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Study of the behavior of *Listeria monocytogenes* in raw milk butter

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Abstract

Soundous El-Hajjaji (2022). Study of the behavior of *Listeria monocytogenes* in raw milk butter. PhD thesis**.** University of Liège - Gembloux Agro-Bio Tech, Gembloux, Belgium, 118 pages, 14 figures, 22 tables.

Abstract

As food is essential to life, food safety is important and a prevalent concern worldwide. *L. monocytogenes* is one of the most dangerous pathogens due to the high mortality rate that causes listeriosis. Considered as a RTE allowing the growth of *L. monocytogenes*, raw milk butter must comply with food safety criteria defined by Regulation (EC) No 2073/2005 imposing the no detection of the pathogen in 25g, unless the manufacturer is able to prove that his product will not exceed the limit of 100 cfu/g throughout the shelf-life.

Studies have shown that the growth potential of *L. monocytogenes* in butter is relatively low and depends on the product characteristics and composition. The main goal of this thesis was to study the behavior of *L. monocytogenes* in raw milk butter through different approaches and determine butter's ability to support survival or growth of *L. monocytogenes*.

First, a description of raw milk butter at both technological and physicochemical level was conducted through a phone survey and on-site monitoring. Four main sequences of maturation were identified (Workshop, Fridge, Workshop-Fridge and Fridge-Workshop) leading to a wide range of pH (from 4.25 to 6.50).

Based on this data, durability studies were conducted on various batches of naturally contaminated raw milk butter. The samples presented low levels of contamination (< 10 log cfu/g). After that, challenge tests and metagenetic analyses were carried out on two inoculated batches for deeper assessement. Two processes were simulated: maturation at 4 °C and at 14 °C. For each batch, analyses were realized on the cream during maturation and on butter during storage. It was found that butter's ability to support the growth of *L. monocytogenes* depends on the way the maturation was carried out. Maturation temperature has a strong influence on raw milk butter subdominant microbiota, which affects the product's characteristics and thus the growth of *L. monocytogenes*. For butter made from cream matured at room temperature (14 °C), no growth of the pathogen was observed. This butter presented low pH values (< 4.8) and high levels of lactic acid bacteria especially *Lactococcus* spp. Butter made from cream matured at refrigerated temperature (4 °C) allowed however the growth of *L. monocytogenes*. This butter presented high pH values (≥ 6.5) and lower levels of *Lactococcus* spp compared to acidic butter.

For acidic butter, not allowing the growth of the pathogen, a request was submitted to FASFC for a revision of food safety criterion. For sweet butter allowing the growth of the pathogen, we tried to adjust mathematical models to our data. However, further tests and more data are needed for better results.

Globally, this thesis contributed to the global knowledge on Walloon raw milk butter and a better understanding of the behavior of *L. monocytogenes* in this product. Further research to acquire more data to develop models integrating different factors and their interactions would be interesting for anticipating the bacterium growth during shelf life.

Key words: Butter, *Listeria monocytogenes*, cream acidification, durability studies, challenge tests, growth potential, metagenetics, predictive microbiology

Résumé

Soundous El-Hajjaji (2022). Etude du comportement de *Listeria monocytogenes* dans le beurre au lait cru. Thèse de doctorat**.** Université de Liège - Gembloux Agro-Bio Tech, Gembloux, Belgique, 118 pages, 14 figures, 22 tableaux.

Résumé

Comme la nourriture est essentielle à la vie, la sécurité sanitaire des aliments est importante et une préoccupation répandue dans le monde entier. *L. monocytogenes* est l'un des agents pathogènes les plus dangereux en raison du taux de mortalité élevé que cause la listériose. Considéré comme un aliment prêt à être consommé permettant la croissance de *L. monocytogenes*, le beurre au lait cru doit respecter les critères de sécurité des denrées alimentaires définis par le règlement (CE) n°2073/2005 imposant la non-détection du pathogène dans 25g, sauf si le fabricant est en mesure de démontrer que son produit ne dépassera pas la limite de 100 ufc/g pendant toute la durée de conservation.

Des études ont montré que le potentiel de croissance de *L. monocytogenes* dans le beurre est relativement faible et dépend des caractéristiques et de la composition du produit. L'objectif principal de cette thèse était d'étudier le comportement de *L. monocytogenes* dans le beurre au lait cru à travers différentes approches et de déterminer la capacité du beurre à soutenir la survie ou la croissance de *L. monocytogenes.*

Dans un premier temps, une description du beurre au lait cru tant au niveau technologique que physico-chimique a été réalisée à travers une enquête téléphonique et des suivis de production. Quatre grandes séquences de maturation ont été identifiées (Atelier, Frigo, Atelier-Frigo et Frigo-Atelier) conduisant à une large gamme de pH (de 4,25 à 6,50).

Sur la base de ces données, des études de vieillissement ont été menées sur différents lots de beurre au lait cru naturellement contaminés. Les échantillons présentaient de faibles niveaux de contamination (< 10 log ufc/g). Des tests de provocation et des analyses métagénétiques ont été effectués par la suite sur deux lots inoculés pour une évaluation plus approfondie. Deux processus ont été simulés : la maturation à 4 °C et à 14 °C. Pour chaque lot, des analyses ont été réalisées sur la crème en cours de maturation et sur le beurre en cours de stockage. Il a été constaté que la capacité du beurre à soutenir la croissance de *L. monocytogenes* dépend de la manière dont la maturation a été effectuée. La température de maturation a une forte influence sur le microbiote dominant du beurre au lait cru, qui affecte les caractéristiques du produit et donc la croissance de *L. monocytogenes*. Pour le beurre produit à partir de crème maturée à température ambiante (14 °C), aucune croissance du pathogène n'a été observée. Ce beurre présentait des valeurs de pH faibles (< 4,8) et des niveaux élevés de bactéries lactiques, en particulier *Lactococcus* spp. Le beurre produit à partir d'une crème maturée à une température de réfrigération (4 °C) a cependant permis la

croissance de *L. monocytogenes.* Ce beurre présentait des pH élevés (≥ 6,5) et des niveaux inférieurs de *Lactococcus* spp par rapport au beurre acide.

Pour le beurre acide, ne permettant pas la croissance du pathogène, une demande a été soumise à l'AFSCA pour une révision du critère de sécurité sanitaire de l'aliment. Pour le beurre doux permettant la croissance du pathogène, nous avons essayé d'ajuster des modèles mathématiques à nos données. Cependant, d'autres tests et plus de données sont nécessaires pour de meilleurs résultats.

Globalement, cette thèse a contribué à la connaissance globale du beurre au lait cru wallon et à une meilleure compréhension du comportement de *L. monocytogenes* dans ce produit. Des recherches plus poussées pour acquérir plus de données pour développer des modèles intégrants différents facteurs et leurs interactions seraient intéressantes pour anticiper la croissance de la bactérie pendant la durée de conservation.

Mots clés : Beurre, *Listeria monocytogenes*, acidification de la crème, études de vieillissement, tests de provocation, potentiel de croissance, métagénétique, microbiologie prédictive

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2. El-Hajjaji, S., Gérard, A., De Laubier, J., Di Tanna, S., Lainé, A., Patz, V., Sindic, M., 2020. Assessment of growth and survival of *Listeria monocytogenes* in raw milk butter by durability tests. Int. J. Food Microbiol. 321, 108541. https://doi.org/10.1016/j.ijfoodmicro.2020.108541
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Communications:

1. El-Hajjaji, S., De Laubier, J., Di Tanna, S., Lainé, A., Patz, V., Sindic, M., 2018. Study of the development of *Listeria monocytogenes* in raw milk butter. 23rd National Symposium for Applied Biological Sciences (NSABS), Brussels, Belgium. http://hdl.handle.net/2268/220786
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I

General introduction

1. Butter production and consumption

Since ancient times, milk and dairy products form a major part of human diet and are a vital source of many nutrients including minerals and vitamins (Belitz et al., 2009; Gaucheron, 2011). Butter is one of the oldest dairy products (Boulongne, 2015). It is a product rich of fat (minimum 80% m/m) and thus a high-energy food, providing approximately 715 calories per 100 grams (The Editors of Britannica Encyclopedia, 2020).

The European Union is a major player in the world dairy sector as the main milk and cheese producer (USDA, 2021). It is also the second butter producer after India. In 2020, the EU produced around two and a half million tons of butter (USDA, 2021). Germany and France are the leading butter producing countries in EU with 24% and 19% of the total butter produced in EU, respectively (CLAL, 2020). These countries are followed by Ireland (12%), Poland (11%), Netherlands (6%) and Belgium (6%).
In Belgium, the overall butter production was over 95000 tons in 2020 which had increased by 21% compared to 2010 (CBL, 2021).
Not only is the EU a leading producer of butter, but the region is also one of the main exporters of butter among other dairy products. Although the volume of butter exported from the EU increased in the last years, the domestic demand and consumption for butter in the EU has remained relatively consistent from 2014 to 2020 (OECD and Food and Agriculture Organization of the United Nations, 2020). In Belgium, the global consumption per capita in 2020 was of 2.4 kg, which had increased by 11.6% compared to 2019 (CBL, 2021).

2. Types of butter and raw milk butter

Butter is a water-in-oil emulsion made from cream by phase inversion occurring during its manufacture. Figure 1 shows the butter making process.

According to its manufacturing process, butter products are broadly classified as sweet butter made from unfermented sweet cream and cultured butter made from cultured ripened cream (Belitz et al., 2009; Hae-Soo et al., 2013). As shown in Figure 1, after skimming, cream undergoes heat treatment. However, in some countries, of north and east Africa for exemple, raw cream (or raw milk) is still used to produce traditional sour cream butter (Ahmed et al., 2016; Alganesh and Yetenayet, 2017; Idoui et al., 2010; Samet-Bali et al., 2009). This method is generally adopted by traditional small-scale on-the-farm producers. The cream naturally acidifies under the effect of lactic acid bacteria naturally present in the cream.
In Belgium, artisanal butter represents 20% of the total production, with 80% produced in Wallonia (APAQ-W, n.d.). Farms specialized in dairy production in Wallonia represent 12.8% of the Walloon exploitations while in Flanders they represent 4% (Nature & Progrès, 2016). In 2018, the Walloon dairy sector represented 25% of the value of Walloon agriculture, i.e., 451 million euros (SPW, 2020).

In the survey carried out by the College of Producers in 2018 among 523 producers – processors, it was found that 68% of respondents use raw milk. Butter was also found to be the most widely produced dairy product (74% of the respondents) (SOCOPRO, 2017). This thesis will focus on raw milk butter (artisanal Walloon butter) which represents the majority of Walloon artisanal production.

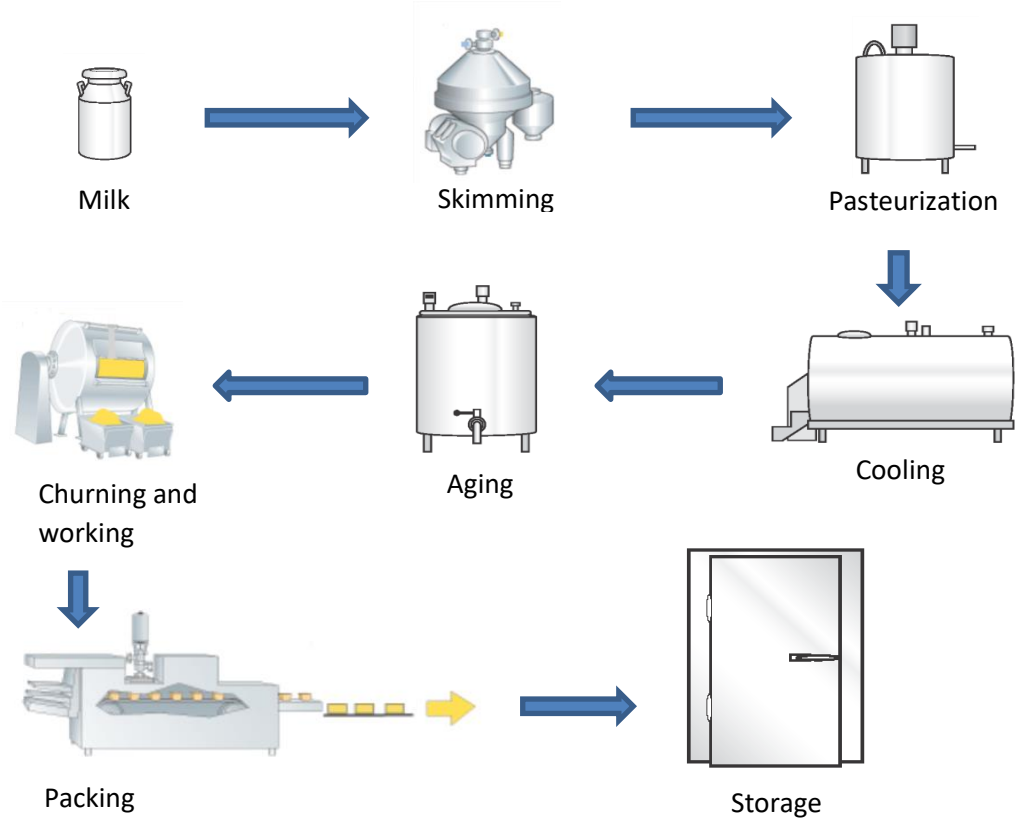

Figure 1: Production process of butter

3. Butter spoilage and contamination

Modern manufacturing methods have minimized the bacterial spoilage of butter. However, defects caused by microbes do occasionally occur. Various types of spoilages and defects have been encountered in butter. Two main types of butter spoilage are surface discoloration and flavor defects such as rancidity and putridity (Ozer and Akdemir-Evrendilek, 2014). These defects have been attributed to *Pseudomonas* and *Flavobacterium* (Budhkar et al., 2014; Ozer and Akdemir-Evrendilek, 2014; Pal et al., 2016). Other microorganisms including yeasts are also known to cause spoilage of butter (Budhkar et al., 2014; Pal, 2014; Sagdic et al., 2010).

Beside spoilage, microorganisms can also be the cause of microbiological hazards associated with butter like *Salmonella*, *Escherichia coli* O157:H7 and *Listeria monocytogenes* (Holliday, 2001). The latter is the subject of this thesis. This bacterium is the causative agent of listeriosis in humans and can affect sensitive populations such as infants, elderly people and those suffering from immune deficiency (Farber and Peterkin, 1991; Jemmi and Stephan, 2006; McLauchlin et al., 2004). Although it is not common, listeriosis has a mortality rate of over 25% (Jordan et al., 2016; McLauchlin, 1996; Saha et al., 2015).

In the following parts, the available information in the scientific literature concerning the occurrence of *L. monocytogenes* in butter is summarized. These parts were adapted from the following published review article:

El-Hajjaji, S., Gérard, A., Sindic, M., 2020. Is Butter A Product at Risk Regarding *Listeria monocytogenes?* - A Review. Food Reviews International 1–12. https://doi.org/10.1080/87559129.2020.1831528

The studies on the behavior of the pathogen in butter and dairy fat products were also covered in this review. Papers were gathered using Google Scholar and Scopus, and with English and French keywords. In addition, we examined summary reports published by European and non-European agencies about zoonosis and food borne outbreaks. We also examined lists of references in all articles we located to ensure that all reports were identified.

4. *Listeria monocytogenes* and listeriosis

Listeria monocytogenes is one of the 20 species belonging to the genus *Listeria* (Doijad et al., 2018; Leclercq et al., 2019; Magalhães et al., 2014; Núñez-Montero et al., 2018; Orsi and Wiedmann, 2016). It is responsible for listeriosis in humans. It can affect sensitive populations such as infants, pregnant women, elderly people and those suffering from immune deficiency (Buchanan et al., 2017; Jemmi and Stephan, 2006; McLauchlin et al., 2004). Most cases of listeriosis were reported in the elderly, especially over 65 years old (EFSA BIOHAZ Panel et al., 2018; Gillespie et al., 2010; Goulet et al., 2008). In 2015, the group comprising people ≥ 75 years old recorded the highest occurrence rate in the EU in the period 2008-2015. The occurrence rates for this age group's men and women were of 2.20 and 1.30 cases per month per million person respectively (EFSA BIOHAZ Panel et al., 2018). In 2019, 2621 confirmed invasive human cases of listeriosis have been reported by European Union members. Representing 0.8% of all the reported cases, listeriosis is one of the less commonly reported zoonosis compared with campylobacteriosis (66.9% of the reported cases) and salmonellosis (26.7% of the reported cases) (European Food Safety Authority and European Centre for Disease Prevention and Control, 2021). Although it is not common, listeriosis has a mortality rate over 25% (Jordan et al., 2016; McLauchlin, 1996; Saha et al., 2015). In 2019, it presented a high hospitalization (92.1%) and the highest mortality rate (17.6%), being the most severe zoonosis reported in Europe

(European Food Safety Authority and European Centre for Disease Prevention and Control, 2021).

The ingestion of contaminated food is the principal route of transmission of *L. monocytogenes* (Swaminathan and Gerner-Smidt, 2007). Various foods have already been identified as potential vectors of *L. monocytogenes*, especially ready-to-eat (RTE) foods, mainly those made from meat and smoked fish, and dairy products. Even though cheese is the most reported dairy product, butter is of concern as well (European Food Safety Authority and European Centre for Disease Prevention and Control (EFSA and ECDC), 2019; Jofré et al., 2016; Pérez-Rodríguez et al., 2017).

