involved in pathways not shared with uncultured *Fusobacterium* sp. from *Herve* cheese, namely ether lipid metabolism, steroid biosynthesis, dichlorobenzene and dichlorobenzoate degradation and phosphate and phosphinate metabolism.

Surpisingly, by homology, potential genes coding for α- or β-galactosidase were not observed on the genome of *Fusobacterium* sp. from *Herve*. Possibly, its presence in cheese could not thus be associated to lactose metabolism.

4. Genes involved in antibiotics resistance

In order to determine potential composition of a growth media to allow isolation and culture of the *Fusobacterim* sp., having a clue on its resistance to antibiotics could be interesting. From proteome analysis, this bacterium could possess 45 identified genes involved in resistance to antibiotics (**Table** 7-11). All types of antibiotics targets were concerned, namely protein synthesis, transcription, metabolic pathways, DNA processing and cell wall biosynthesis. As a reminder, Medium B previously used during attempts to isolate this bacterium was supplemented with erythromycin, neomycin and vancomycin. From the latter table, it was observed that *Fusobacterium* sp. could indeed be resistant to erythromycin, thanks to two genes, namely *macA* and *macB*. However, neomycin and vancomycin were not included in the list.

Table 7-10. Subsystems possessed by uncultured *Fusobacterium* sp. from *Herve* cheese and unshared with other *Fusobacterium* spp.

Subsystem name	N genes	N proteins	Protein names	EC Number
Histidine biosynthesis	9	10	Adenylsuccinate synthetase	EC 6.3.4.4
			ATP phosphoribosyltransferanse	EC 2.4.2.17
			Histidinol dehydrogenase	EC 1.1.1.23
			Histidinol-phosphate aminotransferase	EC 2.6.1.9
			Imidazoleglycerol-phosphate dehydratase	EC 4.2.1.19
			Imidazole glycerol phosphate synthase amidotransferase	/
			Phosphoribosylformimino-5-aminoimidazole carboxamide ribotide isomerase	EC 5.3.1.16
			Imidazole glycerol phosphate synthase cyclase	/
			Phosphoribosyl-AMP cyclohydrolase	EC 3.5.4.19
Cysteine syntesis	4	3	Serine acetyltransferase	EC 2.3.1.30
			Cysteine synthase	EC 2.5.1.47
			Sulfate permease	/
Arginine biosynthesis	7	8	Argininosuccinate synthase	EC 6.3.4.5
			N-acetyl-gamma-glutamyl-phosphate reductase	EC 1.2.1.38
			Glutamate N-acetyltransferase	EC 2.3.1.35
			N-acetylglutamate kinase	EC 2.7.2.8
			N-acetylornithine aminotransferase	EC 2.6.1.11
			Ornithine carbamoyltrnasferase	EC 2.1.3.3
			Argininosuccinate lyase	EC 4.3.2.1

(Continued)

Table 7-10. (Continued)

Subsystem name	N genes	N proteins	Protein names	EC Number
DNA repair through bacterial photolyase	1	1	Deoxyribopyrimidine photolyase type 2	EC 4.1.99.3
2-ketoacid oxydoreductases disambiguation	4	4	Pyruvate:ferredoxin oxidoreductase (beta subunit)	EC 1.2.7.1
			Pyruvate:ferredoxin oxidoreductase (alpha subunit)	EC 1.2.7.1
			Pyruvate:ferredoxin oxidoreductase (delta subunit)	EC 1.2.7.1
			Pyruvate:ferredoxin oxidoreductase (gamma subunit)	EC 1.2.7.1
Citrate lyase	5	4	Malonyl CoA carrier protein transacylase	EC 2.3.1.39
			Citrate lyase alpha chain	EC 4.1.3.6
			Citrate lyase beta chain	EC 4.1.3.6
			Citrate lyase gamma chain	EC 4.1.3.6
Fatty acids synthesis	19	9	Acetyl-CoA carboxyltransferase beta chain	EC 6.4.1.2
			Acetyl-CoA carboxyltransferase alpha chain	EC 6.4.1.2
			KASII	EC 2.3.1.179
			KASIII	EC 2.3.1.180
			Enoyl-reductase	EC 1.3.1.9
			Long-chain fatty acid acyl carrier protein ligase	/
			Malonyl CoA-acyl carrier protein transacylase	EC 2.3.1.39
			Acyl carrier protein	/
			3-hydroxyacyl dehydratase	EC 4.2.1.59
			FadG	EC 1.1.1.100
Adenylylsulfate reductase	4	1	Adenylylsulfate reductase alpha subunit	EC 1.8.99.2

Table 7-11. Genes and proteins involved in antibiotics resistance in uncultured *Fusobacterium* sp. from *Herve* cheese.

Genes	Protein names	EC numbers	Conferring resistance to
Alr	Alanina racemase	EC 5.1.1.1	D-cycloserine
Ddl	D-alanine-D-alanine ligase	EC 6.3.2.4	D-cycloserine
dxr	1-deoxy-D-xylulose 5-phosphate reductoisomerase	EC 1.1.1.267	Fosmidomycin
EF-G	Translation elongation factor G	/	Fusidic acid
EF-Tu	Translation elongation factor Tu	/	Kirromycin, Enacyloxin, Pulvomycin
FabK	Enoyl-[acyl-carrier-protein] reductase [FMN, NADH]	EC 1.3.1.9	Triclosan
FabK-like	Putative FabK-like enoyl-[acyl-carrier-protein] reductase	/	Triclosan
folA, Dfr	Dihydrofolate reductase	EC 1.5.1.3	Trimethoprim, Brodimoprim, Tetroxoprim, Iclaprim
folB	Dihydropteroate synthase	EC 2.5.1.15	Sulfadiazine, Sulfadimidine, Sulfadoxine, Sulfamethoxazole, Sulfisoxazile, Sulfacetamide, Mafenide, Sulfasalazine, Sulfamethizole, Dapsone
GdpD	Glycerophosphoryl diester phosphodiesterase	EC 3.1.4.46	Daptomycin
gidB	16S rRNA (guanine(527)-N(7))-methyltransferase	EC 2.1.1.170	Streptomycin
gyrA	DNA gyrase subunit A	EC 5.99.1.3	Clofazimine, Ciprofloxacin, Gatifloxacin, Levofloxacin, Moxifloxacin, Nalidixic acid, Ofloxacin, Sparfloxacin, Trovafloxacin
gyrB	DNA gyrase subunit B	EC 5.99.1.3	Clofazimine, Gatifloxacin, Ciprofloxacin, Levofloxacin, Moxifloxacin, Nalidixic acid, Ofloxacin, Sparfloxacin, Novobiocin, Coumermycin A1, Clorobiocin, Coumermycin, Trovafloxacin

(Continued)

Table 7-11. (Continued)

Genes	Protein names	EC numbers	Conferring resistance to
Iso-tRNA	Isoleucyl-tRNA synthetase	EC 6.1.1.5	Mupirocin
kasA	KASII	EC 2.3.1.179	Isoniazid, Triclosan
MacA	Macrolide-specific efflux protein	/	Erythromycin
MacB	Macrolide export ATP-binding/permease protein	/	Erythromycin
MurA	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	EC 2.5.1.7	Fosfomycin
PgsA	CDP-diacylglycerol-glycerol-3-phosphate 3-phosphatidyltransferase	EC 2.7.8.5	Daptomycin
rho	Transcription termination factor rho	/	Bicyclomycin
rpoB	DNA-directed RNA polymerase beta subunit	EC 2.7.7.6	Rifamycin, Daptomycin, Rifabutin, Rifampin
rpoC	DNA-directed RNA polymerase beta' subunit	EC 2.7.7.6	Daptomycin
rpsL	SSU ribosomal protein S12p	/	?
S10p	SSU ribosomal protein S10p	/	Tetracyclin, Tigecycline
S12p	SSU ribosomal protein S12p	/	Streptomycin

3. Conclusions

In conclusion, NGS allowed to assemble and to annotate a signifcant part of the genome of uncultured *Fusobacterium* sp. identified in raw milk *Herve* cheese. Although some assembled contigs had limited size, limiting interpretation of their genetic content, evidence was brought that this bacterium could belong to a novel species of the genus *Fusobacterium*, as suggested by ANIm for pairwise genomes comparisons, and by phylogenomic tree. Nevertheless, the description of a novel species requires isolation of the bacterium on growth media, as well as its characterization. At this moment, it was still not possible to isolate the bacterium, despite all tested media and incubation conditions.

Metagenomics and proteome comparison allowed to identify theoeretical differences between this bacterium and other *Fusobacterium* spp., involving unshared PGfams, pathways or subsystems. The list of genes involved in resistance to antibiotics could give keys for the choice of growth media which potentially allow the isolation of this *Fusobacterium*. Similarly, it remained difficult to understand how and why this bacterium was present in cheese, as the presence of *Fusobacterium* spp. in food is not documented, as well as how it was able to survive and even to grow during refrigerated storage to become a subdominant taxon in raw milk *Herve* cheese. Further studies, discussed in the next chapter, could be useful to go deeper with these unsolved questions.

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Chapter 8

General discussion, conclusions and perspectives

General discussion provides a conclusion and a critical view on the work performed and on the results gathered during the thesis. The threat for food safety associated with the presence of *L. monocytogenes* in Belgian artisanal cheeses will be discussed. Some recommendations will be provided to producers and authorities. Potential improvements, limitations of the present work, or alternative pathways that could have been investigated will also be pointed and developed. Some perspectives will be suggested in order to continue this work to answer unsolved questions.

L. monocytogenes is the pathogenic agent responsible for listeriosis, the fifth most occurring foodborne disease in EU, with 2,621 reported cases in 2019. During this year, 11% of the concerned patients died. Although these figures are worrying, they should be moderated, in the way that a significant number of cases are kept under silence. Indeed, it can be assumed that listeriosis is only identified in case of acute symptoms. Patients suffering mild symptoms, including diarrhea, do not necessarily consult their doctor, and these cases are not taken into account. Figures reported by EFSA-ECDC can thus be considered as an underestimation. In addition to that, mainly people at risk suffer acute symptoms and potentially die. These people are thus overrepresented in the reported cases, contributing to an overestimated death rate. Nevertheless, due to the risk for people at risk, it is necessary to keep on research on the growth of *L. monocytogenes* in RTE foods.

Cheeses, especially made from raw milk, are pointed as potential vector of *L. monocytogenes*. Nevertheless, while writing state of the art on this topic in Chapter 1, it was rapidly noticed that the prevalence of the pathogen in raw milk cheese was not necessarily higher than in pasteurized milk cheeses. Furthermore, the behavior of the pathogen in cheese is variable, and highly variety-dependent. In Belgium, when starting to work on this topic in 2017, available knowledge on artisanal cheese varieties and on their characteristics was poor. The main reference was a promotion book edited by APAQ-W (2016), but it was not exhaustive and focused on Wallonia only.

Due to this lack of data, all Belgian artisanal cheese varieties were considered as allowing the growth of *L. monocytogenes*. This involved the obligation for manufacturers to guarantee non-detection of the pathogen in 25 g of cheese before sales. Impact of such precautions on cheese producers is not neglectible. Firstly, in case of detection of *L. monocytogenes* in cheese, producers have to recall sold pieces and to destroy the whole batch, resulting in significant financial losses. This type of news items is shared by FASFC on its website and social networks, and sometimes also by mass media. Impact on producers' reputation is negatively affected. Cheesemakers also have to investigate on the origin of the contamination. A *sine qua non* condition to restart sales is to manufacture three consecutive batches in which *L. monocytogenes* is not detected in 25 g of cheese. This procedure is tricky and generates huge economic and moral consequences. As an example, some producers interviewed during Chapter 3 stopped their activities in relation to this sword of Damoclès. An infamous Belgian example is the case of a producer of raw milk *Herve* cheese, who closed his factory following recurrent contamination of batches with the foodborne pathogen (Bodeux, 2015). For sure, strict food safety

criteria are necessary to protect consumers' health when products actually favor the growth of *L. monocytogenes*.

During this thesis, a prevalence of 1.49% (2 cheeses out of 134) was observed for *L. monocytogenes* in Belgian artisanal cheeses. Author was also contacted several times by producers facing contamination of batches, following FASFC routine analyses or tests included in their self-checking system. It means that the fight against *L. monocytogenes* in RTE foods, and especially in cheese, is still an important issue nowadays. Nevertheless, foreign studies gathered in Chapter 1 already identified cheese varieties not allowing the growth of the pathogen during refrigerated storage, and even permitting a decrease in the levels during shelf-life. An obvious postulate was thus that some Belgian artisanal cheeses could also be able to prevent the growth of *L. monocytogenes*. Consequently, less strict criteria could sometimes be applied for some cheese varieties.

Collection of data on manufacturers, manufacture and final products

The main objective of the present thesis was thus to determine and to understand the fate of *L. monocytogenes* in a panel of artisanal cheese varieties from Belgium, and to precise potential risk for food safety associated with these products.

Before being able to perform expensive experiments, *e.g.* challenge or shelf-life studies, it was essential to fill gaps in the general knowledge of Belgian artisanal cheeses. A first important step was thus to contact as much repertoried producers as possible for a phone survey. While preparing the survey, it was noticed that obtaining an up-to-date list of artisanal cheese producers was a challenge: their number was permanently evolving, with the appearance of new artisans, and the cessation of activities of other farms. Globally, 142 complete answers were gathered (110 and 32 in Wallonia and Flanders, respectively), corresponding to a participation rate of 70% (based on available listings). From this survey, 424 cheese varieties were identified, clustered into 16 major families.

Nevertheless, some limitations of this survey can be enumerated. It was for instance observed that some producers were suspicious and did not want to provide comprehensive answers, especially on manufacturing processes, cheese varieties and used starters. On the one hand, the interviewer was sometimes suspected to be a representative of FASFC trying to collect data on cheesemaking practices. Typically, questions about production volumes and sales were embarrassing. On the other hand, some cheesemakers believed that the interviewer was another producer trying to collect data in order to plagiarize recipes, and they wanted to protect trade secrets. A consequence of this mistrust was the difficulty to convince some producers to answer all questions, resulting in undesirable missing values in the dataset.