Butter is a milk fat product « in the form of a solid, malleable emulsion, principally of the water-in-oil type, derived exclusively from milk and/or certain milk products » with a minimum milk fat content of 80 g/100g (Council Regulation (EC), 1994). As a RTE food, butter must comply with the Commission Regulation (EC) N° 2073/2005 on microbiological criteria for foodstuffs. The latter imposes the no detection of *L. monocytogenes* in 25 g of ready-to-eat (RTE) foods able to support its growth. Foods with a pH value above 4.4 or a water activity higher than 0.92, or a combination other than a pH lower than 5.0 and a water activity lower than 0.94, are considered susceptible to the multiplication of *L. monocytogenes.* For foods with other physico-chemical characteristics and those that are not able to support the growth of *L. monocytogenes*, the regulation imposes a number of counts ≤ 100 cfu/g in the five units comprising the sample. This criterion can also be applied to other products subject to scientific justification (Commission Regulation, 2019, 2005).

To investigate the growth of *L. monocytogenes* in their product, several studies can be performed by the producers including challenge tests and durability tests. Challenge test is a laboratory based study which consists in monitoring the survival and/or growth of *L. monocytogenes* in artificially contaminated food, under given storage conditions (Beaufort, 2011). For this type of study, it is hard to imitate the natural contamination and a fully equipped laboratory is required, especially if the contamination is conducted in raw materials in the beginning of the process. As for durability tests, they are conducted on naturally contaminated samples. They are more realistic, but their implementation is limited in case of low prevalence. Such tests also require large quantity of the product to cover the intra-batch variability. In Europe, the European Union Community Reference Laboratory for *L. monocytogenes* prepared a technical guidance document for conducting shelf-life studies, dedicated to food business operators (Bergis et al., 2021). In Belgium, Federal Agency for the Safety of the Food Chain (FASFC) also published scientific opinion related to challenge-tests and shelf-life studies for *L. monocytogenes* in cheese (FASFC, 2016a). It also published scientific opinions on the growth of *L. monocytogenes* in raw milk butter (FASFC, 2019, 2016b). Another option is to consult the available scientific literature as a comparison and predictive way because each product studied presents specific formulation and physico-chemical characteristics.

5. Source of contamination with Listeria monocytogenes in dairy farms and dairy industry

5.1. Cattle and barn environment

Dairy farms represent a rich reservoir of *L. monocytogenes* (Hati et al., 2018). Poutrel (1994) associated the massive contamination of raw milk by *L. monocytogenes* to its presence on the teats of mammary glands in cows. In a study conducted by Husu et al. (1990), 13.6% and 6.8% of samples taken from the teats before washing and drying were infected with *Listeria spp.* and *L. monocytogenes,* respectively. No isolation was performed after cleaning the udder. Therefore, an adequate cleaning of teats before milking seems important to reduce contamination by *L. monocytogenes*.

The possible sources of milk contamination in dairy farms include animals, but also feed and barn environment. Fox et al. (2009) reported an occurrence of 19% of *L. monocytogenes* in environmental samples (cow feces, milk, silage, soil, water, etc.) collected from 16 farms in Ireland. They also found a correlation between the level of hygiene standards on the farm and the occurrence of the pathogen. Other studies have isolated *L. monocytogenes* from straw, whether used on the floor or as fodder, and silage (Castro et al., 2017; Husu et al., 1990; Papić et al., 2019; Ueno et al., 1996).

The presence of *L. monocytogenes* in silage has been reported by many authors (Arimi et al., 1997; Fenlon, 1985; Ho et al., 2007; Pauly and Tham, 2003; Perry and Donnelly, 1990). Consumption of contaminated silage leads to the excretion of *L. monocytogenes* by cattle, and so increases the risk of milk contamination (Fenlon et al., 1996; Ho et al., 2007; Sanaa et al., 1993; Vilar et al., 2007). Producing intensively fermented silages (pH < 4.4) and applying a long storage period (more than 30 days) allow to inhibit the survival of *L. monocytogenes* (Pauly and Tham, 2003).

5.2. Processing environment

L. monocytogenes can easily colonize the processing environment and the equipment, especially the moist ones (Santorum et al., 2012). Although thermal processes, mainly pasteurization, are often used to reduce the level of microorganism including *L. monocytogenes* to an acceptable one, the transmission of the pathogen through equipment and processing environment is thus possible, leading to post-pasteurization contamination (Casadei et al., 1998; Kasalica et al., 2011). In fact, butter made from pasteurized milk has already been found positive for *L. monocytogenes* and was also reported as the causative agent of a listeriosis outbreak (Lyytikäinen et al., 2000).

L. monocytogenes is also able to produce biofilms on various food processing surfaces, in the presence of another bacteria or not (Colagiorgi et al., 2017; Magalhães et al., 2017; Osman et al., 2014), and especially when surfaces are exposed to an aqueous environment (Flint et al., 1997). As an example, the presence of *Listeria-*

containing biofilms has been reported in milking equipment (Latorre et al., 2010). Biofilms of *L. monocytogenes* can be resistant to sanitizing agents such as peroxide sanitizer, quaternary ammonium compounds and chlorine (Pang et al., 2019; Rodríguez-López et al., 2018; Rodríguez-Melcón et al., 2019). Treatments with enzymes can be used as an alternative to remove biofilms (Lequette et al., 2010).

The pathogen may reach milk as a result of exogenous contamination via equipment and barn environment, or, more rarely, by a direct excretion following the presence of mastitis (Belleflamme et al., 2006; Hassan et al., 2000; Winter et al., 2004). Good practices are therefore needed to prevent the contamination by *L. monocytogenes,* and its growth and survival (Husu et al., 1990; Sanaa et al., 1993).

6. Outbreaks of listeriosis associated with butter

Among dairy products, cheese presents relatively high percentage of occurrence of *L. monocytogenes* and was associated to listeriosis cases in Europe during 2017 (European Food Safety Authority and European Centre for Disease Prevention and Control (EFSA and ECDC), 2018). However, cases of listeriosis associated with butter consumption have also been reported.

Although the organism was not isolated from butter, the first association between consumption of butter and listeriosis occurred in USA (Ryser and Marth, 1999). In Los Angeles County, 11 perinatal listeriosis cases were reported in 1987. Seven of the strains obtained from the victims were of serotype 1/2a, which is one of the predominant serotypes of *L. monocytogenes* causing infections (Datta, 2003). In fact, serotypes 1/2a, 1/2b and 4b are the most associated with human cases of listeriosis. These belong to lineages I and II that harbor the most prevalent clonal complexes (CC) such as CC1,CC2, CC4 and CC6 that are hypervirulent (Datta and Burall, 2018; Maury et al., 2019, 2016; Orsi et al., 2011; Painset et al., 2019). A subsequent case-control study identified butter as a possible food vehicle. No further information is found in the literature.

The second outbreak of listeriosis associated with contaminated butter, occurred in Finland (Lyytikäinen et al., 2000). The epidemic concerned 25 patients, six of whom died. They were mostly immunocompromised and people admitted to a hospital where they consumed contaminated butter for a prolonged period (Maijala et al., 2001). The outbreak was caused by *L. monocytogenes* serotype 3a, which is a rare causative serovar (Lyytikäinen et al., 2000). The strain linked with this outbreak was detected in all samples obtained from the hospital, as well as in the butter and environmental samples from the dairy, especially the screw conveyor of the butter silo and the end of the packaging machine of the 7 and 10 g packaging line. Except for one, all the positive samples had less than 100 cfu/g of *L. monocytogenes* (Lyytikäinen et al., 2000). However, the observed levels of the pathogen increased during the storage at 6.5-7.2 °C of pooled samples of 7, 10 and 500 g packages (4.3 – 5.1, 1.8 – 2.9 and 0.8 – 3.6 log cfu/g respectively) (Maijala et al., 2001). The consumption of a contaminated product with a relatively high dose of the pathogen can cause listeriosis. However, this outbreak led the researchers to make another assumption regarding contamination by *L. monocytogenes,* according to which a prolonged consumption of contaminated

product can cause listeriosis. So, not only the level of *L. monocytogenes* present in food is important, but also the actual amounts of food consumed (Maijala et al., 2001).

In 2003, an outbreak of 17 cases of listeriosis associated with butter, occurred in Northeast England (Advisory Committee on the Microbiological Safety of Food, 2009; Gillespie et al., 2006). A dairy butter was found contaminated by *L. monocytogenes*. The same strain involved in this outbreak was isolated from butter and from a drain at the dairy. It was a serotype 4b (Advisory Committee on the Microbiological Safety of Food, 2003).

7. Occurrence of Listeria monocytogenes in butter

The outbreaks of listeriosis associated with butter have led researchers to take an interest in the contamination of this product. Various studies were conducted in different countries to examine the occurrence of *L. monocytogenes* in butter.

The frequencies of occurrence of *L. monocytogenes* in butter varied between 0.0% and 18.7% (Table 1). As illustrated in Table 1, the highest occurrences were observed for butters made from raw milk, at a small scale (at farms or at small productive centers located in urban areas). These results could be due to the absence of heat treatment during processing stages. Although some strains of *L. monocytogenes* are heat resistant, milk pasteurization at 75 or 76 °C during 16.2 sec can lead to 4.5- to 6.2-D process, and thus provide a reasonable margin of safety (Farber et al., 1992). Studies also reported the effect of heat treatment on the inactivation of *L. monocytogenes* in other dairy product. In double cream for example, 1 log reduction of the pathogen was achieved in 7.9 to 9.5 sec at 68°C, depending on the strain (Casadei et al., 1998; Doyle et al., 2001).

Table 1: Occurrence of *L. monocytogenes* in butter

Country	Sampling origin	Sample type	Sampling period	Number of samples	Occurrence (%)	Levels of the pathogen (cfu/g)	Identification method	References
Belgium	Farms	Raw milk butter	2002	64	18.7	NA	Culture + Accuprobe Reagent Kit	(De Reu et al., 2004)
	Farms	Raw milk butter	2006-2014	362	30.0	10-40	Culture + biochemical tests + count plate	(N'Guessan et al., 2015)
	Primary production and processing centers and stores	Raw milk butter	2008-2015	309	19.1	NA	NA	(FASFC, 2016b)
Hungary	Food stores	Pasteurized milk butter	2001-2006	16	0.0	___	Culture + biochemical tests	(Varga, 2007)
Iran	Retail stores	Traditional butter (raw milk)	2007-2009	25	4.0	NA	Culture + biochemical tests + PCR	(Rahimi et al., 2010)
		Commercial butter (pasteurized milk)		15	0.0	___		

Table 1: Occurrence of *L. monocytogenes* in butter (*Continued*)

Country	Sampling origin	Sample type	Sampling period	Number of samples	Occurrence (%)	Levels of the pathogen (cfu/g)	Identification method	References
Iran	Retail stores	Raw milk butter	2011-2012	18	0.0	___	Culture + biochemical tests + PCR	(Shamloo et al., 2015)
	NA	Traditional butter (raw milk)	2010-2012	70	12.9	NA	Culture + biochemical tests	(Shahbazi et al., 2013)
	Retail markets	Traditional butter (raw milk)	2016	100	1.0	NA	Culture + biochemical tests + PCR	(Akrami-Mohajeri et al., 2018)
Italy	Small scale producers	NA	1987-1988	20	0.0	___	Culture + biochemical tests + API 20 Strep	(Massa et al., 1990)
Northern Ireland	Milk processing centers	Pasteurized milk butter	1989	34	0.0	___	Culture + biochemical tests	(Harvey and Gilmour, 1992)
Turkey	Retail stores	Raw milk butter	2012-2013	100	2.0	NA	Culture + Microbact 12L + PCR	(Aksoy et al., 2018)
	Dairy plants and retail markets	Pasteurized milk butter	NA	10	0.0	___	Culture + biochemical tests	(Aygun and Pehlivanlar, 2006)
	Markets	NA	2013	10	10.0	NA	Culture	(Cardak, 2013)

Table 1: Occurrence of *L. monocytogenes* in butter (*Continued*)

Country	Sampling origin	Sample type	Sampling period	Number of samples	Occurrence (%)	Levels of the pathogen (cfu/g)	Identification method	References
Turkey	NA	NA	2011-2012	20	0.0	___	Culture + biochemical tests + PCR	(Kevenk and Terzi Gulel, 2016)
UK	Production, retail and catering premises	Pasteurized milk butter	2004	3229	0.4	< 10	Culture + AFLP	(Lewis et al., 2006)
	Retail premises	NA	2006-2007	878	0.0	___	Culture + AFLP	(Little et al., 2009)
	Premises	NA	2008-2009	419	0.0	___	Culture + API	(Meldrum et al., 2010)
USA	Dairy plants	NA	1987	30	0.0	___	NA	(Kozak et al., 1996)

NA, Not Available; PCR, Polymerase Chain Reaction; AFLP, Amplified Fragment Length Polymorphism; API, Analytical Profile Index

The high occurrence of *L. monocytogenes* in raw milk butter could also be due to environmental contamination. A correlation between the level of hygiene standards on the farm and the occurrence of the pathogen was reported by Fox et al. (2009). In a study conducted by De Reu et al. (2004) to assess the bacteriological quality of raw milk and raw milk farm products produced in Belgium, 64 butters were examined for *Listeria spp.* and *L. monocytogenes* among other hygiene indicators and pathogenic bacteria. Even though no statistical relationship was found between the hygiene indicators and the absence/presence of *Listeria spp*., it was observed that the contamination with coliforms, *Escherichia coli* and *Staphylococcus aureus* was higher for butter samples containing *Listeria spp*. Suitable hygienic conditions have to be provided to prevent contamination by *L. monocytogenes*, as well as recontamination during production. Even though *L. monocytogenes* has mainly been detected in raw milk butter, it can also be found in processed foods as a result of a post contamination after pasteurization (European Food Safety Authority, 2013).

The levels of contamination of butter by *L. monocytogenes* are generally less than 10 cfu/g (Kozak et al., 1996; Lewis et al., 2006; N'Guessan et al., 2015). Based on quantitative modeling, more than 90% of invasive listeriosis cases are attributable to ingestion of RTE foods containing > 2,000 cfu/g (EFSA BIOHAZ Panel et al., 2018).

8. Growth of Listeria monocytogenes in butter

Poor hygienic production and processing practices result in colonization and transmission of *L. monocytogenes* to food. Once in the product, the behavior of the bacterium is influenced by a variety of factors. For butter, these factors include temperature, pH, salt, water dispersion and the presence of preservatives. The results reported by some studies on the survival, growth, and inactivation of *L. monocytogenes* in butter as affected by these factors are summarized in Table 2.

L. monocytogenes is a psychrotrophic bacterium which has the ability to grow and survive at refrigeration temperatures (Chan and Wiedmann, 2008; Lanciotti et al., 1992; Rosset, 2001). The growth remains to be faster in ambient than chill temperatures. Holliday et al. (2003) found that the growth of *L. monocytogenes* reached a maximum level after 3 days in butter (pH 6.4) stored at 21 °C (6.6 log cfu/g), while it was obtained after 21 days in the same product stored at 4.4 °C (6.3 log cfu/g). The same result was reported by Voysey et al. (2009), who observed a maximum level of *L. monocytogenes* cells (6.3 log cfu/g) after 14 days in butter (pH 6.6) stored at 21 °C, while it was reached after 28 days in butter stored at 8 °C.

The same studies also reported no or slow growth of the pathogen in other samples of butter with a low pH (< 5.8) and/or high salt concentration. The behavior of *L. monocytogenes* in a low pH and high salt concentration is strongly temperature dependent (Cole et al., 1990; Saha et al., 2015). *L. monocytogenes* can grow at salt concentrations up to 10% of NaCl, nevertheless its growth can be slowed down at levels of salt of 2% (Michelon et al., 2016; Voysey et al., 2009). Salting causes a reduction in water activity, and so creates less favorable conditions for the growth of *L. monocytogenes* (Chaplin, 2003; Verraes et al., 2015). Similarly, a pH below 4.4 is

not favorable for the growth of the pathogen (Commission Regulation, 2005; Michelon et al., 2016).

Butter is a water-in-oil emulsion with high fat content. The viability of *L. monocytogenes* varies with fat content (Donnelly and Briggs, 1986; Holliday et al., 2003). Less fat results on a not finely dispersed serum, which allows bacterial growth (Bullock and Kenney, 1969). The sequestering of aqueous phase in oil phase is thus another important characteristic of butter to prevent the growth of *L. monocytogenes*. Coarse butter (largest water droplets size) allows easily the growth of *L. monocytogenes* compared to fine butter (smallest water droplets size) (Michelon et al., 2016; Voysey et al., 2009). An emulsion with droplet size less than 10 μm provide an unfavorable environment for the growth of microorganisms. Smaller is the droplets size, more limited is the area and the quantity of nutrients available for microbial growth in the droplet (Delamarre and Batt, 1999).

Preservatives have also an inhibitory effect on the growth of *L. monocytogenes*. Fat spreads with preservatives like potassium sorbate and sodium benzoate, showed no growth of the pathogen (Holliday et al., 2003). It was reported that potassium sorbate can inhibit microbial growth (Kaul et al., 1981). The adverse effect of preservatives is however influenced by the temperature and pH. For example, sodium diacetate, sodium benzoate and sorbates are more effective at preventing growth of *L. monocytogenes* at lower storage temperatures and pH (FSANZ, 2013).

High levels of lactic acid bacteria, involved in cream maturation, can also inhibit the growth of the pathogen. Lactic acid bacteria produce many antimicrobial substances such as organic acids, hydrogen peroxide, carbon dioxide, diacetyl and bacteriocins (Dortu and Thonart, 2009; Jordan et al., 2016). A variety of bacteriocins have been identified as potential inhibitors against *L. monocytogenes* (Chen and Hoover, 2003; Harris et al., 1989). Their effectiveness is variable depending on the pH. The activity of nisin is for example more pronounced when the pH decreases (Dal Bello et al., 2012; Gallo et al., 2007).

Other parameters can also affect the behavior of *L. monocytogenes* in butter. A fraction of naturally occurring fatty acids of milk (lauric acid ($C_{12:0}$), linoleic acid ($C_{18:2}$) and linolenic acid ($C_{18:3}$)), was found to have an inhibitory effect on *L. monocytogenes* (Petrone et al., 1998; Wang and Johnson, 1992).

Table 2: Studies on the behavior of *L. monocytogenes* in butter

Type of product	Studied parameter (s)	Production and storage conditions	Main conclusions	References
Butter, yellow fat spread and margarine [a]	Temperature and physical abuse	- Surface inoculation with *Salmonella spp., E. Coli* and *L. monocytogenes.* - Storage at 37 °C for 1h under high relative humidity (85%) before storing at 4.4 °C or 21 °C for up to 21 days.	- No growth in the products with low pH and containing preservatives. - Fastest growth of *L. monocytogenes* in the product stored at 21 °C.	(Holliday et al., 2003)
Light butter	Storage temperature	- Inoculation with *L. monocytogenes* and *Yersinia enterocolotica.* - Storage at 4 °C or 20 °C over 32 days.	- Growth of *L. monocytogenes* at 4 °C.	(Lanciotti et al., 1992)
Milk Fat Products (MFP)[b] and churned butter	Water droplet size (WDS)	- Surface inoculation of the MFP with *L. monocytogenes.* - Inoculation of the pasteurized cream churned to make butter with *Listeria innocua* [c]. - Storage at 8 °C for up to 42 days.	- Water droplet size, pH and preservatives are key elements to prevent growth of *L. monocytogenes.*	(Michelon et al., 2016)
Butter	WDS, salt level and storage temperature	- Inoculation of the cream churned to make butter with different WDS with *L. monocytogenes.* - Inoculation into the butter using the "mix" method for salt and temperature experiment.	- *L. monocytogenes* was able to grow easily in coarse butter (large droplet size). - Salt had an adverse effect on the growth of *L. monocytogenes.*	(Voysey et al., 2009)

	- Storage at 8 °C for WDS and salt level experiments. - Storage at 8 °C or 21 °C for temperature experiment.	- Growth of *L. monocytogenes* in butter stored at 8 °C was comparable to that at 21 °C.

[a] Fat spreads are water-in-oil emulsions derived from vegetable and/or animal fats.(Council Regulation (EC), 1994)

[b] Milk fat products are water-in-oil emulsions obtained from milk, with fat contents less than 80 g/100g.(Council Regulation (EC), 1994)

[c] *Listeria innocua* is apathogenic *Listeria* species used as a surrogate for *L. monocytogenes* given their genetic similarit

9. Conclusion

The available information on the occurrence and the level of contamination of *L. monocytogenes* in butter is limited. Reviewing the published studies, butters made from raw milk were the most contaminated by the pathogen, which could be due to the absence of thermal treatment during processing stages and/or due to an environmental contamination. Whether it is via the cattle, the equipment or the barn environment, the risk of contamination with *L. monocytogenes* in dairy farms is high. Processing environments also present a potential source of contamination with the pathogen. Good hygiene conditions are therefore needed to prevent the transmission of the pathogen to the food.

Studies have shown that the growth potential of *L. monocytogenes* in butter is relatively low. Various factors can influence the behavior of the pathogen in butter. pH and water droplets size and distribution are, among other parameters, the most important, as they are more susceptible to slow down the growth of the pathogen.

This review revealed that most of the studies focused on a specific parameter or evaluated products with a particular formulation. Therefore, to accurately assess if butter should be considered "at risk" regarding *L. monocytogenes*, it would be of interest to study a panel of butters with different characteristics. It would also be of interest to work in conditions that reflect the reality.

II

Objectives

1. Objective

The general objective of this work was to study the behavior of *L. monocytogenes* in raw milk butter and assess the possibility of considering butter as a product not allowing the growth of *L. monocytogenes* during shelf-life according to the EC 2073/2005.

To expound the above main objective, the specific aims of this thesis were as follows:

- Review the production process of raw milk butter in Wallonia (Belgium).
- Describe the physico-chemical characteristics and bacterial composition of raw milk butter.
- Determine the risk factors of raw milk butter contamination with *L. monocytogenes*.
- Assess the growth and survival of *L. monocytogenes* during raw milk butter production and storage.
- Determine the factors affecting the behavior of *L. monocytogenes* in raw milk butter.
- Develop a simulation model for the growth of *L. monocytogenes* in raw milk butter.

To attain these objectives, four experimental studies were conducted. Figure 2 shows the technology roadmap of this research.

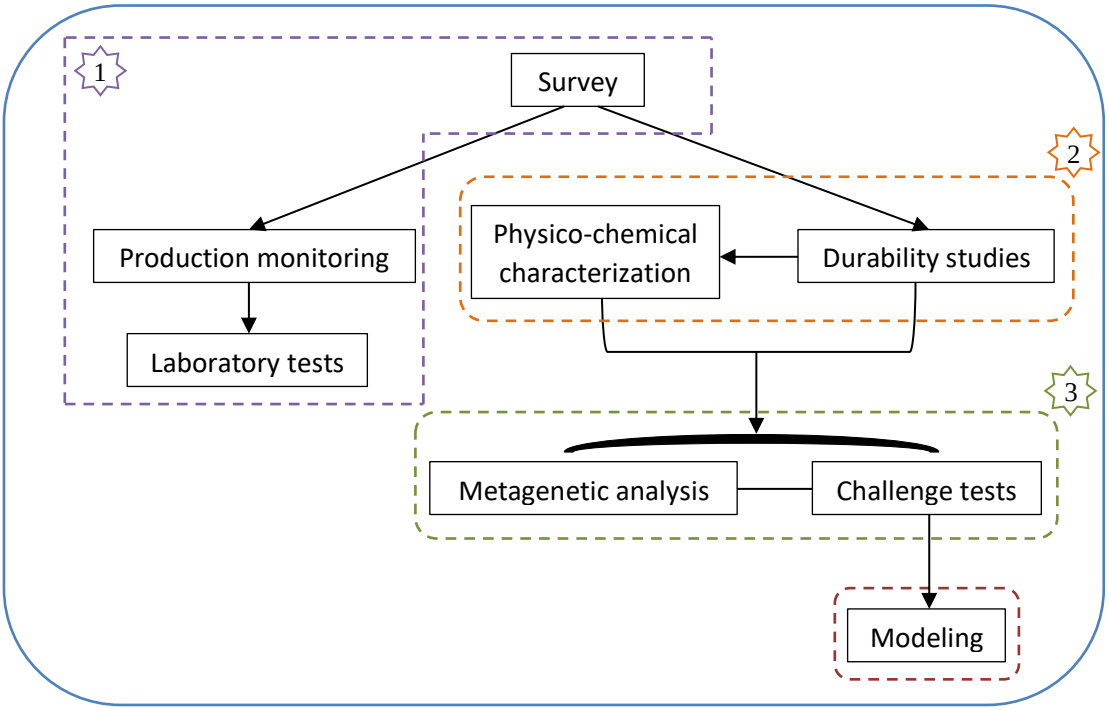

Figure 2: Technology roadmap of this thesis
The numbers refer to the experimental studies

2. Thesis outline

The rest of this thesis is a compilation of three published papers presented in the following chapters. Chapter three deals with raw milk butter along the process of fabrication and describes the procedures and practices used in local production (experimental study 1). Chapter four presents the findings from durability studies conducted to assess growth and survival of *L. monocytogenes* in raw milk butter during shelf life (experimental study 2). Chapter five addresses the relation between bacterial content of raw milk butter and *L. monocytogenes*, depending on cream maturation temperature (experimental study 3). A general discussion and a conclusion are presented in chapter six.

III

Overview of the local production process of raw milk butter in Wallonia

Drafted from:

El-Hajjaji, S., Gérard, A., De Laubier, J., Di Tanna, S., Lainé, A., Patz, V., Sindic, M., 2019. Overview of the local production process of raw milk butter in Wallonia (Belgium). Int. J. Dairy Technol. https://doi.org/10.1111/1471-0307.12608

El-Hajjaji, S., De Laubier, J., Di Tanna, S., Lainé, A., Patz, V., Sindic, M., 2020. Effect of maturation temperature and starter cultures on the rate of cream acidification. 25th National Symposium for Applied Biological Sciences (NSABS), Gembloux, Belgium

This chapter presents the first experimental study which is dedicated to the description of butter production process in Wallonia (Belgium). A general diagram was first generated through a survey that covered the steps related to dairy farming and those related to manufacturing process. Then, field monitoring and laboratory tests were carried out to determine the key steps in the manufacturing process.

Abstract

This study aims to describe the procedures and practices used in local production of raw milk butter. The demand for local products is increasing; hence there is a need to describe the practices used in the artisanal production of raw milk butter. Therefore, a survey of 147 raw milk butter producers was carried out. The results from the survey indicate that there is not one single way to produce butter at artisanal level. In terms of maturation, six temperature sequences were distinguished. The rate of cream acidification varied depending on time and temperature conditions applied for cream maturation.

Key words: Local production, butter, raw milk, survey, acidification

1. Introduction

Butter is considered one of the first dairy products in history (Boulongne, 2015). According to the codex standard for butter (FAO/WHO, 1971), it is "a fatty product derived exclusively from milk and/or products obtained from milk, principally in the form of an emulsion of the type water-in-oil " that contains a minimum milk fat content of 80 g/100 g. To produce one kilogram of butter, 22 litres of milk are needed (Boulongne, 2015).

For centuries, butter was manufactured at a small scale. After standing raw milk overnight in bowls, cream was separated from raw milk and then churned in wooden churns. However, in the nineteenth century, mechanization and scale-up of the butter-making process were introduced. Batch churning was replaced in dairies by continuous churning processes, and pasteurization and starter cultures were introduced (Budhkar et al., 2014; Deosarkar et al., 2016).

In recent years, consumers have lost confidence in industrial food products. Consequently, the need to return to local products and local trade has increased (SPW, 2017a). In France, where people are fond of local products, consumption of butter in some areas like Bretagne, Poitou-Charentes or Rhône-Alpes is an integral part of the diet (Raiffaud, 2017).

Belgium also has a rich tradition of regional products. In Wallonia, 14.1% of the farms are specialized in milk production, which represents 23.5% of the value of Walloon agricultural and horticultural production (SPW, 2017b).

The amount of milk processed by the dairy industry in Belgium has increased strongly since 2006, which has led to an increase in the production of dairy products such as milk powder, cheese and butter (CBL, 2016).

Compared to industrially produced butter, there is a lack of data regarding artisanal butter production. The objective of this work was to describe the practices used in the local production of raw milk butter.

2. Materials and methods

A survey was carried out in Wallonia (Belgium), from October to December 2016, in order to generate an accurate picture of the production of raw milk butter in this region. The survey was established to consider the characteristics related to dairy farming and those related to the manufacturing process. The first ones consisted of information about the production system such as the number and breed of cows in production, and the milking equipment used. The dairy farming part also included questions related to stalling and feeding. The manufacturing process part dealt with the data for each step, from skimming to packaging. Information like the temperature, time and place of maturation and the equipment used for churning and moulding were collected in this section.

Walloon dairy farmers producing raw milk butter were listed by gathering data from the Walloon Agency for the promotion of quality in agriculture (APAQ-W) available online, and from the Laboratory of Quality and Safety of Agro-food Products (Gembloux Agro-Bio Tech) through the 'DiversiFerm' project. A total of 211 raw milk butter producers, still in activity, were identified. In fact, 67 of the listed producers stopped butter production.

Following the survey, butter production was monitored in 20 farms from April to September 2018. These were chosen in a way to compare the different methods for cream maturation identified. Cream maturation was monitored using FC2022 HALO probe (HANNA instruments, Woonsocket, USA) which allows simultaneous recording of temperature and pH. The probe was plunged in cream, directly after skimming. It was programmed to take a measurement every 30 minutes until day of churning.

In addition to that, four comparative tests of maturation were conducted in laboratory to work in more controlled conditions. The combinations were made considering storage temperature and addition of starter cultures as main factors. For each test, the same cream was used. All the creams came from the same producer. Cream pH and temperature measurements were recorded every 30 minutes for 5 days. Lactic acid bacteria (LAB) counts were also determined before and after maturation according to ISO 15214.

3. Results

Of 211 producers approached, 147 responded to the survey, representing a participation rate of 70%.

3.1. Breeding and milk production

In Wallonia, two breeding methods are found: rearing either one or several breeds, representing 70% and 30% of producers, respectively. Holstein is the breed raised

most commonly, among others, namely Belgian blue, Jersey, Montbeliard, and Normande (48%, 9%, 3%, 3% and 1% of producers, respectively).

As for the size of the herd in production, 69% of producers have fewer than 60 cows with 31% raising fewer than 40 cows (Table 3). Depending on this factor, the weekly production of butter strongly varies from one group to another. The average quantity of butter produced for the small herd size group (fewer than 20 cows) is 19 kg per day of manufacture, against 67 kg for the large herd size group (more than 60 cows in production).

Table 3: Survey data regarding breeding and milk production

Parameter	Modality	Number of producers (n = 147)	Proportion (%)
Number of cows in production	fewer than 20	11	7.5
	20-40	34	23.1
	40-60	56	38.1
	more than 60	41	27.9
	NA	5	3.4
Milking equipment	pipeline	31	21.1
	bucket milker	8	5.4
	robot	6	4.1
	milking parlour	97	66.0
	NA	5	3.4
Stalling system	loose	100	68.0
	tied up	28	19.1
	cubicles	10	6.8
	NA	9	6.1
Stalling area	fully straw	26	17.7
	partly straw	88	59.9
	duckboard	28	19.0
	NA	5	3.4

The essential ingredient to make butter is cream, which is obtained from milk. The majority of the producers (91%) milk twice a day. As for the milking equipment used, parlour and pipeline were well represented (65% and 21%, respectively).

Regarding litter and feed, 77% of the producers have stalls with straw, and the majority (90%) gives silage to the cattle when they are inside. Furthermore, nearly all the producers take the animals out to pasture during the summer season. More than half of the respondents (57%) give a supplement with silage foods to the cows at least at some point in this season.

3.2. Butter production process

The butter production at farms goes through well-defined steps. Separated from the milk by skimming, the cream, cultured or not, is maintained at a certain temperature and then churned. The butter grains obtained are then washed and kneaded. Finally, butter is moulded and packaged. Figure 3 summarizes the butter-making process.

3.2.1. Skimming

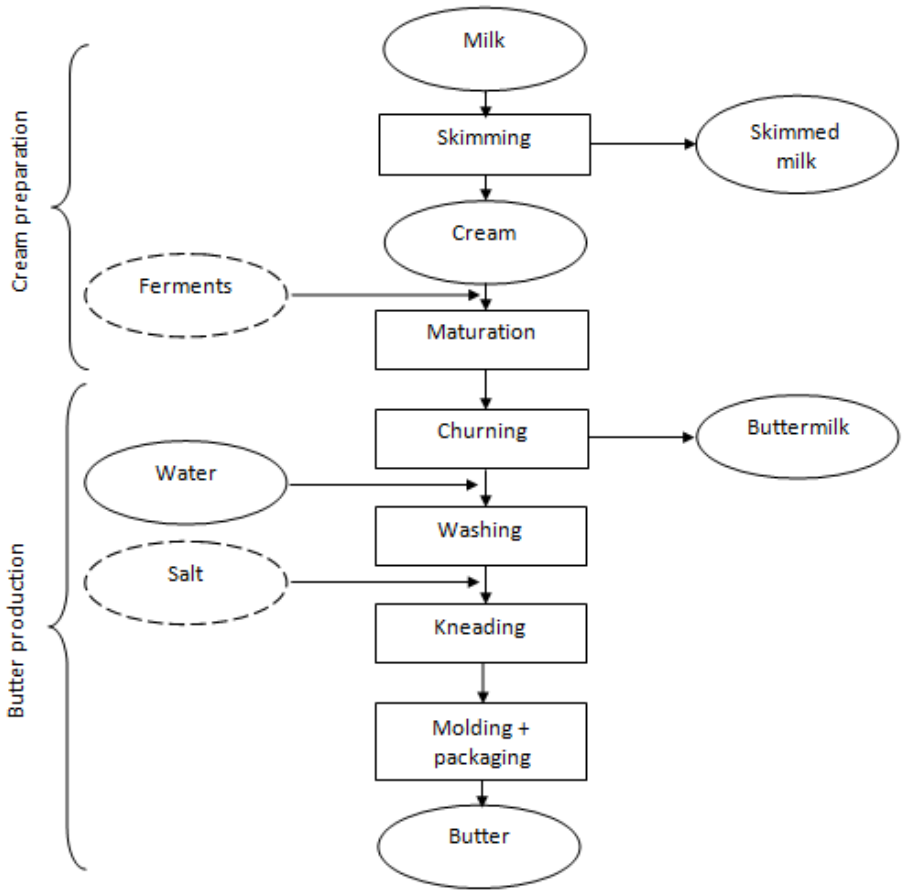

Figure 3: Raw milk butter production process

The first step in making butter is skimming. For 93% of producers, this is an operation that takes place during milking.