The choice of phone calls to perform the survey in this sector could be questionable. Appointments in face-to-face with producers in farms or cheese factories could have allowed to build more confidence, but this approach would have

been much more time consuming. Futhermore, it is not guaranteed that producers would be enclined to welcome strangers in their exploitation. A third option could have been to design an online survey but, to author's opinion, participation rate would have been lower. It was indeed observed during the thesis that most producers were still not comfortable with computer technologies. All put together, phone survey was the best alternative, despite precited drawbacks.

The survey allowed the identification of major cheese types, in terms of occurrence, namely UACC, GSHC and SPSHC, accounting together for more than 60% of listed varieties. Knowing most prevalent types was an important factor, as it must be kept in mind that an objective of this work was to generate data on the growth of *L. monocytogenes* useful for as much producers as possible. Sampling was based on this variable. Nevertheless, an alternative factor should have been considered, namely production volume in kilograms or transformed milk liters. This factor would have provided more realistic relative proportions of each cheese family. As an example, *Maquée* was produced in nearly all participating factories, *i.e.* its occurrence was really high, but annual production volume was relatively marginal, in comparison with volumes of GSHC and SPSHC. This variable was part of the survey, but data were not exploitable for several reasons, namely (a) producers did not know production volumes by cheese variety, (b) producers knew annual milk volume transformed into cheese, as well as yields (~50% for UACC, ~10% for other types of cheese), but did not know the proportion of each variety, and (c) producers did not want to communicate their production volume.

Despite this inconvenience, the survey fullfiled its objectives, allowing the identification of a majority of producers and varieties. More importantly, it allowed the establishment of a first contact with producers, sometimes raising interest for the present project.

After acquiring this general knowledge on cheese manufacturers' profile and on cheese varieties, a characterization of these products was necessary. Two fundamental aspects were investigated, namely manufacturing process and cheese physicochemical characteristics. Among the 424 cheeses varieties repertoried during the survey, 65 were selected for further investigations, *i.e.* 15.3%. Marginal cheese families, including *Ricotta*, *Mascarpone*, *Mozzarella* and blue-veined cheeses, were not included in the sampling plan. The latter one was designed to include varieties from all Belgian provinces, all milk animal origins and all milk heat treatments.

All concerned factories were visited and the first steps of manufacture were monitored, *i.e.* from milk seeding to initiation of curd draining. Throughout this process, data were collected, being both qualitative (precise names of commercial starters and inclusion or not of specific manufacturing steps, *i.e.* curd cutting, whey removal, salting, brining, shaping, draining, pressing,…) and quantitative (pH and temperature at various steps, amount of rennet, …). It is known that essential physico-chemical changes occur during draining/pressing and ripening. At the beginning of ripening, LAB from starters are dominant in curd (Irlinger *et al.*, 2015). Irlinger and Spinnler (2020) described pH evolution during ripened cheese manufacture and ripening. They reported that pH decrease is really limited during

curdling for mixed and enzymatic curds, with a loss of approximately 0.2 pH units only. Most pH changes occur during pressing or draining steps, as well as during ripening, where pH is increased due to activities of ripening microflora.

Methodology used during Chapter 4 of this thesis allowed an accurate assessment of pH evolution during UACC manufacture, with data collected during curdling, at the end of this step, and at the end of draining. Nevertheless, in the case of ripened cheeses manufactured from mixed curds, pH was only measured during curdling and directly after shaping. Ideally, pH evolution should also have been recorded during draining, pressing and ripening. For practical reasons, it was not possible. During SH/HC manufacture, curd is often pressed for several hours, *e.g.* 1 h for *Raclette*, from 3 to 5 h for *Gouda*, 6 to 18 h for *Cheddar* and *Manchego*, and up to 20 h for *Abondance* (Goudédranche *et al.*, 2001; 2002). Similarly, unpressed cheeses, including UACC, MRSC and SRSC, are drained for hours. Ripening conditions, including temperature, air flow, and percentage of relative humidity, can impact cheese safety (Callon *et al.*, 2011). Ripening duration is variable between cheese varieties, ranging from two weeks to several months, and sometimes several years for some old *Gouda* or *Comté* (Goudédranche *et al.*, 2001; 2002). Collecting such data was out of the scope of this thesis, as it focused on RTE food placed on the market. Nevertheless, monitoring of pH and a_w during the whole manufacturing process could be a clue to increase knowledge on the fate of *L. monocytogenes* during cheese production.

Following visits in factories, samples were collected at the end of manufacture for physico-chemical characterization. Measured parameters included: a_w in cheese cores, pH in cores and on the surface, salt content, fat content and dry matter. Globally, data were coherent with available literature, summarized by the review paper presented in Chapter 1. Only three cheese varieties had pH or a_w sufficiently low to prevent the growth of *L monocytogenes*, according to Regulation (EC) No 2073/2005. Nevertheless, it should be suggested to producers to systematically measure pH of their products at the end of manufacture. Indeed, this information could be very useful for FASFC inspectors in order to determine the risk for food safety associated to an eventual contamination with *L. monocytogenes*. It also allows faster decisions, in comparison with analysis performed by laboratories.

An objective of this work was to use all collected qualitative and quantitative data to develop a new classification tool for Belgian artisanal cheeses, based on clustering approaches. Various approaches were tested, in collaboration with expert statisticians. Obtained clusters closely corresponded to major cheese families, as described by Profession Fromager (2020), and did not provide any supplemental precisions. As a consequence, this classification was considered throughout the thesis. Despite this disappointment, collected data were sufficient to characterize Belgian artisanal cheeses and allowed the selection of representative varieties to perform challenge studies with *L. monocytogenes*.

L. monocytogenes was detected in two of the considered batches, namely a MRSC and a SPSHC. Contamination level was really high in the spoiled MRSC, *i.e.* 4.68 $\log_{10}$ cfu/g. As described in Chapter 4, ten remaining cheese wheels from this

batch were seized, and three enumerations of *L. monocytogenes* were performed on each sample. This procedure enlightened the issue of sampling procedure for microbial enumeration. Indeed, on the one hand, *L. monocytogenes* was not detected in five pieces but, on the other hand, levels up to 6 $\log_{10}$ cfu/g were observed in other samples. Furthermore, a great heterogeneity was observed between triplicates for a given wheel, ranging for instance from levels < 1 to > 4 $\log_{10}$ cfu/g. These observations raise the question of the likelihood, during routine controls, to erroneously conclude that products are free of *L. monocytogenes*. For the present case study, chance to conclude that the batch was safe was 53.3% (16 enumerations out of 30 did not detect *L. monocytogenes*). Current sampling procedure could be flawed and a revision should be on the agenda in order to take this inter- and intra-cheese variability into account and to ensure the guarantee of food safety. It is now obvious that *L. monocytogenes* can enter a VBNC state when it faces adverse environmental conditions (Falardeau *et al.*, 2021). In this case, the pathogen is not detectable and enumerable using conventional plating techniques recommended by all reference methods. A transition to molecular techniques should be considered in the future.