According to the survey, half of the respondents skim more than four times per week. By doing so, creams of different ages are used for the butter production. The age of the cream varies depending on the time between skimming and production. Figures 4 and 5 represent the number of producers and the extent to which they use old and fresh creams, respectively. A wide variation in age is noticed in the oldest creams compared to the youngest ones that are used in making butter. For 76% of the respondents, the youngest creams are matured for a maximum of 48 hours.

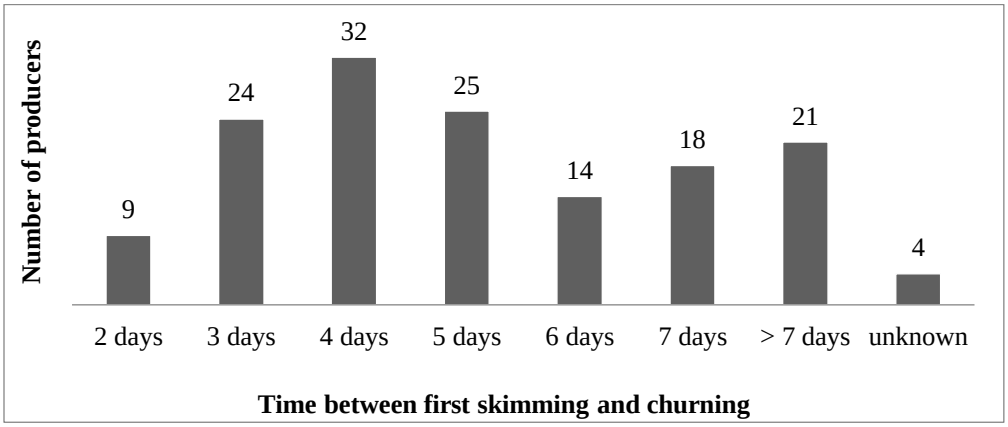

Figure 4: Number of producers by time between first skimming and butter production

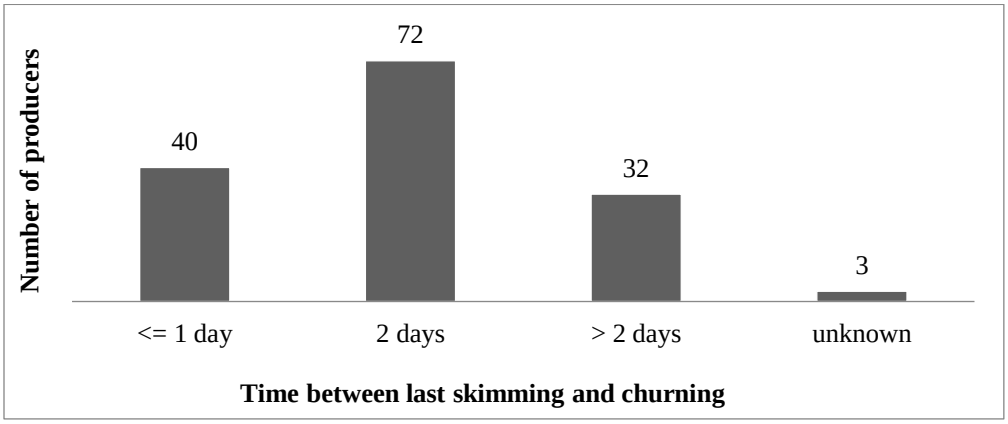

Figure 5: Number of producers by time between last skimming and butter production

After skimming, starter cultures may be added. In Wallonia, a production without the addition of ferments for cream maturation is most commonly adopted (68%). For those who use ferments, only 1% use a natural ferment (matured cream), while others use mesophilic starter cultures.

It was also observed that pH decreased faster in creams with starters compared with naturally matured creams. However, the results showed that even the cream without ferments can reach a low pH (<= 4.8) if it is matured for at least 48 hours at a favorable temperature (>= 14 °C) (Figure 6b).

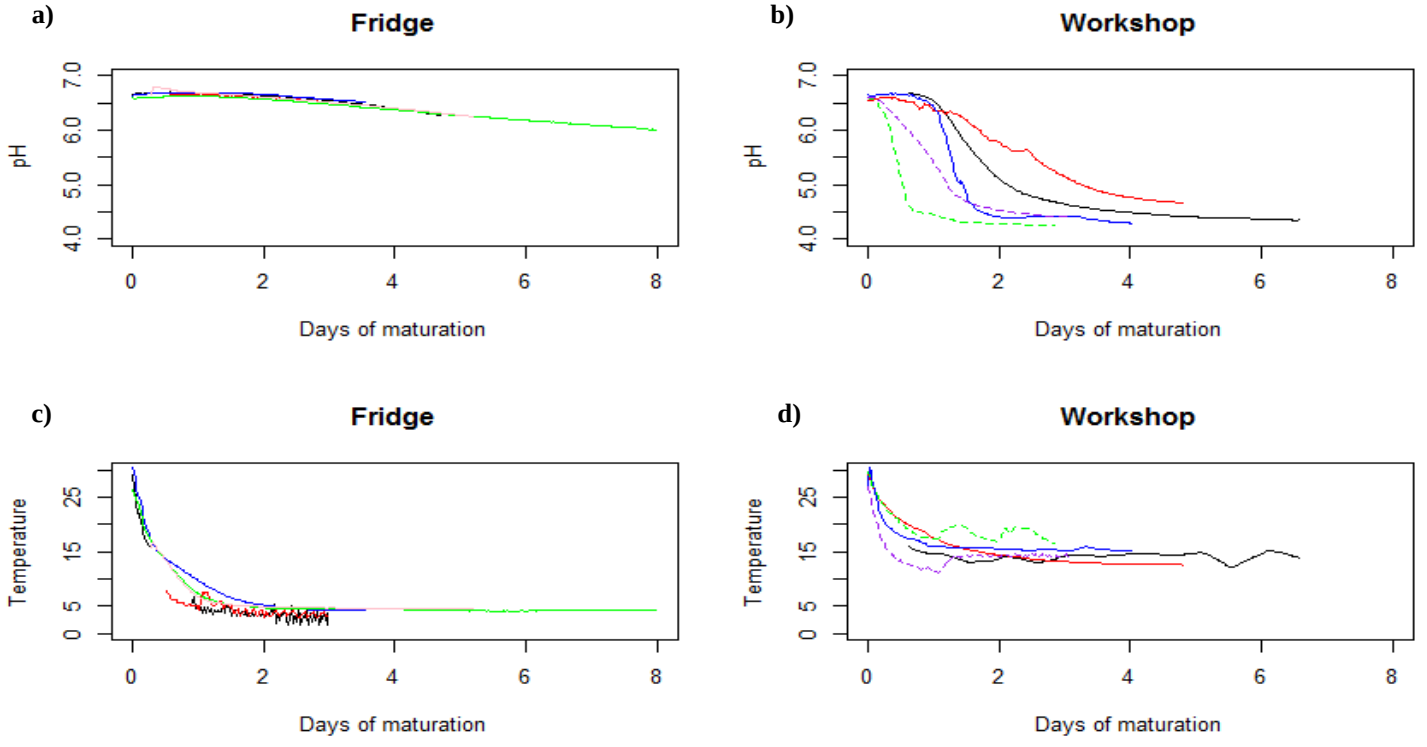

Figure 6: Evolution of cream pH (a and b) and temperature (c and d), in farms, in function of storage place. Each color represents a different cream (continuous line: cream without starters, dotted line: cream with starters)

3.2.2.Maturation

The conditions of cream maturation can be very variable between producers. Taking the time, place and temperature of cream storage into account, six storage sequences were distinguished (workshop, workshop-fridge, workshop-fridge-workshop, fridge, fridge-workshop and fridge-workshop-fridge). The distribution of the producers according to these sequences is shown in Table 4.

Table 4: Number of producers by sequence of maturation

Sequence of maturation	Number of producers (n = 147)	Proportion (%)
workshop	41	27.9
workshop-fridge	37	25.2
workshop-fridge-workshop	2	1.4
fridge	38	25.8
fridge-workshop	20	13.6
fridge-workshop-fridge	3	2.0
unknown	6	4.1

The fridge and workshop terms were chosen depending on the variation of temperature. The term fridge is used for a temperature that varies between 2 and 7 °C, whereas the production workshop term is used for a temperature between 8 to 23 °C. For a two-place sequence such as workshop-fridge, the storage time in the second place represents 75% of the total time, and for a three-phase sequence, storage time partition is ¼, ¼ and ½, respectively.

As shown in Figure 6 (a and b), cream pH evolution in farms was different when maturation was performed under refrigerated temperatures compared to maturation at room temperature. Regardless of the length of maturation, acidification of creams in the fridge was very slow. Indeed, the mean pH value of creams only decreased from 6.65 ± 0.04 to 6.27 ± 0.24. The mean value of creams temperature at the end of maturation was 3.9 ± 0.5 °C. For creams at room temperature, pH suddenly dropped after a lag phase. The mean pH values recorded before and after maturation were of 6.62 ± 0.05 and of 4.39 ± 0.16, respectively. The average value of matured creams temperature was 14.5 ± 1.5 °C. The same findings were observed in the tests conducted in the laboratory (Table 5). In test 4, for example, where creams without

starters were studied, the pH value of the refrigerated cream was higher than the pH of the cream stored at 14 °C.

3.2.3. Churning

To turn the oil-in-water emulsion of the cream into a water-in-oil emulsion, the churning stage is fundamental in the artisanal butter process. While 34% of producers use a stainless-steel churn, a wooden churn is still present in 66% of the farms.

Regarding the cream volume in the churn, 87% of the producers fill the churn more than half full, of which 41% fill it to more than three quarters of the total volume.

3.2.4. Washing, kneading, and moulding

After churning comes washing. This step involves eliminating buttermilk residues by adding water to the churn. The majority of producers (84%) use tap water, the remainder use well water.

The time spent on washing butter varies by producer; however, more than half of the producers (52%) do three washes and, generally speaking, 95% of the producers do a maximum of four washes. Over two thirds (69%) of the producers use, each time, a volume of water lower than the volume of cream initially present in the churn, and 6% add as much water as cream.

To make the butter more homogeneous and to have good water dispersion, 93% of the producers proceed to kneading. Of these, more than two thirds (69%) do a quick kneading in less than 5 minutes.

The methods of moulding the butter and the material used to do it vary from one producer to another. The most commonly used are wooden moulds, pallets or hand moulding (37%, 24% and 26%, respectively).

Once produced and moulded, the butter is packaged with butter paper and then refrigerated.

Table 5: Results of pH and LAB for the 4 tests conducted in the laboratory

	Test 1			**Test 2**			**Test 3**			**Test 4**		
	Before maturation	After maturation		Before maturation	After maturation		Before maturation	After maturation		Before maturation	After maturation	
		Fridge-NSC	Fridge-SC		Workshop-NSC	Workshop-SC		Fridge-SC	Workshop-SC		Fridge-NSC	Workshop-NSC
pH	6.61	6.32	5.82	6.57	4.38	4.31	6.57	5.64	4.37	6.60	6.03	5.35
LAB (log cfu/g)	5.49± 0.21	5.98±0.33	6.48± 0.00	4.93± 0.09	7.48 ± 0.00	7.48± 0.00	5.87± 0.22	6.48± 0.00	7.48± 0.00	3.85± 0.03	5.94± 0.05	7.48 ± 0.00

SC: With starter cultures
NSC: Without starter cultures

4. Discussion

4.1. Breeding and milk production

The findings from this study indicate that Holstein is the most prevalent breed in Walloon dairy farms, while a smaller proportion of producers raise Jersey or Normande cattle. It is undeniable that production capacity and milk fat composition are different from one breed to another (Lindet, 1907). Jersey and Normande are among the breeds most suitable for butter production, as they give fatter milk (Boulongne, 2015; Lindet, 1907). The composition of milk fat varies also according to season and lactation stage (Chen et al., 2014).

Feed is also an important factor regarding milk composition. The procedure adopted at this stage appears to be common among all producers, especially for taking the cattle to pasture. Meadows constitute 42% of the agriculture area used in Belgium (STATBEL, 2017). Grass is known to be the most suitable food for milk cows. The milk produced is rich in fat and proteins (O'Callaghan et al., 2016).

The use of grazing lands is however, limited to a certain period. To have good productivity, cows must be abundantly nourished at all times. The use of other feeds is therefore required, which explains why producers give silage as complement. Maize silage is the feed used most commonly on dairy farms in Belgium (Haesaert et al., 2002). Fodder corn amounts to 62% of forage crops (STATBEL, 2017). Concentrated feeds, like cereals, protein crops and soybean meal, are another important complement given by producers. They are characterized by high levels of dry matter and energy, and so are used to balance nitrogen and energy levels in the basic ration and to support dairy production (Cuvelier and Dufrasne, 2015).

4.2. Cream preparation

Once the milk is obtained, it is advisable to skim it very quickly after milking in order to keep a temperature favourable for skimming (Dunand, 2010). According to the survey, producers abide by this advice since the skimming part seems to take place during milking. The use of cream separator has made getting the cream from milk much easier and faster (Pouriau, 1895). It should be noted that some countries of north and east Africa still adopt the old method of making butter from naturally fermented sour milk. Raw milk is left overnight at ambient temperature to coagulate. The fermented milk is then churned in a skin bag or a clay pot (Ahmed et al., 2016; Alganesh and Yetenayet, 2017; Idoui et al., 2010; Samet-Bali et al., 2009).

In industrial butter production, as milk collected from dairies is transported under refrigeration to the processing plant, it is heated to 65 °C before skimming to bring it to a favourable temperature (Robinson, 2005). However, in accordance with the Ministerial Order of 1 February 2007 with regard to the methods of quality control of raw cow milk (Anonymous, 2007), milk is no longer considered raw when it is heated to more than 40 °C.

More and less ripened creams were found to be used in the making of raw milk butter, with a minimum of less than 24 hours of ripening. Boulongne (2015) mentioned in her book that the cream must be left for about 2 to 3 days to become acidic enough for churning. If the cream has not reached a relatively high acidity, and so does not contain enough solid fat, churning becomes difficult (Chandan et al., 2015; Walstra et al., 2006). The introduction of ferments decreases the maturing time required (FAO, 1998). This approach appears to be adopted less in Wallonia as a matter of tradition. In France, adding starter cultures is a key step in the butter production process (Raiffaud, 2017). These are composed of selected bacterial strains with the proper physiological and metabolic features (Leroy and De Vuyst, 2004; Wouters et al., 2002).

In the industrial process, the cream undergoes other operations like standardization, pasteurization and deodorization before being cultured (Boutonnier, 2007; Deosarkar et al., 2016; Lapointe-Vignola, 2002).

Wide variation was observed in terms of cream maturation, for which six sequences were distinguished. For more than half of the producers, cream is preserved at low temperature (2 to 7 °C) for at least 75% of maturation time. The temperature sequence of the physical maturation influences both the amount of fat solidified by crystallization and the degree of fat globule agglomeration, and so affects the butter consistency and flavour (Boutonnier, 2007; Walstra et al., 2006). Cream storage temperature also impacts pH evolution. Butter made with cream matured at low temperature is firmer (Dunand, 2010; Lindet, 1907) and less acidic. In fact, the decrease in pH is associated with the growth of LAB (Ewe and Loo, 2016). During cream fermentation, LAB convert lactose into lactic acid, resulting in a lower pH (Siezen and Bachmann, 2008). As shown in Table 5, LAB counts after maturation was higher in creams matured at 14 °C (Workshop) compared to those matured at 4 °C (Fridge). The temperature of maturation impacts the growth of LAB and thus the rate of acidification. Silva et al., (2018) found that the maximum growth rate of LAB increased with incubation temperature. The growth rate at 4 °C was the lowest. The same result was reported by Rosso et al., (1995) who observed no growth below 5 °C. Indeed, LAB used in dairy fermentation include mesophilic species with a minimum growth temperature ranging from 5 to 10 °C, and an optimum at 30 °C (Anonymous, 2003; Wouters et al., 2002). Therefore, the most advantageous temperature for the maturation of the cream varies between 14 and 19 °C (Lindet, 1907). Adjustment of cream cooling and ageing conditions is however, possible depending on season and fat composition (Chandan et al., 2015).

4.3. Butter production

The cream and churn temperature at churning are also important. If too low, the agglomeration is done badly and slowly; if too high, it may cause excessive fat loss in buttermilk (Chirade et al., 2000; FAO, 1998). Generally, the churn temperature is similar to that of the cream after maturation. In Wallonia, it varies between 8 and 14 °C, which is the recommended temperature to maintain (FAO 1998).

Other factors affect both the rate and efficiency of the churning process, such as filling level and churning time (Walstra et al., 2006). For the first one, it appears from this study that the churns of most of the producers are quite full. It is recommended that the cream does not reach half of the inner capacity of the churn, as the cream will be stirred instead of being churned and so the churning will last longer (Dunand, 2010; FAO, 1998). However, Dunand (2010) specified that this factor is less important when a churn with a rotating propeller is used. For the second one, the butter grains have to be large enough so that their firmness will not be, to some extent, affected by washing (Walstra et al., 2006).

Water used in washing must be of excellent microbiological and chemical quality and at the same temperature as butter (FAO, 1998). In this study, tap water is used by most producers. It was also found that the average number of washings is three. The FAO (1998) reported that one or two washes are sufficient, if the cream used is of good quality.

It appears from this study that producers perform a quick kneading. In literature, slightly over-working butter is preferred to under-working, and increased speed preferred to working at slow speed (Walstra et al., 2006). That allows having smaller moisture droplets, which is important for the keeping quality of butter as it limits the growth of microorganisms (Michelon et al., 2016).

Microbial growth can also be avoided or delayed in the presence of salt. Majority of the producers (91.8%) make salted butter in addition to unsated butter mainly for the flavour. Beside imparting a salty taste in a product and enhancing the flavour of other ingredients, salt acts as a preservative by reducing the water activity of the substrate (Elias et al., 2020; Man, 2007; Ravishankar and Juneja, 2014). In a study conducted by Tola et al. (2018) to evaluate traditional butter preservation techniques, salted butter presented the least deteriorated microbial and organoleptic properties after a prolonged storage time.

5. Conclusion

Information on traditional handling and processing practices for obtaining butter from cows' milk are limited. This study highlighted the traditional knowledge transmitted over generations. Walloon butter is a typical indigenous product, less standardized than industrial butter. It is made from raw milk without any heat treatment and, unlike in France, without much use of starter cultures.

Butter production at farms comes down to well-defined steps. However, each step presents a lot of variation, which may lead to butters with various physico-chemical characteristics, mainly in terms of pH, water activity and water distribution compared to industrial butters. It would be interesting to check this aspect, especially because these parameters could affect the development of microorganisms such as *Listeria monocytogenes*.

In the phase of maturation, six temperature sequences were distinguished. The thermal conditions to which the cream is subjected affect its rate of acidification. Maturation at refrigeration temperature does not lead to a significant pH decrease.

By its different characteristics, raw milk butter meets the needs of a wide range of consumers. It is therefore important to maintain the knowledge about butter artisanal production.

IV

Assessment of growth and survival of *Listeria monocytogenes* in raw milk butter by durability tests

Drafted from:

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In the previous chapter, it was found that raw milk butter production in Wallonia presents a big variability, especially in terms of maturation. It was also found that the latter is key step that affects the rate of cream acidification and thus pH butter. It was also found from the literature (chapter I) that the growth of *L. monocytogenes* in butter is affected by its characteristics including pH.

To cover this variability, durability studies were conducted in the second experimental study to assess the growth and survival of *L. monocytogenes* in raw milk butter at the end of storage. All the samples were naturally contaminated and presented various characteristics reflecting thus the reality as presented in this chapter. This chapter is also dedicated to the description of raw milk butter physico-chemical characteristics. Samples were collected from Walloon market and analyzed for pH, a_w and water distribution. Even thought data of a_w has been included in this study, the measurement of this parameter in butter is not relevant according to the standard ISO 18787:2017 about the determination of water activity in foodstuffs.

Abstract

Butter is a complex matrix characterized by a high fat content. Existing publications on the behavior of *Listeria monocytogenes* in this type of food reported contrasted results. This study was performed to provide further information and data about raw milk butter's ability to support survival or growth of *L. monocytogenes.* Durability tests were performed on naturally contaminated samples of raw milk butter with various physico-chemical characteristics. At the end of shelf life, no growth of *L. monocytogenes* was observed in the studied butters, regardless of their physico-chemical characteristics (pH, a_w, water dispersion index and salt concentration) and the initial level of contamination. The number of positive samples and the colony counts of *L. monocytogenes* were even decreased at the end of the storage period.

Key words: pathogen, dairy product, storage, growth potential, intrinsic factors

1. Introduction

During the period 2008-2016, the European Union knew an increase of confirmed cases of listeriosis, which was reported as the most severe zoonosis (European Food Safety Authority and European Centre for Disease Prevention and Control, 2017). Listeriosis is a foodborne infection characterized by gastroenteritis, meningitis, septicemia, abortion and sometimes death. Its lethality rate is over 25% (Buchanan et al., 2017; Jordan et al., 2016). High risk populations, i.e. pregnant women, newborn, immunocompromised individuals and the elderly in particular, are the most susceptible to listeriosis (Gillespie et al., 2010; Goulet et al., 2008; McLauchlin et al., 2004). *Listeria monocytogenes* is the causative agent of this infection. It has the ability to grow in a wide range of temperature (-1.5 °C to 45 °C) with an optimum between 30 °C and 37 °C, and at pH levels between 4.4 and 9.6 (Buchanan et al., 2004; Magalhães et al., 2014). It can also survive in high salt concentrations (up to 10% of NaCl) (Cole et al., 1990; Liu et al., 2005).

The European Commission regulation (EC) N° 2073/2005 on microbiological criteria for foodstuffs has established safety criteria for ready-to-eat (RTE) foods other than those intended for infants and for special medical purposes, as regards *L. monocytogenes*, depending on their characteristics (pH and water activity), the possible growth of *L. monocytogenes* and the stage where the criterion applies. If growth is not possible, the regulation imposes a number of counts ≤ 100 cfu/g in the five units comprising the sample (n=5). This criterion is also applied for products with pH ≤ 4.4 or $a_w \leq 0.92$, products with pH ≤ 5.0 and $a_w \leq 0.94$, and products with a shelf life of less than five days. It can also be applied to other products subject to scientific justification. Otherwise, the regulation imposes a no detetction of this pathogen in 25g (n=5) before the food has left the immediate control of the producer, unless the latter is able to demonstrate that his product will not exceed the limit 100 cfu/g throughout the shelf-life (Commission Regulation, 2019, 2005).

In a number of studies, it is reported that *L. monocytogenes* can be present in butter (made of raw or pasteurized milk), and that listeriosis outbreaks have been caused by contaminated butter in USA (Ryser and Marth, 1999), Finland (Lyytikäinen et al., 2000) and England (Advisory Committee on the Microbiological Safety of Food, 2003). Based on these previous findings, butter can be considered as RTE food potentially allowing growth of this pathogen. These records have led researchers to take interest in the behavior of *L. monocytogenes* in butter. Existing publications are not sufficient to determine butter's ability to support survival or growth of *L. monocytogenes.*

The purpose of this study was to assess growth and survival of *L. monocytogenes* in raw milk butter during shelf life.

2. Materials and methods

This study was conducted in two parts. In the first one, durability tests were performed on naturally contaminated samples of butter. Both physico-chemical and microbiological characteristics were determined. In the second part, samples of raw milk butter were collected from the Walloon market and were analyzed for physico-chemical characteristics only.

2.1. Durability tests

2.1.1. Samples

Twenty different batches of raw milk butter, with no preservatives, naturally contaminated with *L. monocytogenes* were collected from 20 different farms in Wallonia. A certain procedure was to be followed: (a) detection of *L. monocytogenes* following a request for analysis by the producer or the authority, (b) requesting a permission to take the contaminated batches once being informed, and (c) contacting the laboratory. This whole procedure took at least one week. Only batches that were no more than 14 days old were considered, in order to have a significant evolution of *L. monocytogenes* over time.

The samples were sent refrigerated (max 7 °C) to the food laboratory of CdL (Comité de Lait, Battice, Belgium) for durability test.

2.1.2.Storage conditions

Depending on the age of the butter upon arrival at the laboratory, different preservation conditions were applied. If the samples were more than seven days old, they were stored at 12 °C until the end of shelf life. Otherwise, they were kept at 7 °C until the seventh day after the production, and then stored at 12 °C to simulate a break in the cold chain. These storage temperatures and periods were chosen to reflect the foreseeable conditions of distribution and storage as advised by the "EURL-Lm technical guidance document for conducting shelf-life studies on *L. monocytogenes* in ready-to-eat foods" (Beaufort et al., 2014). A storage period of 30 days, from the moment of manufacture, was chosen for the samples of raw milk butter in order to cover most of those encountered in the market.

2.1.3.Microbiological and physico chemical analyses

For each batch of raw milk butter, physico-chemical (pH, water activity, NaCl content based on sodium determination in serum phase and water distribution) and microbiological characteristics (*L. monocytogenes* (detection and enumeration), *Escherichia coli*, coagulase positive *Staphylococci*, *Pseudomonas* spp., total aerobic flora, yeasts and molds) were determined at the reception of the samples ("day 0") and at the end of the shelf life (30 days after the day of manufacture: "day 30"). A batch of butter consists of several subunits on which the repetitions of the analyses are carried out. For *L. monocytogenes*, 30 samples are analyzed at "day 0" and 30 at "day 30". All analyses were performed according to standard methods. Table 6 summarizes the parameters analyzed with the number of repetition and the method applied for each parameter.

2.1.4.Statistical analysis

Confidence interval: The estimated proportion of units exceeding 100 cfu/g and the confidence interval associated were determined using a Bayesian calculator. The calculation was based on the central confidence interval.

Growth potential: it is an estimation of the difference between the median of count results at the end of shelf-life in log cfu/g and the median of results at the beginning. Before the log transformation, some conditions were applied to the raw quantitative data relative to *L. monocytogenes*. An enumeration value of 9 cfu/g was fixed in case of < 10 cfu/g (the limit of enumeration of the method). On the other hand, a value of 0.04 cfu/g (1 cfu/25g) was used if an absence of *L. monocytogenes* was found in 25g.

Statistical analyses were carried out with R software, version 3.3.3. To evaluate the significant differences and mean values, Student test or Wilcoxon test were applied depending on the normality of data. Statistical significance was defined when a p-value was below 0.05.

The relationship between the intrinsic factors at "day 0" and *L. monocytogenes* was estimated using Pearson correlation coefficient.

Table 6: Physico-chemical and microbiological parameters analyzed during each durability study, number of repetition and method applied for each parameter.

Parameter	Number of samples	Day of analysis*	Method
Temperature (°C)	1	"day 0"	/
Water dispersion	1	"day 0"	ISO 7586
pH	5	"day 0" and "day 30"	ISO 7238
Water activity (a_w)	1	"day 0" and "day 30"	ISO 21807
Salt (% of NaCl in mg/100 mg, water phase)	3	"day 0" and "day 30"	ISO 8070 - Sodium determination
L. monocytogenes (presence/absence in 25g)	30	"day 0" and "day 30"	Vidas LMO II
L. monocytogenes (cfu/g)	30	"day 0" and "day 30"	ISO 11290-2
Escherichia coli (cfu/g)	3	"day 0" and "day 30"	ISO 16649-2
Coagulase positive *Staphylococcus* (cfu/g)	3	"day 0" and "day 30"	ISO 6888-2
Pseudomonas spp. (cfu/g)	3	"day 0" and "day 30"	ISO 11059
Total aerobic flora at 22 °C (cfu/g)	3	"day 0" and "day 30"	Tempo AC
Yeasts (cfu/g)	3	"day 0" and "day 30"	ISO 6611
Molds (cfu/g)	3	"day 0" and "day 30"	ISO 6611

* "day 0" corresponds to the day of the first analysis after reception of the samples
"day 30" corresponds to the day 30 after production

2.2. Physico-chemical characterization of raw milk butters from the market

In order to ascertain that the intrinsic factors of the samples analyzed by storage are representative of those encountered in the Walloon market, 144 raw milk butters were collected from 61 different farms in Wallonia. The collection was organized over two periods. The first one occurred between December 2017 and January 2018, and the second one occurred between May and June 2018. The samples were transported refrigerated to the laboratory LARECO (LAboratoire de REcherches et de COnseils, Marche-en-Famenne, Belgium) where pH and water activity (a_w) analysis were performed on each sample according to ISO 7238 and ISO 21807 respectively. A water dispersion test (Wator test) was also carried out according to ISO 7586. To determine the number and size of the water droplets, the processing and analysis of the images of indicator paper "wator", scanned beforehand in a resolution of 600 dpi (dots per inch), was carried out with ImageJ 1.51s Freeware (Rueden et al., 2017; Schindelin et al., 2012). When necessary, the droplets contour was defined manually using the "eraser" tool, and the white holes in the black spots were filled with the command "fill holes". The size of the water droplets was expressed by the Feret's diameter which is the distance between two parallel tangents on opposite sides of the profile of a particle (Merkus, 2009). The mean number and the mean size of the droplets for each group were then calculated.

Statistical analyses were carried out with R software, version 3.3.3. To evaluate the significant differences and mean values, Student test (normally distributed data as indicated by Shapiro Wilk test, $p > 0.05$) or Wilcoxon test (non normally distributed data) were applied. Statistical significance was defined when a p-value was below 0.05.

3. Results

3.1. Physico-chemical characterization

3.1.1. Durability tests

Contaminated samples of butter were collected for durability studies. The physico-chemical and microbiological characteristics were both determined.

The analyzed samples presented a wide variation in terms of pH. The pH values obtained at "day 0" ranged from 4.47 to 6.15, with a mean value of 5.12 ± 0.47 (Table 7). However, a significant decrease of pH values was observed at the end of shelf life ("day 30") with a mean value of 4.85 ± 0.41.

Table 7: Physico-chemical characteristics of raw milk butters at "day 0" and "day 30"

Parameter		"day 0"				"day 30"			
		Mean ± SD	**Median**	**Min**	**Max**	**Mean ± SD**	**Median**	**Min**	**Max**
pH		5.12 ± 0.47	5.07	4.47	6.15	4.85 ± 0.41	4.77	4.12	5.65
a_w		0.97 ± 0.02	0.97	0.93	1.00	0.97 ± 0.01	0.97	0.94	0.99
NaCl	Salted	0.72 ± 0.37	0.70	0.19	1.43	0.68 ± 0.34	0.65	0.15	1.23
	Unsalted	0.14 ± 0.29	0.03	0.01	1.05	0.12 ± 0.29	0.03	0.02	0.90

The values of a_w ranged from 0.93 to 1.00 with a mean value 0.97 ± 0.02 at "day 0", and from 0.94 to 0.99 with a mean value 0.97 ± 0.01 at "day 30".

Within the batches of raw milk butter collected, 40 % were salted. The maximum salt content observed was 1.43% (mg/100 mg) of NaCl.

Regarding water dispersion, all the samples were classified high in the grading scale (scale units 1 and 2A), as they presented a lot of relatively large water droplets.

3.1.2.Raw milk butters from the market

Additional raw milk butter samples from all over Wallonia were collected for physico-chemical characterization. The pH of the raw milk butter samples ranged from 4.25 to 6.50 with an average of 5.12 ± 0.61 (Table 8). The values of pH of raw milk butters collected in the first period were not different from those collected in the second period (p-value 0.39). Also, no difference was found between these samples and those from the durability tests (p-value 0.50).

Table 8: pH and a_w of raw milk butters collected from the market during the two periods

Parameter	Period of December and January (n = 75)				Period of May and June (n = 69)			
	Mean ± SD	**Median**	**Min**	**Max**	**Mean ± SD**	**Median**	**Min**	**Max**
pH	5.10 ± 0.63	4.90	4.25	6.50	5.15 ± 0.58	4.95	4.40	6.50
a_w	0.97 ± 0.02	0.98	0.91	0.99	0.98 ± 0.01	0.98	0.93	1.00

For water activity, the values obtained for raw milk butters varied from 0.91 to 1.00 with a mean value of 0.98 ± 0.02.

The water dispersion values of butter samples found using the grading scale presented in the standard are listed in Table 9. More than half of the samples were classified "high" in the grading scale (scale units 1 and 2A). These are characterized by many droplets (about 5 droplets/cm²) with relatively large size (about 2 mm). An example is shown in Figure 7.

Table 9: Results of water dispersion, expressed by scale units, of raw milk butters collected from the market

Scale units	Number of droplets / cm²	Droplets size (cm)	Frequency	Percentage (%)
1	5 ± 3	0.23 ± 0.05	64	44.4
2A	5 ± 2	0.18 ± 0.04	55	38.2
2B	5 ± 2	0.15 ± 0.02	15	10.4
2C	NA	NA	0	0.0
3A	2 ± 1	0.11 ± 0.01	3	2.1
3B	2 ± 2	0.14 ± 0.06	5	3.5
3C	1 ± 0	0.08 ± 0.00	1	0.7
4	NA	NA	0	0.0
5	0.1 ± 0	0.09 ± 0.00	1	0.7

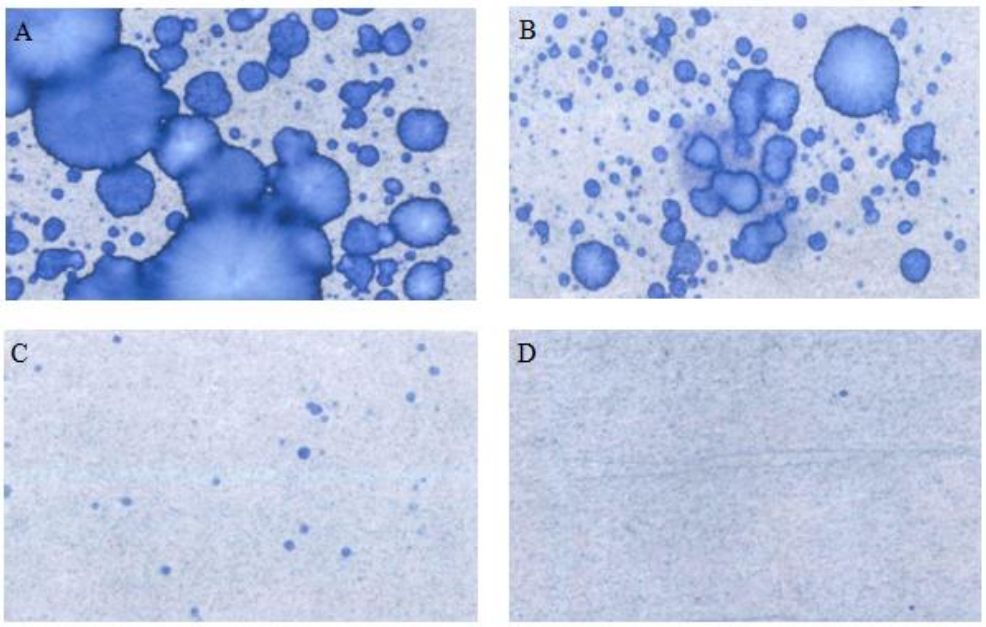

Figure 7: Examples of the spots obtained for water distribution and their classification using the grading scale. A: scale unit 1, B: scale unit 2A, C: scale unit 3B, D: scale unit

3.2. Microbial profile of raw milk butter samples analyzed by durability tests

Beside *L. monocytogenes, Escherichia coli*, coagulase positive *Staphylococcus*, *Pseudomonas* spp., total aerobic flora, yeasts and molds were also analyzed. *E. coli* and *Staphylococcus* are generally used as hygienic indicators to examine food processing, while *Pseudomonas* spp., yeasts and molds are related to food spoilage.