Results of challenge studies and opinion on available guidelines and standards

Despite this observation, sampling for enumeration of *L. monocytogenes* during challenge studies was performed according to available guidelines (EURL *Lm*, 2014; FASFC, 2016), as a goal of these experiments was to produce results useful for producers and taken into account by food safety agencies. This objective was reached, as scientific commitee of FASFC published an advice (08-2020), and FASFC itself a new circular PCCB/S3/1636380 (FASFC, 2020a; 2020b). The latter one allowed a revision of food safety criterion for UACC with pH < 5.0 at the end of manufacture, provided that producers systematically record pH for each batch. In these circumstances, UACC is considered as belonging to category 1.3 of Regulation (CE) No 2073/2005, *i.e.* RTE food not allowing the growth of *L. monocytogenes*, and a contamination up to 100 cfu/g is tolerated before sales. Producers have thus to invest in a pH-meter, with a precision of at least 0.1 pH units.

Possessing a pH-meter is not sufficient. A tutorial on how to adequately use this apparatus should be provided to producers, including calibration and, more importantly, how to perform an accurate measurement. Advice should also be given on the choice of the adequate probe(s). Indeed, hardnesses of *Maquée* or SPSHC are not the same, and the purchase of multiple probes would probably be necessary. Explanations on the recording of data and on maintenance, cleaning and disinfection of probes could also be a great help for producers. This information could be provided by FASFC by updating sectorial guide G-034 (2012), or via organisms accompanying producers, like DiversiFerm. Finally, it can be said that the choice of the threshold value of pH 5.0 could be questionable, although it allows a certain degree of freedom. Indeed, FASFC suggests via its guide G-034 (2012), in an example dedicated to UACC, that pH at the end of draining is a critical control point

that should be included in HACCP. As such, it is suggested that pH > 4.6 at this step should be considered as doubtful and that the efficacy of starters should be checked (FASFC, 2012).

For other types of cheese, challenge studies performed during this thesis did not allow the implementation of new global food safety criteria. SRSC and MRSC should generally be considered as a potential threat for food safety. Indeed, δ of *L. monocytogenes* was generally > 3 $\log_{10}$ cfu/g for these cheeses, when EURL *Lm* (2014) considers RTE food as at risk when δ is above the threshold value of 0.5 $\log_{10}$ cfu/g. For GSHC and SPSHC, variability was observed between factories, between batches, but also between pieces within a given batch. Nevertheless, some varieties did not allow the growth of the pathogen during challenge studies. An official report was provided to concerned producers, granting them a revision of food safety criteria. Globally, from these experiments, it can be concluded that it is extremely hazardous to determine intuitively the potential risk associated with the presence of *L. monocytogenes* in ripened cheese based on their characteristics. All the work performed did not allow the identification of key indicators helping for this decision.

The best advice that could be provided to producer is to fund challenge studies on their products in order to potentially beneficiate from individual revisions of food safety criteria because, in the current state, it will never be reasonable to make a global decision for these products. A solution to get such a global revision could be that producers selling similar varieties converge on common specifications, as it is for instance the case in foreign countries for PDO cheese. In the latter case, process and ripening could be standardized and be identical in all dealing farms, resulting in an increased chance to be able to make challenge studies useful for several producers. Nevertheless, from field experience, it can be doubtful that Belgian producers will be encline for such an initiative. In addition to that, this could result in an impoverishment of the diversity of Belgian artisanal cheese. To date, *Herve* remains the only Belgian PDO cheese.

In the USA, FDA imposed that raw milk cheeses are ripened for at least 60 days before sales, hypothesizing that ripening allows a decrease in the levels of *L. monocytogenes* (Arias-Roth *et al.*, 2021). Although this approach is interesting, its implementation in Belgium seems utopic, as most cheese varieties are not ripened for such a long time. As an example, usual ripening time for SPSHC and MRSC is 3 to 4 weeks and 2 weeks, respectively. Furthermore, Falardeau *et al*. (2021) raise doubts concerning the effectiveness of this extended ripening.

Although challenge studies are expensive, they remain the easiest way to change things. These costs could frighten producers, also provided that results of challenge studies are valuable for one variety only, meaning that producers manufacturing SRSC, MRSC and SPSHC have to perform challenge studies for each cheese. However, although performing a challenge study is anything but a guarantee to benefit from a revision of the criteria, the game is worth the candle. It should also be reminded that the tolerance of 100 cfu/g in cheese does not mean that investigations are not necessary in case of presence of *L. monocytogenes* in cheese or in the

factory. Manufactured products must be as safe as possible, and the target must remain the non detection of the pathogen in 25 g of cheese. In case of contamination, it remains essential to identify the origin of the contamination, in particular if other dairy products are manufactured in the same workshop. Good manufacturing practices must always be applied, as well as self-checking and HACCP. This is also the conclusion of FASFC scientific commitee for these cheese types.

It is often recommended to people at risk to avoid the consumption of raw milk cheese. Nevertheless, from state of the art and from results of this thesis, it is not clear whether raw milk cheeses represent a bigger threat than pasteurized milk cheeses. As aldready explained, post-pasteurization and post-processing steps represent a major contamination pathway. In the case of pasteurized milk cheeses, the pathogen does not have to face up to competitive microbiota. Such products should thus be considered as as dangerous as raw milk cheeses for pregnant women and old or immunosuppressed people.

Concerning the variability observed for GSHC and SPSHC, Lahou and Uyttendaele (2017) already reported this phenomenon. As mentioned in Chapter 5, the recommended method for δ calculation does not allow to take this variability into account. Through scientific advices on raw milk butter and artisanal cheeses, FASFC suggests to calculate δ using the lowest value at day-0 and the highest at end of shelf-life (FASFC, 2019; 2020a). This method is a worst case. A less stringent alternative is proposed by Nederlandse Voedsel- en Warenautoriteit (NVWA; De Loy-Hendrickx *et al.*, 2018). The latter one distinguishes two cases allowing to take into account intra-batch variability. In the first scenario, *i.e.* when standard deviation of levels of *L. monocytogenes* at end of shelf-life is $< 0.5 \log_{10}$ cfu/g, intra-batch variability is considered neglectible, and δ is calculated using method detailed by EURL *Lm* (2014). The second scenario concerns challenge studies with standard deviation at end of shelf-life $> 0.5 \log_{10}$ cfu/g, meaning that behavior of *L. monocytogenes* is not uniform between test units. For instance, levels of the pathogen could have been decreased in two samples but have been increased in the last one. In this case, the highest enumeration at end of shelf-life is used for calculation of δ.

All abovementioned approaches are compared in **Table** 8-1, based on challenge study SH12 from Chapter 5. Following guidelines from EURL *Lm* (2014), it was concluded that this variety did not allow the growth of *L. monocytogenes*. Highest δ among batches was indeed -0.05 $\log_{10}$ cfu/g. Nevertheless, it can be observed from **Table** 8-1 that δ differed between methods of calculation. Using alternative methods, it must be concluded that this cheese variety represents a high risk for food safety, as δ of 2.52 and 2.75 $\log_{10}$ cfu/g were calculated using NVWA and FASFC methodologies, respectively. In other words, levels higher than 10,000 cfu/g were observed at end of shelf-life, from cheeses contaminated around 100 cfu/g at day-0. Consequently, such products could cause harmful health problem for people at risk.