The results of *E. coli* at "day 0" showed that only 19 % of the samples were below 1.0 log cfu/g, while 14 % were between 1.0 and 2.0 log cfu/g and 67 % of the samples exceeded 2.0 log cfu/g. The mean number of colonies detected at "day 0" was 3.0 log cfu/g. Concerning *Staphylococcus*, 44 % of the samples exceeded the threshold limit of enumeration 1.0 log cfu/g. Overall, at the end of the storage period, a decrease in *E. coli* and *Staphylococcus* was observed.

It appears also from the results that butter samples have a relatively high total bacterial count, reaching 7.7 log cfu/g. The data displayed in Table 10 indicate that yeasts and molds counts at "day 0" ranged from 1.0 to 7.5 log cfu/g and from 0.9 to 4.7 log cfu/g respectively. The samples showed a significant increase in yeasts and molds counts at the end of storage period compared to "day 0". The samples had also high counts of *Pseudomonas* spp. that reached 7.6 log cfu/g.

Table 10: Microbial profile of raw milk butter samples at "day 0" and "day 30"

Parameter	**"day 0" (log cfu/g)**				**"day 30" (log cfu/g)**			
	Mean ± SD	**Median**	**Min**	**Max**	**Mean ± SD**	**Median**	**Min**	**Max**
Escherichia coli	3.01 ± 1.33	3.23	0.95	4.70	2.25 ± 1.60	1.15	0.95	5.48
Coagulase positive *Staphylococci*	1.93 ± 1.26	0.95	0.95	4.70	1.35 ± 0.85	0.95	0.95	4.17
Total aerobic flora	7.17 ± 0.63	7.69	5.48	7.69	7.17 ± 0.68	7.34	5.48	8.32
Yeasts	3.27 ± 1.27	3.04	1.00	7.48	4.91 ± 0.80	4.70	2.85	6.48
Molds	2.02 ± 1.04	1.78	0.95	4.70	3.12 ± 1.26	3.26	0.95	5.70
***Pseudomonas* spp.**	5.73 ± 1.46	6.46	2.00	7.61	5.61 ± 1.29	5.98	3.00	7.74

3.3. Behavior of L. monocytogenes in raw milk butter samples analyzed by durability tests

For each batch of butter naturally contaminated with *L. monocytogenes*, 30 samples were analyzed at the beginning and at the end of the storage period. *L. monocytogenes* was detected in 66 % (398 presences) of the samples analyzed at "day 0". Of these, 40 % had a contamination level of less than 1.0 log cfu/g, 16% between 1.0 and 2.0 log cfu/g, and the remaining 10 % had a contamination level beyond the critical limit of 2.0 log cfu/g. The results of the latter samples were not interpreted with the rest, since the objective behind the durability test consisted in verifying that the limit of 100 cfu/g is not exceeded at the end of the storage period. It was found that high level of *L. monocytogenes* is correlated with high pH and a_w values (correlation coefficient of 0.39 and 0.29 respectively). In contrast, salt had an inverse effect on *L. monocytogenes* (correlation coefficient of -0.17), compared to pH and a_w. However, no statistical relationship was found (p value > 0.05).

At the end of the storage period, no growth of *L. monocytogenes* was observed in any of the batches. An estimated growth potential of 0.0 was the highest value obtained. For the batches with a contamination level at the beginning below 2.0 log cfu/g, the estimated proportion of units exceeding this value at the end of shelf life was 0.0 % with a confidence interval at 95 % of [0.0 % - 0.6 %]. A decrease of *L. monocytogenes* was also observed in the samples exceeding 2.0 log cfu/g with a highest estimated growth potential value of -0.3 (Table 11). It was found that growth potential is positively correlated with pH and a_w values (correlation coefficient of 0.41 and 0.16 respectively). In contrast, high salt content implies low growth potential (correlation coefficient of -0.41). However, no statistical relationship was found (p value > 0.05).

Table 11: Results of durability tests realized on raw milk butter about *L. monocytogenes*

ID	Presence in 25g at "day 0" (n=30)	Presence in 25g at "day 30" (n=30)	N >= 100 cfu/g at "day 0"	N >= 100 cfu/g at "day 30"	Enumeration at "day 0" (median in log cfu/g)	Enumeration at "day 30" (median in log cfu/g)	Growth potential (log cfu/g)
EV_01	4	2	0	0	-1.40	-1.40	0.00
EV_02	25	0	0	0	1.00	-1.40	-2.40
EV_03	1	0	0	0	-1.40	-1.40	0.00
EV_04	12	1	0	0	-1.40	-1.40	0.00
EV_05	23	1	0	0	0.95	-1.40	-2.35
EV_06	28	25	0	0	0.95	0.95	0.00
EV_07	21	1	0	0	0.95	-1.40	-2.35
EV_08	24	2	0	0	0.95	-1.40	-2.35
EV_09	22	0	0	0	0.95	-1.40	-2.35
EV_10	19	17	0	0	0.95	0.95	0.00
EV_11	29	30	28	16	2.62	2.00	-0.62
EV_13	12	0	0	0	-1.40	-1.40	0.00
EV_14	23	18	0	0	1.00	0.95	-0.05
EV_15	7	0	0	0	-1.40	-1.40	0.00
EV_16	30	30	30	30	2.60	2.23	-0.37
EV_17	15	1	0	0	-0.22	-1.40	-1.18
EV_18	30	30	2	0	1.60	1.30	-0.30
EV_19	30	23	0	0	0.95	0.95	0.00
EV_20	22	24	0	0	0.95	0.95	0.00
EV_21	21	6	0	0	0.95	-1.40	-2.35

4. Discussion

In this study, the behavior of *L. monocytogenes* was investigated in a range of raw milk butters with various physico-chemical characteristics, in order to determine whether or not this product supports the growth of the pathogen. Durability studies were performed on naturally contaminated samples stored for 30 days at conditions that reflected the reality.

The findings of this study showed that, in most of the contaminated samples, the levels of *L. monocytogenes* in raw milk butter were low (< 10 cfu/g). The same result were reported by Kozak et al. (1996), Lewis et al. (2006) and N'Guessan et al. (2015). It was also found that, not only *L. monocytogenes* did not grow in this product, but it even decreased. Yet the samples showed pH and a_w values favorable for the growth of the pathogen (Tables 12 and 13). Indeed, *L. monocytogenes* has optimal growth rates at $a_w \geq 0.98$ and a pH value between 6.00 and 8.00, while growth stops below a_w of 0.92 and pH of 4.40 (Buchanan et al., 2004; Hitchins and Whiting, 2001). However, the durability test samples had pH and a_w values that ranged from 4.47 to 6.15 and from 0.93 to 1.00 respectively, which were relatively similar to those observed in the market samples (Tables 7 and 8).

The results relative to the growth of *L. monocytogenes* in butter were in accordance with those reported by Michelon et al. (2016) who observed no growth of the pathogen in the tested samples of churned butters and commercial milk fat products (pH < 5.80). The levels of the bacterium remained however stable during shelf life. This may be explained by the fact that the products studied by Michelon et al. (2016) were made from pasteurized cream, which reduced the microbial concentration and so, the nutritional competition. The same reason could explain the increase of *L. monocytogenes* in "sweet cream whipped salted butter" reported by Holliday et al. (2003). The product was made from pasteurized cream with absence of preservatives.

The size and distribution of water droplets was another characteristic to observe regarding bacterial growth. Bullock and Kenney (1969) found that bacterial counts after the storage period were three to four times higher in the low fat dairy spreads with large serum droplets (> 50 microns), compared to the products with small droplets (3 to 20 microns). Studies have also demonstrated that water droplets size and distribution is a key parameter in preventing the growth of *L. monocytogenes* (Michelon et al., 2016; Voysey et al., 2009). Voysey et al. (2009) observed that *L. monocytogenes* grew easily in coarse butter with large water droplets size. In this study, butter samples had in general large water droplets (about 2 mm), which is favorable for the growth of microorganisms. However, no growth was observed in any of the samples. This could be due to the fact that the initial level of *L. monocytogenes* of the contaminated samples was much lower than that used by Voysey et al. (2009).

In this study, the samples showed various microbial profiles in terms of *E. coli*, *Staphylococcus*, *Pseudomonas* spp., total aerobic flora, yeasts and molds. De Reu et al. (2004) noted that the high colony counts of the hygiene indicators coliforms, *E. coli* and *Staphylococcus aureus* are related to the presence of *Listeria* spp. in raw milk

butter, although no significant statistical relationship was found. Unlike *E. coli* and *S. aureus*, an increase in yeasts and molds involves a decrease in *L. monocytogenes*. According to a study conducted by Goerges et al. (2006), all tested yeasts had an inhibitory potential on *L. monocytogenes*. The authors related this result to the competition for nutrients. *Pseudomonas* spp. was also reported as an effective competitor of *L. monocytogenes* (Farrag and Marth, 1989). This psychrotrophic bacterium showed exhibited wide spectrum antimicrobial activity against *L. monocytogenes* among other Gram positive bacteria (Cheng et al., 1995; Freedman et al., 1989; Gram, 1993). The findings of this study showed that the presence and the levels of *L. monocytogenes* in the samples decreased regardless of the levels of the other bacteria. This result could be due to the presence of other microorganisms like lactic acid bacteria. Ahamad and Marth (1989) have reported that lactic acid had an inhibitory effect on *L. monocytogenes*. Bacteriocins, one of the many antimicrobial substances produced by lactic acid bacteria, have also been identified as exhibiting activity against *L. monocytogenes* (Chen and Hoover, 2003; Dortu and Thonart, 2009; Jordan et al., 2016).

5. Conclusion

No growth was observed in the samples of naturally contaminated butter analyzed with durability test. The number of contaminated samples and the colony counts of *L. monocytogenes* even decreased at the end of the storage period. The durability tests performed show that raw milk butter does not allow the growth of the pathogen regardless of its physico-chemical and microbiological characteristics. Nevertheless, an analysis on raw milk butter with high pH value (pH > 6.2) would be interesting to support these findings. This study suggested that the behavior of *L. monocytogenes* in raw milk butter could be affected by other parameters like the microbiota, especially lactic acid bacteria. It would be of interest to study the evolution of the pathogen in butter compared to that of microbiota.

Table 12: Physico-chemical and microbiological characteristics of the batches at “day 0”

ID	Starter culture	pH at "day 0" (mean ± SD)	a_w at "day 0"	NaCl at "day 0" (mean ± SD in %NaCl)	*L. monocytogenes* at "day 0" (mean ± SD in log cfu/g)	*E. coli* at "day 0" (mean ± SD in log cfu/g)	*Staphylococci* at "day 0" (mean ± SD in log cfu/g)	Total aerobic flora at "day 0" (mean ± SD in log cfu/g)	Yeasts at "day 0" (mean ± SD in log cfu/g)	Molds at "day 0" (mean ± SD in log cfu/g)	*Pseudomonas* at "day 0" (mean ± SD in log cfu/g)
EV_01	Yes	5.02 ± 0.06	0.97	0.19 ± 0.00	-1.08 ± 0.81	NA	NA	NA	NA	NA	NA
EV_02	Yes	4.72 ± 0.06	0.93	0.52 ± 0.00	0.76 ± 1.01	NA	NA	NA	NA	NA	NA
EV_03	Yes	4.62 ± 0.13	0.94	0.02 ± 0.00	-1.32 ± 0.43	0.95 ± 0.00	0.95 ± 0.00	5.48 ± 0.00	NA	4.70 ± 0.00	NA
EV_04	No	5.49 ± 0.22	0.96	0.03 ± 0.00	-0.43 ± 1.21	0.95 ± 0.00	1.93 ± 0.22	7.69 ± 0.00	3.80 ± 0.10	3.02 ± 0.06	7.24 ± 0.54
EV_05	No	5.40 ± 0.17	0.98	1.35 ± 0.07	0.41 ± 1.01	3.21 ± 0.02	3.03 ± 0.05	7.69 ± 0.00	3.02 ± 0.25	1.55 ± 0.13	5.55 ± 0.72
EV_06	No	6.12 ± 0.04	0.97	0.02 ± 0.01	0.80 ± 0.60	3.88 ± 0.10	0.95 ± 0.00	7.62 ± 0.12	1.36 ± 0.39	1.46 ± 0.28	7.23 ± 0.15
EV_07	No	4.60 ± 0.09	0.97	0.65 ± 0.03	0.25 ± 1.10	3.91 ± 0.13	1.03 ± 0.13	7.59 ± 0.17	4.48 ± 0.00	0.95 ± 0.00	2.10 ± 0.18
EV_08	Yes	4.72 ± 0.14	0.95	0.98 ± 0.07	0.48 ± 0.96	3.17 ± 0.15	1.68 ± 0.59	7.69 ± 0.00	2.80 ± 0.35	2.26 ± 0.83	4.87 ± 0.12

Table 12: Physico-chemical and microbiological characteristics of the batches at “day 0” (*Continued*)

ID	Starter culture	pH at "day 0" (mean ± SD)	a_w at "day 0"	NaCl at "day 0" (mean ± SD in %NaCl)	*L. monocytogenes* at "day 0" (mean ± SD in log cfu/g)	*E. coli* at "day 0" (mean ± SD in log cfu/g)	*Staphylococci* at "day 0" (mean ± SD in log cfu/g)	Total aerobic flora at "day 0" (mean ± SD in log cfu/g)	Yeasts at "day 0" (mean ± SD in log cfu/g)	Molds at "day 0" (mean ± SD in log cfu/g)	*Pseudomonas* at "day 0" (mean ± SD in log cfu/g)
EV_09	No	4.60 ± 0.08	0.97	0.32 ± 0.05	0.33 ± 1.06	3.26 ± 0.07	0.95 ± 0.00	7.69 ± 0.00	2.55 ± 0.05	1.75 ± 0.05	4.64 ± 0.11
EV_10	No	5.42 ± 0.04	0.96	0.72 ± 0.05	0.09 ± 1.15	4.48 ± 0.00	3.65 ± 0.06	7.69 ± 0.00	3.25 ± 0.20	1.06 ± 0.10	6.23 ± 0.30
EV_11	No	5.42 ± 0.27	0.98	0.05 ± 0.01	2.51 ± 0.74	2.51 ± 0.04	4.70 ± 0.00	7.69 ± 0.00	3.23 ± 0.18	1.00 ± 0.00	6.48 ± 0.00
EV_13	No	4.54 ± 0.05	1.00	0.06 ± 0.01	-0.46 ± 1.17	1.26 ± 0.24	0.95 ± 0.00	7.69 ± 0.00	4.48 ± 0.00	3.62 ± 0.06	2.49 ± 0.50
EV_14	No	5.40 ± 0.06	0.95	0.83 ± 0.09	0.58 ± 1.12	4.48 ± 0.00	3.53 ± 0.08	7.69 ± 0.00	2.38 ± 0.22	1.46 ± 0.41	5.83 ± 0.16
EV_15	No	4.88 ± 0.08	0.97	1.02 ± 0.04	-0.85 ± 1.01	2.33 ± 0.08	0.95 ± 0.00	6.69 ± 0.00	4.07 ± 0.08	1.10 ± 0.17	6.48 ± 0.00
EV_16	No	5.20 ± 0.09	0.99	0.03 ± 0.00	2.57 ± 0.08	4.48 ± 0.00	0.95 ± 0.00	6.69 ± 0.00	3.00 ± 0.33	2.31 ± 0.24	6.48 ± 0.00
EV_17	Yes	5.20 ± 0.19	0.97	0.30 ± 0.04	-0.16 ± 1.26	0.95 ± 0.00	0.95 ± 0.00	6.69 ± 0.00	2.24 ± 0.04	2.33 ± 0.14	5.75 ± 0.12

Table 12: Physico-chemical and microbiological characteristics of the batches at “day 0” (*Continued*)

ID	Starter culture	pH at "day 0" (mean ± SD)	a_w at "day 0"	NaCl at "day 0" (mean ± SD in %NaCl)	*L. monocytogenes* at "day 0" (mean ± SD in log cfu/g)	*E. coli* at "day 0" (mean ± SD in log cfu/g)	*Staphylococci* at "day 0" (mean ± SD in log cfu/g)	Total aerobic flora at "day 0" (mean ± SD in log cfu/g)	Yeasts at "day 0" (mean ± SD in log cfu/g)	Molds at "day 0" (mean ± SD in log cfu/g)	*Pseudomonas* at "day 0" (mean ± SD in log cfu/g)
EV_18	No	5.50 ± 0.15	0.98	0.02 ± 0.01	1.48 ± 0.32	1.88 ± 0.09	0.95 ± 0.00	6.69 ± 0.00	6.72 ± 0.66	2.00 ± 0.00	6.48 ± 0.00
EV_19	No	5.85 ± 0.10	0.99	0.03 ± 0.00	0.95 ± 0.00	3.26 ± 0.04	2.82 ± 0.19	6.69 ± 0.00	2.09 ± 0.28	0.95 ± 0.00	6.48 ± 0.00
EV_20	No	4.67 ± 0.10	0.98	0.03 ± 0.01	0.37 ± 1.09	4.70 ± 0.00	3.74 ± 0.13	6.69 ± 0.00	4.05 ± 0.43	3.19 ± 0.37	6.70 ± 0.00
EV_21	No	4.83 ± 0.07	0.99	0.02 ± 0.01	0.26 ± 1.10	4.48 ± 0.00	0.95 ± 0.00	6.69 ± 0.00	2.01 ± 0.51	1.73 ± 0.05	6.32 ± 0.14

Table 13: Physico-chemical and microbiological characteristics of the batches at “day 30”

ID	pH at "day 30" (mean ± SD)	a_w at "day 30"	NaCl at "day 30" (mean ± SD in %NaCl)	*L. monocytogenes* at "day 30" (mean ± SD in log cfu/g)	*E. coli* at "day 30" (mean ± SD in log cfu/g)	*Staphylococci* at "day 30" (mean ± SD in log cfu/g)	Total aerobic flora at "day 30" (mean ± SD in log cfu/g)	Yeasts at "day 30" (mean ± SD in log cfu/g)	Molds at "day 30" (mean ± SD in log cfu/g)	*Pseudomonas* at "day 30" (mean ± SD in log cfu/g)
EV_01	4.93 ± 0.05	0.95	0.15 ± 0.00	-1.24 ± 0.60	NA	NA	NA	NA	NA	NA
EV_02	4.53 ± 0.02	0.97	0.44 ± 0.00	-1.40 ± 0.00	NA	NA	NA	NA	NA	NA
EV_03	4.16 ± 0.04	0.97	0.02 ± 0.00	-1.40 ± 0.00	0.95 ± 0.00	0.95 ± 0.00	5.48 ± 0.00	NA	5.70 ± 0.00	NA
EV_04	4.61 ± 0.05	0.98	0.02 ± 0.01	-1.32 ± 0.43	0.95 ± 0.00	0.95 ± 0.00	7.56 ± 0.19	4.48 ± 0.00	4.43 ± 0.42	6.00 ± 0.00
EV_05	5.27 ± 0.08	0.96	1.19 ± 0.04	-1.32 ± 0.43	1.07 ± 0.20	2.35 ± 0.16	7.09 ± 0.54	5.99 ± 0.09	3.65 ± 0.30	6.45 ± 0.32
EV_06	5.54 ± 0.04	0.98	0.02 ± 0.00	0.59 ± 0.91	3.86 ± 0.08	0.95 ± 0.00	7.53 ± 0.17	4.60 ± 0.15	1.85 ± 0.35	7.57 ± 0.22
EV_07	4.67 ± 0.06	0.97	0.65 ± 0.01	-1.32 ± 0.43	1.22 ± 0.42	0.95 ± 0.00	7.69 ± 0.00	4.48 ± 0.00	0.95 ± 0.00	6.23 ± 0.30
EV_08	4.55 ± 0.11	0.96	0.88 ± 0.03	-1.24 ± 0.60	0.95 ± 0.00	0.95 ± 0.00	6.64 ± 0.37	4.60 ± 1.52	3.34 ± 0.12	3.00 ± 0.00
EV_09	4.53 ± 0.07	0.97	0.36 ± 0.04	-1.40 ± 0.00	0.97 ± 0.03	0.95 ± 0.00	7.26 ± 0.17	4.48 ± 0.00	4.48 ± 0.00	3.00 ± 0.00
EV_10	5.35 ± 0.04	0.96	0.52 ± 0.01	-0.07 ± 1.19	5.48 ± 0.00	0.95 ± 0.00	8.01 ± 0.27	5.48 ± 0.00	1.47 ± 0.58	5.84 ± 0.10
EV_11	4.79 ± 0.03	0.99	0.05 ± 0.01	1.99 ± 0.11	1.81 ± 0.29	4.10 ± 0.06	7.96 ± 0.07	4.60 ± 0.39	3.48 ± 0.00	5.97 ± 0.09
EV_13	4.33 ± 0.02	0.98	0.05 ± 0.00	-1.40 ± 0.00	0.95 ± 0.00	0.95 ± 0.00	6.48 ± 1.06	4.51 ± 0.53	4.24 ± 0.47	3.71 ± 0.05
EV_14	5.38 ± 0.05	0.94	0.90 ± 0.09	0.01 ± 1.17	3.57 ± 0.18	0.95 ± 0.00	7.44 ± 0.23	4.48 ± 0.00	2.21 ± 0.49	6.67 ± 0.11

Table 13: Physico-chemical and microbiological characteristics of the batches at "day 30" (*Continued*)

ID	pH at "day 30" (mean ± SD)	a_w at "day 30"	NaCl at "day 30" (mean ± SD in %NaCl)	*L. monocytogenes* at "day 30" (mean ± SD in log cfu/g)	*E. coli* at "day 30" (mean ± SD in log cfu/g)	*Staphylococci* at "day 30" (mean ± SD in log cfu/g)	Total aerobic flora at "day 30" (mean ± SD in log cfu/g)	Yeasts at "day 30" (mean ± SD in log cfu/g)	Molds at "day 30" (mean ± SD in log cfu/g)	*Pseudomonas* at "day 30" (mean ± SD in log cfu/g)
EV_15	4.74 ± 0.06	0.96	1.00 ± 0.01	-1.40 ± 0.00	0.95 ± 0.00	0.95 ± 0.00	7.31 ± 0.14	5.48 ± 0.00	2.16 ± 0.10	6.12 ± 0.12
EV_16	4.94 ± 0.04	0.98	0.02 ± 0.01	2.19 ± 0.08	4.14 ± 0.25	0.95 ± 0.00	7.69 ± 0.00	5.06 ± 0.16	3.19 ± 0.26	5.76 ± 0.15
EV_17	4.92 ± 0.05	0.97	0.28 ± 0.02	-1.32 ± 0.43	0.95 ± 0.00	0.95 ± 0.00	6.77 ± 0.32	4.70 ± 0.00	3.08 ± 0.15	5.62 ± 0.14
EV_18	5.20 ± 0.06	0.98	0.02 ± 0.01	1.29 ± 0.31	1.14 ± 0.29	0.95 ± 0.00	6.64 ± 0.17	5.97 ± 0.11	2.00 ± 0.00	5.98 ± 0.07
EV_19	5.47 ± 0.11	0.98	0.02 ± 0.01	0.41 ± 1.01	2.82 ± 0.15	1.84 ± 0.26	6.95 ± 0.11	3.98 ± 0.86	2.05 ± 0.05	6.22 ± 0.21
EV_20	4.40 ± 0.13	0.98	0.03 ± 0.00	0.48 ± 0.96	5.17 ± 0.16	2.59 ± 0.37	7.69 ± 0.00	6.48 ± 0.00	4.16 ± 0.28	6.63 ± 0.39
EV_21	4.56 ± 0.04	0.99	0.02 ± 0.00	-0.93 ± 0.96	3.49 ± 0.84	0.95 ± 0.00	6.83 ± 0.35	4.13 ± 0.30	3.68 ± 0.94	4.52 ± 0.90

V

Study of the bacterial profile of raw milk butter, made during a challenge test with *Listeria monocytogenes*, depending on cream maturation temperature

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In the previous chapter, durability studies were used to assess the growth of *L. monocytogenes* in raw milk butter. Based on the results, no growth of *L. monocytogenes* was observed at the end of shelf life regardless of the product's physico-chemical characteristics. However, no sample presented a pH > 6.2 and no intermediate analyzes was conducted during storage. The third experimental study was thus carried out to evaluate the growth potential of *L. monocytogenes* in raw milk butter during storage. To compare between the worst-case scenario (most favorable) and the least favorable conditions for the growth of *L. monocytogenes*, challenge tests were conducted simulating a maturation at 4 °C and 14 °C.

The previous results also showed that it is essential to investigate the bacterial profile of raw milk butter. In parallel with challenge tests, metagenetic analysis was thus carried out. This research further extends our knowledge on the behavior of *L. monocytogenes* in raw milk butter and the factors affecting its growth.

Abstract

Bacteria can play different roles and impart various flavors and characteristics to food. Few studies have described bacterial microbiota of butter. In this study, next-generation sequencing was used to determine bacterial content of raw milk butter, processed during a challenge test, depending on cream maturation temperature and on the presence or not of *L. monocytogenes*. Two batches were produced. pH and microbiological analyses were conducted during cream maturation and butter storage. DNA was also isolated from all samples for 16S rDNA amplicon sequencing analysis. For butter made from cream matured at 14 °C, a growth potential of *L. monocytogenes* of - 1.72 log cfu/g was obtained. This value corresponds to the difference between the median of counts at the end of storage and the median of counts at the beginning of storage. This butter (pH value of 4.75 ± 0.04) was characterized by a dominance of *Lactococcus*. The abundance of *Lactococcus* was significantly higher in inoculated samples than in control samples (p value <0.05). Butter made from cream matured at 4 °C (pH value of 6.81 ± 0.01) presented a growth potential of 1.81 log cfu/g. It was characterized by the abundance of psychrotrophic bacteria mainly *Pseudomonas.* This study demonstrated that cream maturation temperature impacts butter microbiota, affecting thus product's characteristics and its ability to support or not the growth of pathogens like *L. monocytogenes*.

Key words: 16S rDNA sequencing, metagenetics, growth potential

1. Introduction

Listeria monocytogenes is the causative agent of listeriosis, a severe foodborne disease with high mortality rate. In 2019, 300 deaths were reported in Europe due to listeriosis, representing a case fatality rate of 17.6% (European Food Safety Authority and European Centre for Disease Prevention and Control, 2021). Most cases of listeriosis arise from the ingestion of contaminated food, especially ready-to-eat (RTE) (Jofré et al., 2016; Pérez-Rodríguez et al., 2017).

As a RTE food, butter is also prone to contamination by *L. monocytogenes*. However, its ability to support survival or growth of the pathogen depends on its

formulation and characteristics (Holliday et al., 2003; Michelon et al., 2016; Voysey et al., 2009). Many intrinsic and extrinsic factors such as temperature, pH and water activity (a_w), were shown to affect the growth of *L. monocytogenes* in food (Fernandez et al., 1997; Hayman et al., 2008; Nyhan et al., 2018; Schvartzman et al., 2011). RTE foods with pH ≤ 4.4 or $a_w \leq 0.92$ or pH ≤ 5.0 and $a_w \leq 0.94$ do not support the growth of *L. monocytogenes* (Commission Regulation, 2005). In Wallonia (Belgium), no growth of *L. monocytogenes* was observed during storage of naturally contaminated samples of raw milk butter, even though they presented pH and a_w values theoretically allowing the growth of the pathogen (El-Hajjaji et al., 2020a).

The presence of antimicrobials or competitive microbiota can also inhibits the growth of *L. monocytogenes* (Al-Zeyara et al., 2011; Brandt et al., 2010; Goerges et al., 2006; Murdock et al., 2007). Lactic acid bacteria (LAB), for example, have shown an inhibitory effect on *L. monocytogenes* in various food matrices (Amézquita and Brashears, 2002; Arqués et al., 2005; Koo et al., 2012; Teixeira de Carvalho et al., 2006). To study food microbiota, traditional methods based on cultivation, isolation and identification of bacteria based on their morphological characteristics were often used. Nowadays, newer and automated methods are adopted, including sequencing of the 16S rRNA gene (Phumudzo et al., 2013). Over the past decade, next-generation sequencing technologies evolved rapidly and led to an improved representation of samples biodiversity (Shokralla et al., 2012).

To our knowledge, published studies of food microbial ecology have focused on plant-, meat- and fish-derived fermented foods, milk, fermented milk and cheese. Studies of bacterial communities of butter have rarely been conducted. The objective of this study was to use next-generation sequencing to analyze bacterial content of raw milk butter, processed during a challenge test, depending on cream maturation temperature and on the presence or not of artificially inoculated *L. monocytogenes*.

2. Materials and methods

2.1. Listeria monocytogenes cultures

To consider the growth variability between strains, a cocktail of two strains (ATCC 19114 and 12MOB105LM of a culture collection, provided by Quality Partner sa (Herstal, Belgium)) was used in this study. The second strain was isolated from a dairy product. Cryobeads containing respective strains were incubated at 37 °C for 18 h in 9 ml brain heart infusion (BHI). A subculture was prepared by diluting 1 ml of this culture into 9 ml of BHI and incubated at 7 °C for 7 days. A cocktail was prepared by mixing the same volume from each culture. Dilutions of the mixed cultures were then made until obtaining a concentration of 10^5 cfu/ml.

2.2. Butter manufacture

Two batches of raw milk butter were manufactured in a pilot unit (Food Science Department, Faculty of Veterinary Medicine, University of Liège, Liège, Belgium). The batches were produced at the same day and using the same batch of cream obtained from a dairy farm directly after skimming. For each batch of butter, 20 l of cream were used. Half of the cream was inoculated with 5 ml of the cocktail of strains

to obtain a contamination level of 50 cfu/ml. Remaining cream was used to manufacture control samples. Creams were then incubated for 3 days of maturation, either at 4 °C (first batch, B1) or at 14 °C (second batch, B2). These two temperatures of maturation were selected to represent the two most common and opposite practices (maturation in fridge (4 °C) or workshop (14 °C)) followed by raw milk butter producers in Wallonia (El-Hajjaji et al., 2019). The matured creams were churned until phases' separation. After buttermilk removal, grains of butter were washed three times with cold water (12 to 14 °C) and finally kneaded and packed into blocks of 250 g. Butter was stored at 9 °C for 30 days. No starter cultures were added so as not to affect the initial microbiota. It is also the most adopted practice in Wallonia (El-Hajjaji et al., 2019). No salt was added neither.

2.3.Microbiological and physico-chemical analyses

For all analyses, three different samples of cream and/or butter per batch were submitted each time. All samples (inoculated and non-inoculated) were analyzed for total mesophilic microbiota, LAB and pH according to ISO 4833, ISO 15214 and ISO 2917 methods, respectively. Analyses were conducted at D'0 (before maturation), D'1 (after 1 day of maturation), D'2 (after 2 days of maturation) and D'3 (after 3 days of maturation) for cream samples and at D0 (before storage), D7 (after 7 days of storage), D14 (after 14 days of storage) and D30 (after 30 days of storage) for butter samples. For the latter samples, a_w was also determined at the beginning (D0) and the end of the storage period (D30), using the ISO 21807 method.

L. monocytogenes was enumerated in inoculated samples at D'0, D'1, D'2 and D'3 for cream, and D0, D7, D14 and D30 for butter. The enumeration was conducted according to RAPID' L.mono (Bio-Rad, Hercules, CA, USA) method with a detection limit of 10 cfu/g. For control samples, only the detection of the bacteria in 25 g was performed at the beginning of cream maturation and at the beginning of butter storage.

2.4.DNA extraction and sequencing

DNA extraction and sequencing were carried out on three different samples of cream and/or butter per batch each time. DNA was isolated from each sample using the FastDNA Spin Kit (MP Biomedicals, Santa Ana, CA, USA), following the manufacturer's recommendations. DNA was eluted into DNase-free water and its concentration and quality were evaluated using a NanoDrop ND-1000 spectrophotometer (ThermoFisher, Wilmongton, USA).

DNA samples were stored at -20 °C until use in 16S rRNA gene amplicon sequencing analysis.

Library preparation and sequencing analysis were carried out by DNA Vision S.A. (company, Gosselies, Belgium) using Illumina technology. Library preparation was done by amplifying the V1-V3 region of the 16S rRNA gene. The forward and reverse primer sequences used in this study, including the Illumina adapters, were

5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGAGTTTGATCCTGGCTCAG-3' and 5'-

GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGATTACCGCGGCTGCT GG-3', respectively.

2.5.Bioinformatics analysis

The analysis of the sequencing data was conducted using Mothur software package for trimming, length and quality filtering, and the removing of chimeras (Schloss et al., 2009). The sequences that passed the quality check were aligned to the SILVA alignment database at genus level (Quast et al., 2012). The final reads were then clustered into operational taxonomic units (OTUs) at a 0.03 distance unit cutoff.