Table 8-1. Comparison of three methods to calculate δ during challenge studies with intra-batch variability > 0.5 $\log_{10}$ cfu/g at the end of shelf-life.

Batch ID	Time	*L. monocytogenes* enumerations ($\log_{10}$ cfu/g)	δ EURL *Lm* (2014)	δ FASFC (2020a)	δ NVWA (De Loy-Hendrickx *et al.*, 2018)
Batch 1	Day-0	1.90 2.04 2.00	**-0.05**	0.36	-0.05
	End of shelf-life	1.78 1.95 2.26			
Batch 2	Day-0	2.08 1.85 2.20	-0.08	**2.75**	**2.52**
	End of shelf-life	2.00 4.60 1.90			
Batch 3	Day-0	2.08 2.00 2.20	-0.13	1.18	1.10
	End of shelf-life	1.78 1.95 3.18			

Legend: δ written in bold represents the highest value obtained by method of calculation.

The author would like to express its opinion on the most appropriate method for δ calculation. All put together, guidelines should be based on the method suggested by NVWA and detailed in the book of De Loy-Hendrickx *et al.* (2018). Indeed, in case of intra-batch variability at the end of shelf-life < 0.5 $\log_{10}$ cfu/g, principle of this method stays the same as the one currently recommended by EURL *Lm* (2014). In case of intra-batch variability at the end of shelf-life > 0.5 $\log_{10}$ cfu/g, this method considers the phenomenon and provides a more realistic picture of the potential risk associated to the presence of *L. monocytogenes* in the concerned RTE food. It is not necessary to use the lowest value at day-0 for the calculation of δ, although it was suggested by FASFC scientific experts (2020a). Indeed, challenge studies are based on artificially contaminated cheeses, and the variability in contamination levels is limited in these circumstances. Furthermore, EURL *Lm* (2014) demands to restart the whole challenge study when standard deviation > 0.5 $\log_{10}$ cfu/g is observed at day-0.

Recently, ISO 20976-1 standard was published, providing guidelines to perform microbial challenge studies (ISO, 2019). This method introduced a fourth approach for the calculation of δ. It is asked to perform enumerations of *L. monocytogenes* at intermediate time points throughout shelf-life, and to use the highest value observed, minus median initial contamination. This means that δ is not necessarily based on intial and final situations, and that this calculation takes into account eventual peaks

during storage. Neverthless, this protocol requires only one sample for each sampling point (except for day-0 where three samples remain compulsory), meaning that it does not consider intra-batch variability, which was a major concern identified during the present work. Another novelty was introduced by this standard. Indeed, if challenge study is performed simultaneously on three batches, it is not necessary to consider three cheeses at day-0, and a single wheel/piece per batch is sufficient. In this case, standard deviation < 0.3 $\log_{10}$ cfu/g is asked between batches at day-0.

Improvement of challenge studies and opinion on alternative methods

Exploring the growth of *L. monocytogenes* in cheese using artificial contamination, as it is the case during challenge studies, could raise criticism. Inoculation procedure is subject to debate, as reviewer's remarks beared witness on it. As for all models, some drawbacks can be enumerated. It is indeed difficult to mimic natural contaminations, for instance by reproducing the exact physiological state of *L. monocytogenes* cells. Similarly, inoculation of the pathogen is responsible for a sudden modification of cheese microbiota and of equilibria between bacterial populations. To limit the extent of this phenomenon, inoculum volume cannot represent more than 1% of the cheese weight. Physico-chemical characteristics of the inoculation medium, *i.e.* pH, a_w and salt content, are also adapted to be closer to cheese characteristics, minimizing the impact of inoculation procedure.

Another drawback of artificial inoculation is that, using a syringe, cells cannot be perfectly dispersed in cheese matrix. Consequently, *L. monocytogenes* can be overrepresented in some cheese parts and absent from others. During the present work, to get around this problem, it was decided to homogenize the whole cheese ten-fold in Half-Fraser broth (Led Techno, Heusden-Zolder, Belgium) for enumeration of *L. monocytogenes*, instead of samples of 25 g only. This practice is now compulsory, according to ISO 20976-1.

It can be argued that durability studies, based on naturally contaminated RTE foods, avoid abovementioned drawbacks. Nevertheless, from author's experience acquired during the thesis, several disadvantages of this alternative can be listed:

- It is extremely difficult to perform a large-scale study based on durability experiments. This approach is more appropriate for a case-by-case study. Indeed, during four years of work, only seven naturally contaminated batches were reported to the author, directly via producers or via DiversiFerm and FASFC. In other words, it means that only seven durability studies would have been performed during this period. A lot of time would thus be necessary to produce significant data. Worse, the seven spoiled batches corresponded to seven cheese varieties. Durability studies would have provided only informative data, as it is not possible to draw conclusions for a variety or a family based on one batch. At least three contaminated batches are necessary to potentially lead to a potential revision of food safety criterion. It is neither likely nor desirable for cheesemakers to have three contaminated batches for a

same variety. It looks smarter for them to fund challenge studies on artificially contaminated samples to know the risk associated to their production before an effective contamination occurs, accompanied by all legislative and reputational consequences.

- The achievement of durability studies is dependent on contamination level. As mentioned in Chapter 4, observed levels of *L. monocytogenes* in naturally spoiled samples were never adequate to perfom valid durability studies. Indeed, *L. monocytogenes* levels were either too low (contaminated SPSHC had levels under the enumeration limit, *i.e.* < 10 cfu/g), either too high (contaminated MRSC had levels up to $3,400,000$ cfu/g). All batches mentioned in Chapter 5 also had levels > 100 cfu/g at day-0. In case of too low initial contamination, for instance 10 cfu/g, variability in the physiological state of individual cells can bias the conclusions (Francois *et al.*, 2006). On the opposite, when initial *L. monocytogenes* levels are > 100 cfu/g, shelf-life study is not useful, as such batches are improper for sales and consumption.

- Although artificial inoculation procedure could be responsible for heterogeneous contaminations within cheese, enumerations performed on naturally contaminated pieces during this study (see Chapter 4) converged to the conclusion that it is similar in the nature. Worse, this heterogeneity was also observed between cheeses within a given batch. Controlled artificial inoculation used during challenge studies allowed more repeatability. When repeatable inoculation procedure failed, *i.e.* when standard deviation of contaminations at day-0 is $> 0.5 \log_{10}$ cfu/g, a new assay must be performed (EURL *Lm*, 2014). This criterion is still much stricter in ISO 20976-1 standard, where variability at day-0 must be $< 0.3 \log_{10}$ cfu/g. Challenge studies should thus result in more repeatable results and conclusions.

People who reviewed the article on challenge studies suggested to consider cheeses manufactured from artificially contaminated milk. In theory, this approach should allow the obtention of more realistic and homogeneous contaminations in cheese. Changes in cheese matrix and microbiota engendered by inoculation procedure are indeed avoided. It also allows a monitoring of the contamination with *L. monocytogenes* during manufacture, ripening and storage. Considering these aspects, one could think that this approach is the panacea for assessing growth of *L. monocytogenes* in cheese. Nevertheless, as for shelf-life studies, several drawbacks can be listed. First of all, in cheese factories, most cases of contamination are associated to post-processing handlings, due to cross-contamination from manufacturing or storage environments to cheese surface. The discussed approach does not take this scenario into account. Indeed, it considers cheeses contaminated as a result of the use of milk spoiled with *L. monocytogenes*, omitting other contamination pathways. Consequently, it does not allow a comprehensive understanding of the fate of the pathogen in the studied cheese varieties, considering all potential spoilage routes. In addition to that, the implementation of such challenge studies is extremely difficult, as enumerated hereafter:

- Cheese manufacture requires a processing plant, or at least pilot-scale installations. In the case of milk contaminated with *L. monocytogenes*, processing plant has to be biosafety level 2, as handling of the pathogen is not tolerated under less strict biosafety conditions (FASFC, 2016). Similarly, ripening rooms, cellars or chambers must comply with these biosafety aspects. Performing a large-scale study as the one presented in this thesis would rapidly become a logistical puzzle, as the amount of cheeses to store during ripening would rapidly increase. These difficulties could however be avoided by using strains of *L. innocua*, as no risks associated to this species are repertoried (Ramaswamy *et al.*, 2007).
- As for challenge studies, initial cheese contamination level should be around 100 cfu/g. For each variety, extra experiments have to be performed to determine adequate milk contamination level. During manufacture, levels of the pathogen do not remain constant, generally increasing during curdling and pressing, but decreasing during ripening of SHC. A proportion of bacterial cells are also eliminated with whey. Variations in the behavior of *L. monocytogenes* are specific to cheese families, and probably to cheese varieties (Alshaibani *et al.*, 2020; Chon *et al.*, 2020; Giacometti *et al.*, 2020). Consequently, a specific inoculum should be determined for each variety, representing a time consuming and expensive procedure, as it would be necessary to perform the whole manufacturing process several times to identify adequate milk contamination resulting in 100 cfu/g of *L. monocytogenes* in cheese, before being able to effectively perform a challenge study.
- Cheese manufactured during this type of experiment would not be comparable to real samples. Indeed, each cheese has its own tipicity, linked to milk, starters, water, and production and ripening environments. This is the so-called *terroir* effect already mentioned in Chapter 6 dedicated to metagenetics (Turbes *et al.*, 2016). Tipicity is also associated to producer's know-how. Despite the use of identical recipes, samples manufactured in a pilot-scale processing plant would not be comparable to cheeses produced in dairy farms, in terms of microbiota and/or physico-chemical characteristics. Indeed, it is nearly impossible to reunite all conditions met in real processing plants and ripening cellars. Consequently, food safety authorities would probably not tolerate the transfer of results and conclusions obtained during such studies to real samples. The usefulness of this type of studies for cheese producers would be limited, although these could be interesting at a scientific point of view.
- Such a procedure does not allow to mimic all potential scenari of contamination, only considering entry of *L. monocytogenes* in cheese processing through spoiled milk. In this case, manufacturing cheeses from artificially contaminated milk does not allow to mimic a contamination during post-processing steps through environment.

Other remarks could also be pointed regarding methodology used during challenge studies. For some varieties, it was not possible to perform experiments on whole

cheese wheels, especially in case of high weight cheeses, *e.g.* GSHC. As a reminder, a challenge study for one batch required at least 12 pieces, comprising six controls and six artificially contaminated samples, according to EURL *Lm* (2014) guidelines. Working on whole pieces for such varieties was not possible from a logistical point of view; cold rooms and fridges representing a limiting factor. Moreover, most producers did not agree to provide samples for free, and researchers had to pay for samples, limiting room of manoeuvre with budget available, knowing that minimal cheese price is generally around 15 €/kg. During this thesis, selected strategy was thus to cut cheese in pieces. Working on whole cheeses would probably provide more realistic growth data, as gas diffusion and dehydration are not the same between whole wheels and cut pieces. Cutting procedure is also responsible for a transfer of bacteria, yeasts and moulds from crust to paste, modifying natural cheese paste microbiota and possibly impacting growth of *L. monocytogenes*. Nevertheless, it can be replied that cheese are generally stored in pieces at consumer's level, and that the use of cheese pieces for challenge studies is tolerated by ISO 20976-1.

Another track for improvement of these experiments could be to perform distinct challenge studies for cheese cores and crusts, mimicking diverse scenarios of contamination. Cheese cores are likely to be contaminated due to spoiled raw milk or contaminated cheese vats. The presence of *L. monocytogenes* on cheese surface is associated to contamination during post-processing steps, including shaping, brining, ripening, packaging or handling. Depending on farms and cheese factories, the most appropriate place of inoculation should be choosen in relation to the most likely transmission route. Besides scenarios of contamination, it should also be reminded that core and rind represent distinct environments, with their own physico-chemical and microbiological characteristics. Inoculating both core and rind, as it was the case during this thesis for SRSC and MRSC, did not allow to know which of these environments was or were favorable for the growth of *L. monocytognes*. An extended scientific knowledge would have been acquired by performing two challenge studies in parallel for all surface-ripened varieties.

Based on remarks previously stated, cutting procedure also plays a role in the transmission of *L. monocytogenes* from cheese crust to paste (Bernini *et al*., 2016). Cheeses are rarely sold wholly and are generally cut before packaging or at retail. Considering that the most prevalent contamination route is the spoilage of cheese surface during post-processing steps, the risk of transfer of *L. monocytogenes* to cores during cutting procedure should be considered (Back *et al*., 1993). Challenge studies could be performed by artificially inoculating cutting surfaces of cheese pieces or slices, representing a third possible place of inoculation, with surface (crust) and center of cheese (core), in ordrer to increase knowledge on the fate of *L. monocytogenes* in various cheese varieties.

Another factor which could increase knowledge on this fate is the deeper monitoring of *L. monocytogenes* levels during cheese shelf-life, as now suggested by ISO 20976-1. This standard recommends focussing on five points of the shelf-life, including day-0 and end of shelf-life. This would allow the production of more accurate and exploitable growth curves. In the present work, by only considering

two points, behavior of *L. monocytogenes* is supposed linear, or at least monotonic. For sure, one can imagine that the real growth curve is not linear. For instance, the pathogen could grow in cheese after inoculation, during early stages of storage, before decreasing to levels lower than the initial inoculum, in relation to dehydration or evolution of cheese microbiota. It is also possible that, at given point(s) of shelf-life, samples do not fullfil food safety criterion, *i.e. L. monocytogenes* levels ≤ 100 cfu/g. As a consequence, enumerating the pathogen more frequently during storage could increase accuracy of challenge studies conclusions, especially on the risk for food safety. Periodic data are also essential for the development of growth models for *L. monocytogenes* in cheese. An important application of challenge studies should be to provide growth data acquired directly on cheese matrix, allowing the rise of more accurate predictive tools, in comparison to currently available models, relying on data acquired *in vitro*, and not considering matrix effect.

Nevertheless, challenge studies doen not allow to consider intrinsic variability associated to cheese artisanal manufacture. This variability can be linked to the process, but also the seasonal variability in milk composition. As an instance, some ripening cellars are warmer during summer season than during winter season. Furthermore, it should be kept in mind that challenge studies provide growth data for one or more *L. monocytogenes* strains, but that behavioural differences between strains could be observed. Consequently, results could be a function of selected strains.

Factors affecting the growth of *L. monocytogenes* in artisanal cheeses

Physico-chemical parameters analyzed during challenge studies, as well as qualitative variable relating to process and ripening, did not allow to understand variability in δ of *L. monocytogenes* in SRSC, GHSC and SPSHC. Measured parameters included pH, a_w and salt, dry matter and water contents. Values recorded during this thesis did not allow to consider these variables as key factor to assess the growth of *L. monocytogenes* in the concerned cheese varieties. For SRSC, no significant differences between varieties were reported. As a reminder, three batches of a *Herve* cheese allowed to decrease levels of contamination during shelf-life, while all other batches (nine) allowed the growth of the pathogen. Similarly, for GSHC and SPSHC, growth potentials were variable between varieties, but also between batches for given varieties. Extra parameters could have been studied in order to explain these differences. Wemmenhove *et al.* (2018) reported that a concentration of undissociated lactic acid > 6.35 mM was sufficient to inhibit the growth of *L. monocytogenes* in *Gouda* cheese. This chemical compound could alter bacterial membranes and favor the efficiency of antibacterial molecules, including bacteriocins produced by resident microbiota (Possas *et al.*, 2021). A systematic determination of undissociated lactic acid concentrations could have been interesting. Concentration of other organic acids, including sorbic, acetic and citric acids could also represent hurdles to the growth of the pathogen, as well as melting

salts like phosphates (Ostergaard *et al*., 2014, Martinez-Rios *et al*., 2020; Possas *et al*., 2021).

It was opted for another strategy during this thesis, based on the hypothesis that cheese microbiota could play a significant role in the variability of growth potential. A recent paper published by Panebianco *et al*. (2021) observed that autochtonous LAB species isolated from Calabrian dairy products could exert an inhibitive activity against *L. monocytogenes*. Complex microbial consortia could also self-protect cheese (Callon *et al*., 2014; Mayo *et al*., 2021). Surprising results obtained during challenge studies on Belgian cheeses could be explained by similar phenomena. Consequently, microbiota of 31 cheese varieties was investigated using metagenetics, based on PCR amplification and Illumina sequencing of V1-V3 regions of bacterial 16S rRNA gene. One batch was considered for each cheese factory, including three samples at day-0, and three samples at end of shelf-life. Sampling was performed by collecting core and rind simultaneously. In total, 1,697 unique OTUs were identified, belonging to 15 phyla and 277 genera. In all cheese types, *Lactococcus* was dominant, but co-dominance with *Streptococcus* was observed in SRSC and SPSHC. An interesting observation was that the relationship between starter cultures and dominant population was not always obvious. Differences in bacterial communities were observed between samples from the same type of cheese, but sometimes also within a given batch. Exploring cheese bacterial communities allowed drawing a new hypothesis: the presence of an unknown *Fusobacterium* in a batch of *Herve* cheese, with a relative abundance > 10%, could explain the behavior of *L. monocytogenes* in this cheese variety. Regarding SPSHC and GSHC, it was not possible to make new hypothesis, based on cheese microbiota, in order to explain variability observed during challenge studies.

Various elements could have allowed an improvement of knowledge acquired during this step dedicated to the study of cheese microbiota. Provided that three batches were available for most cheese varieties, it could have been interesting to study all these samples using metagenetics. As such, the work only provides an instant caption of cheese bacterial community. Seasonality in bacterial communities has already been reported in *Adobera* cheese (Ruvalcaba-Gomez *et al*., 2021). Assessing stability of cheese microbiota over time could have allowed to confirm potential differences between factories, and to suggest the influence of *terroir* effect. It could also define a core microbiota for each cheese factory. Robustness of correlation analyses would also be increased.

All studied samples were dominated by LAB genera, namely *Lactococcus* and *Streptococcus*. Although Mayo *et al*. (2021) mentioned that relative abundance of LAB in cheese is generally > 90%, cumulated relative abundance of *Lactococcus* and *Streptococcus* was higher than 99% in most GSHC and SPSHC samples from this thesis, preventing to visualize subdominant or minor taxa potentially explaining differences in behavior of *L. monocytogenes* between varieties. A solution could have been to increase sampling effort, which was 6,000 sequences in this work, or to consider all sequence reads. Another option allowing to consider these taxa could be to remove major LAB species from the analysis, including *L. lactis* and

S. thermophilus. In a way, this approach was already applied when evaluating community structure (β-diversity) in Chapter 6, as both species were not included in Yue & Clayton dissimilarity matrices used to build NMDS. An alternative could be to remove sequence reads corresponding to these dominant species when performing bioinformatics, considering only sequences from other OTUs. Nevertheless, efficiency of this approach would be limited by the number of sequence reads generated by Illumina sequencing procedure. Another way could be to mask *L. lactis* and *S. thermophilus* DNA sequences directly during PCR amplification steps.

For increased exhaustivity, eukaryotic microorganisms, including yeasts and moulds, should also be studied. These fungi are indeed hosted on naturally ripened cheese surfaces, *i.e.* in crusts of SRSC, MRSC and SPSHC. These organisms play important functions during cheese ripening and rind formation, including lactate metabolization and NH_3 formation, resulting in pH increase allowing the growth of less acid-tolerant bacteria (Frölich-Wyder *et al.*, 2018). Exhaustive characterization of cheese microbiota should thus include these microorganisms. For this purpose, internal transcribed spacer (ITS) regions or 18S and 26S rRNA genes can be targeted (Ceugniez *et al.*, 2017; Afshari *et al.*, 2020). In their review gathering 33 studies on cheese surfaces, Irlinger *et al.* (2015) identified 39 fungal genera. As an example, regarding yeasts and moulds, respectively, only four and two genera were observed by Ceugniez *et al.* (2017) on rinds of *Tomme d'Orchies*. Most frequent yeasts observed on cheese surfaces belong to genera *Candida, Debaryomyces*, *Galactomyces*, *Geotrichum*, *Kluyveromyces, Pichia* and *Yarrowia*. *Penicillium*, *Scoplariopsis* and *Fusarium* are the most observed moulds genera (Irlinger *et al.*, 2015; Gonçalves Dos Santos *et al.*, 2017). Inhibition exerted by yeasts on the growth of *L. monocytogenes* has already been reported, especially from two species isolated from SRSC, namely *Candida intermedia* and *Kluyveromyces marxianus* (Goerges *et al.*, 2006). *Yarrowia lipolytica* is also mentioned as potential inhibitive species (Falardeau *et al.*, 2021). This observation provides an additional argument for the in-depth characterization of cheese eukaryotic microbiota.

In the way this work was performed, it is impossible to determine if identified taxa were located in cheese core or rind, as sampling was performed by collecting both parts simultaneously. This information is important, as these two parts represent distinct ecosystems. Rind has higher pH and lower a_w than core. It is also exposed to oxygen. Consequently, microorganisms able to survive or grow are not common between both. In this work, anaerobes were observed at day-0 or at end of shelf-life, including *Bifidobacterium*, *Faecalibacterium*, *Prevotella* and *Fusobacterium*. It could have been interesting to identify if these OTUs were located in cores or rinds. Similarly, Dugat-Bony *et al.* (2016) observed that psychrophilic bacteria were dominant in rind samples. It could have been interesting to confirm this observation.

Metagenetics based on 16S rRNA gene sequencing does not distinguish dead and alive bacteria. As suggested by an expert who reviewed the article on this topic, it could be thought that observed anaerobes corresponded to dead bacterial cells which were still present in cheese at the end of ripening and during storage. A more appropriate technique could be to identify metabolically active bacterial cells using

RNA-seq for metatranscriptomics data (Afshari *et al*., 2020). Besides providing a confirmation of the activity of particular bacterial genera, this approach could allow the understanding of their roles in cheese ecosystem during refrigerated storage.

All put together, metagenetics only brought one interesting information, in relation to the main objective of this thesis, *i.e.* the understanding of factors influencing the growth/no growth of *L. monocytogenes* in cheese: the identification of the unexpected presence of a likely novel species of *Fusobacterium* in three batches of a *Herve* cheese. As a reminder, this cheese variety did not allow the growth of *L. monocytogenes*, although physicochemical environment was highly in favor of the pathogen. This bacterium could thus be a key factor to understand this surprising observation. Nevertheless, prior to assess its ability to act on the growth of *L. monocytogenes*, an essential step was to isolate this bacterium for further characterization. This step was tricky, and it was still not possible to succeed. Various approaches have been tested, with growth media based on available papers on other *Fusobacterium* spp., working under anaerobic conditions and at several temperatures. PCR assays confirmed the presence of the targeted bacterium in all samples, as well as its ability to grow in BHI supplemented with antibiotics (neomycin and/or erythromycin). The tricky point was thus to gather all conditions necessary for its growth on plates. The fact that *Fusobacterium* sp. was able to grow into liquid media confirmed that bacterial cells were not dead. This hypothesis was doubtful, as its relative abundance was dramatically increased during storage of the first batch of *Herve* cheese at 8°C. As discussed in Chapter 7, various hypotheses could explain its inability to be isolated on growth media, even non-selective, *e.g.* VBNC state and auxotrophy.

Another approach that could have been tried to isolate this *Fusobacterium* sp. is the use of fluorescence activated cell sorting, also known as FACS or flow cytometry. Briefly, it should have required to specifically tag the targeted bacterium with a biomarker, and to sort cheese suspension in order to keep only bacteria of interest. By disposing of sorted cells, it would have been easy to:
- Perform the PCR specific to *Fusobacterium* genus;
- Amplify and sequence V1-V3 regions of 16S rRNA gene of these bacteria and align this query with sequence obtained during metagenetics, allowing to confirm the isolation of expected species;
- Perform WGS and characterize the novel species;
- Assessing the ability of the *Fusobacterium* sp. to inhibit the growth of *L. monocytogenes in vitro*.

Metagenomics allowed to acquire a significant part of the genome of the *Fusobacterium* sp. The completeness of 100% should be considered with caution, as some contigs were of small size. Nevertheless, at least two approaches, namely average nucleotide identity and phylogenomic tree, demonstrated that it probably belonged to a novel species of the genus *Fusobacterium*. Proteome comparison with other *Fusobacaterium* spp. revealed differences in metabolic pathways, subsystems and resistance to antibiotics. Thanks to the latter information, it could be possible to choose growth media potentially allowing the growth of *Fusobacterium* sp. Isolation

of the bacterium is indeed necessary to describe and characterize the novel species. It is also impossible to assess its inhibitory activity against *L. monocytogenes* without bacterium isolation. Despite data acquired through proteome analysis, it remained impossible to understand how this bacterium survived cheese manufacture, ripening and storage, and how it could grow during these steps to become subdominant taxa of this *Herve* cheese. A pathway to explore is the potential of this bacterium to digest milk oligosaccharides.

To go further on this bacterium, it could be interesting to investigate its origin in cheese processing environment. For this purpose, swab samples could be collected on the whole production line, from curdling to ripening. Raw material samples could also be collected, including milk, water and brine. After DNA extraction from all samples, qPCR specific to *Fusobacterium* spp. could be performed. In case of detection in raw milk, it could finally be interesting to investigate in the farm delivering milk to the concerned cheese factory.

Conclusion

Globally, this thesis contributed to the global knowledge on Belgian artisanal cheeses. On the one hand, it allowed getting a global picture on varieties found in this country, on their manufacturing processes, as well as on their physico-chemical and microbiological characteristics. On the other hand, growth data for *L. monocytogenes* were acquired for a range of variety. The latter data, obtained through challenge studies, allowed an official revision of microbiological criteria for UACC with pH ≤ 5 by FASFC, instead of the commonly accepted threshold value of pH 4.4. The thesis pointed the issue associated to inter- and intra-batch variability regarding growth of the pathogen in ripened cheeses, especially concerning SPSHC and GSHC. The difficulty to consider this variability while calculating δ was also underlined. As a consequence, official guidelines provided by EURL *Lm* should be revised to consider this problem. Further, this work contributed to the knowledge of Belgian artisanal cheese microbiota, and enlightened the presence of an unknown bacterium of the genus *Fusobacterium* in a raw milk PDO *Herve* cheese. Notably, the latter cheese showed surprising results during growth experiments, with an unability for *L. monocytogenes* to grow during shelf life in three batches. These observations opens interesting perspectives that should be investigated during the next months.

Besides, this thesis was not able to identify new markers explaining the growth/no growth of *L. monocytogenes* in ripened Belgian artisanal cheese varieties, although a range of likely factors were investigated during these four years of work, *i.e.* pH, a_w, dry matter, salt content, manufacturing process and microbiota. Despite the fact that some factors were not included in this work, *e.g.* concentrations of organic acids, a possible conclusion is that the fate of *L. monocytogenes* is probably not governed by one of this factor, but well by their complex interaction. Due to this complexity, it is utopic to envisage the transfer of knowledge from one cheese variety to another, as confirmed by challenge studies. Results of challenge studies and build growth models based on them should remain a case-by-case

approach. This conclusion could open the door to novel approaches, including biocontrol strategies. The combined use of bacteriocins and bacteriophages could be interesting, as it was already demonstrated that efficacy of their exclusive use was limited. Similarly, the addition of a protective strain is not the panacea, its efficacy being highly cheese-dependent. Furthermore, it is more likely a microbial consortium rather than individual species that can influence the growth of *L. monocytogenes*. In a study performed by Maoz *et al.* (2003), none of 400 species isolated from an inhibitory consortium had an antilisterial activity. Again, this implies extremely complex ecological interactions, involving nonspecific and specific competition. The presence of *L. monocytogenes* in ripened cheeses will thus remain a hot topic in the next years, as well as the understranding of its fate. The development of consensus methods for detection and enumeration of the pathogen and for assessing its growth using challenge studies will also be necessary.

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