2.6.Statistical analysis

Calculation of the growth potential: The growth potential (δ) is the difference between the median of the log cfu/g counts at the end of the storage and the median at the beginning (EURL *Lm* method). If δ is higher than 0.5 log cfu/g, it is assumed that the food is able to support the growth of *L. monocytogenes*, and *vice versa* if the δ is lower than 0.5 log cfu/g (Beaufort et al., 2014). The growth potential was also calculated using the FASFC method (2019) reported by Gérard et al. (2020) as the difference between the highest value at the end of storage and the lowest at D0 (FASFC method).

Bacterial diversity: To evaluate bacterial richness and diversity of the samples, data sets were subsampled using Mothur to obtain the same number of reads per sample. Richness was assessed using number of OTUs and Chao1 estimator, while diversity was assessed using the Shannon diversity index and Inverse Simpson index.

Bacterial population dissimilarity: Difference of profiles was examined using non-metric multidimensional scaling (NMDS) based on Yue & Clayton theta index (Yue and Clayton, 2005). Statistical differences in the bacterial populations between samples were highlighted using analysis of molecular variance (AMOVA). Differences were considered significant when p-values were lower than 0.05. The function "metastats" of Mothur software was then used to determine which OTUs were differentially represented between the samples.

3. Results

3.1.Characterization of creams and butters

The physico-chemical and microbial characteristics of creams and butters during maturation and storage are summarized in Tables 14 and 15, respectively.

At the beginning, the pH values of the two batches of cream were 6.77 ± 0.01 and 6.75 ± 0.01, respectively. During maturation, the pH of B2 cream (maturation at 14 °C) decreased significantly compared to B1 (maturation at 4 °C). The values obtained at the end of maturation were 4.58 ± 0.01 and 6.76 ± 0.01, respectively. After churning, washing and kneading, pH values undergo a slight increase reaching 4.75 ± 0.04 and 6.81 ± 0.01 for B2 and B1 butters, respectively. During storage, both B1 and B2 butter samples showed a decrease in pH. At the end of the storage period, average

pH was 4.52 ± 0.02 and 5.39 ± 0.03 for B2 and B1, respectively. Regarding a_w, the two batches presented a value of 0.98 ± 0.00.

Regarding the behavior of total microbial counts and LAB during cream maturation, results showed a gradual increase in the samples of B1, to reach a mean value of 7.25 ± 0.04 and 5.13 ± 0.11 log cfu/g at the end of maturation, respectively. However, levels of total microbial counts and LAB in B2 samples increased suddenly after one day of maturation to reach 8.30 ± 0.00 and 8.03 ± 0.47 log cfu/g, respectively.

In control butters, levels of total microbial counts and LAB increased by 1.26 (from 5.46 to 6.72 log cfu/g) and 2.31 log units (from 4.43 to 6.75 log cfu/g) in B1 samples during storage, respectively, while it decreased by 1.1 (from 7.31 to 6.21 log cfu/g) and 0.76 log units (from 7.44 to 6.68 log cfu/g) in B2 samples, respectively.

Table 14: pH and microbiological characteristics (averages ± standard deviations) of cream samples during maturation

Cream samples	Inoculation	Day of sampling	pH	Total microbial counts (log cfu/g)	LAB (log cfu/g)	*L. monocytogenes* (log cfu/g)
Cream_B1	Blank	D'0	6.77 ± 0.01	4.11 ± 1.05	3.88 ± 0.06	/
		D'1	6.80 ± 0.01	6.13 ± 0.05	4.65 ± 0.05	/
		D'2	6.78 ± 0.01	7.19 ± 0.02	4.47 ± 0.05	/
		D'3	6.76 ± 0.01	7.25 ± 0.04	5.13 ± 0.11	/
	Inoculated	D'0	NA	4.95 ± 0.04	4.29 ± 0.03	2.19 ± 0.14
		D'1	NA	6.27 ± 0.17	5.42 ± 0.30	2.80 ± 0.13
		D'2	NA	6.96 ± 0.10	5.31 ± 0.11	3.01 ± 0.16
		D'3	NA	7.25 ± 0.06	5.47 ± 0.23	3.75 ± 0.06
Cream_B2	Blank	D'0	6.75 ± 0.01	5.09 ± 0.08	3.93 ± 0.10	/
		D'1	6.69 ± 0.01	8.30 ± 0.00	8.03 ± 0.47	/
		D'2	5.34 ± 0.04	8.22 ± 0.07	8.23 ± 0.07	/
		D'3	4.58 ± 0.01	7.88 ± 0.03	7.88 ± 0.02	/
	Inoculated	D'0	NA	5.14 ± 0.15	4.38 ± 0.12	1.75 ± 0.39
		D'1	NA	8.30 ± 0.00	8.30 ± 0.00	4.26 ± 0.08
		D'2	NA	7.90 ± 0.05	8.23 ± 0.08	5.00 ± 0.11
		D'3	NA	8.14 ± 0.27	8.00 ± 0.26	5.18 ± 0.04

B1: cream maturation at 4 °C, B2: cream maturation at 14 °C

D'0: cream before maturation, D'1: cream after 1 day of maturation, D'2: cream after 2 days of maturation, D'3: cream after 3 days of maturation.

NA: Not Available

Table 15: pH, aw and microbiological characteristics (averages ± standard deviations) of butter samples during storage

Butter samples	Inoculation	Day of sampling	pH	a_w	Total microbial counts (log cfu/g)	LAB (log cfu/g)
Butter_B1	Blank	D0	6.81 ± 0.01	0.98 ± 0.00	5.46 ± 0.14	4.43 ± 0.37
		D7	5.60 ± 0.09	/	7.27 ± 0.04	5.82 ± 0.10
		D14	5.60 ± 0.03	/	7.11 ± 0.09	6.49 ± 0.05
		D30	5.39 ± 0.03	0.96 ± 0.00	6.72 ± 0.08	6.75 ± 0.22
	Inoculated	D0	NA	0.98 ± 0.00	5.48 ± 0.17	4.08 ± 0.00
		D7	NA	/	7.33 ± 0.16	5.75 ± 0.11
		D14	NA	/	7.25 ± 0.05	6.49 ± 0.08
		D30	NA	0.97 ± 0.01	6.57 ± 0.01	6.55 ± 0.07
Butter_B2	Blank	D0	4.75 ± 0.04	0.98 ± 0.00	7.31 ± 0.06	7.44 ± 0.23
		D7	4.58 ± 0.11	/	7.28 ± 0.06	7.22 ± 0.03
		D14	4.47 ± 0.03	/	6.97 ± 0.39	6.62 ± 0.02
		D30	4.52 ± 0.02	0.97 ± 0.00	6.21 ± 0.38	6.68 ± 0.16
	Inoculated	D0	NA	0.98 ± 0.00	6.96 ± 0.34	7.14 ± 0.47
		D7	NA	/	7.04 ± 0.33	7.20 ± 0.03
		D14	NA	/	6.24 ± 0.41	6.15 ± 0.36
		D30	NA	0.97 ± 0.00	4.38 ± 0.10	4.22 ± 0.03

B1: cream maturation at 4 °C, B2: cream maturation at 14 °C

D0: butter before storage, D7: butter after 7 days of storage, B14: butter after 14 days of storage, D30: butter after 30 days of storage.

NA: Not Available

3.2.Growth potential of L. monocytogenes

As shown in Table 14, the level of *L. monocytogenes* increased by 1.56 log cfu/g after maturation for cream stored at 4 °C (B1) and by 3.43 log cfu/g for cream stored at 14 °C (B2). The levels obtained for the two batches were 3.75 ± 0.06 and 5.18 ± 0.04 log cfu/g, respectively. After production, a decrease in contamination levels was observed. The levels of *L. monocytogenes* in butter samples at D0 were respectively 1.49 ± 0.43 log cfu/g and 3.34 ± 0.64 log cfu/g for B1 and B2 (Figure 8).

The representation of the behavior of *L. monocytogenes* in butters during storage is presented in Figure 7. After 30 days of storage, growth potentials of 1.81 and 2.60 log cfu/g were obtained for B1 butter using EURL *Lm* and FASFC methods, respectively. This product therefore allowed the growth of *L. monocytogenes* unlike B2 butter. The second batch presented growth potentials of - 1.72 (EURL *Lm* method) and -1.47 log cfu/g (FASFC method). *L. monocytogenes* was not detected in control samples.

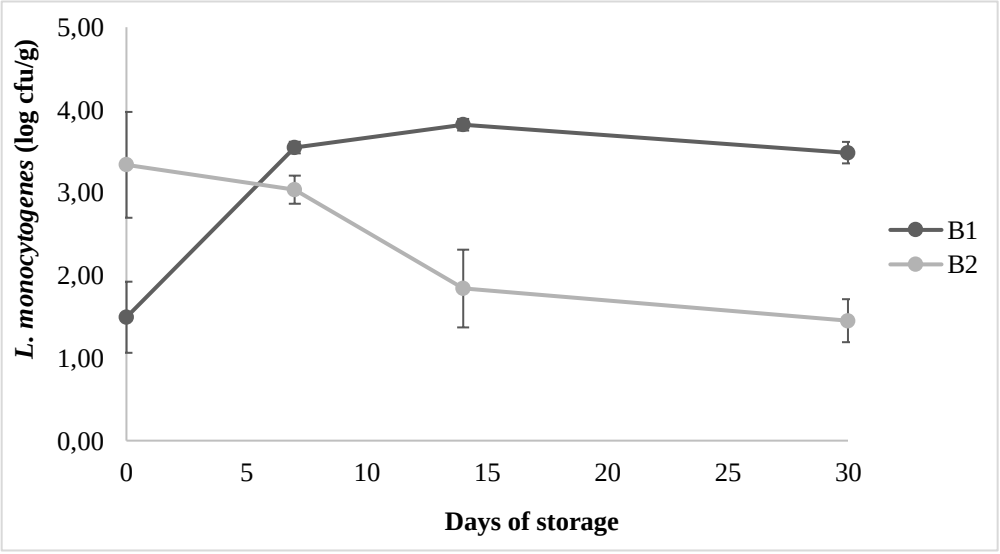


Figure 8: Evolution of levels of *L. monocytogenes* in the two batches of raw milk butter during storage

B1: First batch with cream matured at 4 °C, B2: Second batch with cream matured at 14 °C
Three samples were analyzed each time. Each point represents the mean value of the three measurements and the vertical line represents the standard deviation

3.3.Bacterial diversity in cream and butter

The number of OTUs, the bacterial diversity and richness estimators according to type of samples are presented in Tables 16 and 17. The highest number of OTUs in all cream samples was encountered at D'0. However, B2 cream samples (maturation at 14 °C) showed a decrease in number of OTUs throughout maturation, while the number remained relatively high in B1 cream samples (maturation at 4 °C). B2 cream

samples also showed a low diversity at the end of maturation compared to B1 cream samples.

Table 16: Richness and diversity indices (averages ± standard deviations) of cream samples

Sample	Inoculation	Day of sampling	Number of OTUs	Chao1 index	Shannon index	Inverse Simpson index
Cream_B1	Blank	D'0	228 ± 123	1255 ± 489	4.21 ± 1.34	32 ± 33
		D'1	290 ± 18	1495 ± 75	5.10 ± 0.14	78 ± 19
		D'2	297 ± 2	1643 ± 46	5.22 ± 0.02	109 ± 14
		D'3	306 ± 17	2111 ± 205	5.29 ± 0.08	135 ± 13
	Inoculated	D'0	307 ± 10	1536 ± 100	5.10 ± 0.07	51 ± 7
		D'1	293 ± 28	1624 ± 353	5.17 ± 0.22	106 ± 38
		D'2	279 ± 26	1713 ± 386	5.11 ± 0.18	107 ± 31
		D'3	244 ± 16	985 ± 763	4.88 ± 0.12	76 ± 11
Cream_B2	Blank	D'0	300 ± 25	1625 ± 172	5.01 ± 0.21	47 ± 16
		D'1	272 ± 72	2474 ± 1494	4.95 ± 0.47	87 ± 55
		D'2	102 ± 8	763 ± 58	2.86 ± 0.09	8 ± 1
		D'3	82 ± 7	840 ± 9	2.55 ± 0.09	7 ± 0
	Inoculated	D'0	289 ± 39	1551 ± 358	4.92 ± 0.41	48 ± 22
		D'1	194 ± 25	1358 ± 263	4.20 ± 0.29	28 ± 8
		D'2	73 ± 12	774 ± 175	2.16 ± 0.15	4 ± 0
		D'3	85 ± 10	901 ± 120	2.29 ± 0.12	4 ± 0

B1: cream maturation at 4 °C, B2: cream maturation at 14 °C

D'0: cream before maturation, D'1: cream after 1 day of maturation, D'2: cream after 2 days of maturation, D'3: cream after 3 days of maturation.

The difference between the two batches continued to be observed in butter samples. The number of OTUs and diversity indices were higher in B1 than in B2 butter samples.

Table 17: Richness and diversity indices (averages ± standard deviations) of butter samples

Sample	Inoculation	Day of sampling	Number of OTUs	Chao1 index	Shannon index	Inverse Simpson index
Butter_B1	Blank	D0	239 ± 57	1338 ± 224	4.17 ± 0.64	25 ± 12
		D7	329 ± 4	2134 ± 348	4.96 ± 0.06	50 ± 9
		D14	287 ± 3	2433 ± 334	4.60 ± 0.15	31 ± 12
		D30	278 ± 39	1706 ± 210	4.58 ± 0.18	31 ± 2
	Inoculated	D0	251 ± 17	1764 ± 487	4.38 ± 0.23	28 ± 12
		D7	312 ± 18	2250 ± 713	4.87 ± 0.09	52 ± 6
		D14	299 ± 26	1723 ± 132	4.79 ± 0.25	52 ± 17
		D30	278 ± 18	2328 ± 621	4.61 ± 0.11	36 ± 2
Butter_B2	Blank	D0	116 ± 8	1144 ± 341	2.72 ± 0.05	7 ± 0
		D7	107 ± 17	608 ± 69	2.58 ± 0.17	6 ± 1
		D14	103 ± 3	678 ± 171	2.57 ± 0.04	6 ± 0
		D30	94 ± 17	829 ± 398	2.30 ± 0.18	5 ± 1
	Inoculated	D0	94 ± 4	677 ± 29	2.14 ± 0.03	4 ± 0
		D7	120 ± 27	932 ± 449	2.42 ± 0.30	4 ± 1
		D14	77 ± 3	550 ± 270	1.93 ± 0.08	4 ± 0
		D30	75 ± 8	1629 ± 383	1.77 ± 0.08	3 ± 0

B1: cream maturation at 4 °C, B2: cream maturation at 14 °C

D0: butter before storage, D7: butter after 7 days of storage, B14: butter after 14 days of storage, D30: butter after 30 days of storage.

3.4. Bacterial composition of cream and butter

As no co-sequencing of mock communities was conducted, the error rate due to the biases introduced in sequencing was not assessed. The presented results are thus an estimation of the community composition of the samples.

Three major bacterial phyla (Proteobacteria, Firmicutes and Bacteroidetes), representing more than 90% of relative abundance, were identified in all samples. In B1 cream samples, Proteobacteria were dominant throughout maturation with a continuous increase of their relative abundance to reach 85% at D'3. The same result was observed in B2 cream samples for the first two days. However, at D'2 the relative abundance of Firmicutes increased significantly to reach 80% at D'3. There were no significant differences in bacterial relative abundance between blank and inoculated samples. The dominance of Proteobacteria and Firmicutes continued to be observed in B1 and B2 butter samples during storage, respectively.

At the genus level (Figure 9), 138 bacterial genera were detected in cream samples before maturation (D'0) of which 22 had an average relative abundance ≥ 1%, representing 72% of the total reads. *Undibacterium* (11%), *Ralstonia* (8%), *Acinetobacter* (6%), *Lactococcus* (4%), *Burkholderia* (3%) and *Aeromonas* (1%) were among the most abundant. After the first day of maturation, the bacterial profiles for B1 and B2 cream samples were different. For B1 cream samples, percentages of reads of *Acinetobacter, Pseudomonas* and *Aeromonas* increased during maturation to reach at the end 30%, 12% and 9% of relative abundance, respectively. In terms of relative abundance, these major genera were followed by *Lactococcus* (5%), *Undibacterium* (3%) and *Ralstonia* (2%). As for B2 cream samples, the number of genera detected at the end of maturation was half that of B1 cream samples (32 and 66, respectively) with the dominance of *Lactococcus* (74%) followed by *Acinetobacter* (8%) and *Aeromonas* (4%).

In butter samples (Figure 10), there were more genera detected in B1 than in B2 samples. After production, 69 bacterial genera were detected in B1 butter samples, of which 15 were more abundant (with average relative abundance ≥ 1%) namely *Acinetobacter* (15%), *Pseudomonas* (12%), *Lactococcus* (12%), *Undibacterium* (9%) and *Ralstonia* (7%). As for B2 butter samples, 36 genera were identified of which 9 presented an average relative abundance ≥ 1%. Representing 73% of the total reads, *Lactococcus* was the most abundant one. During storage, psychrotrophic bacteria, mainly *Pseudomonas* increased to be the most dominant in B1 butter samples, while *Lactococcus* continued to be dominant in B2 butter samples.

There were no significant differences in bacterial profile between blank and inoculated samples (AMOVA, p value 0.6). However, the abundance of *Lactococcus* was significantly higher in B2 inoculated samples than in blank samples (p value <0.05).

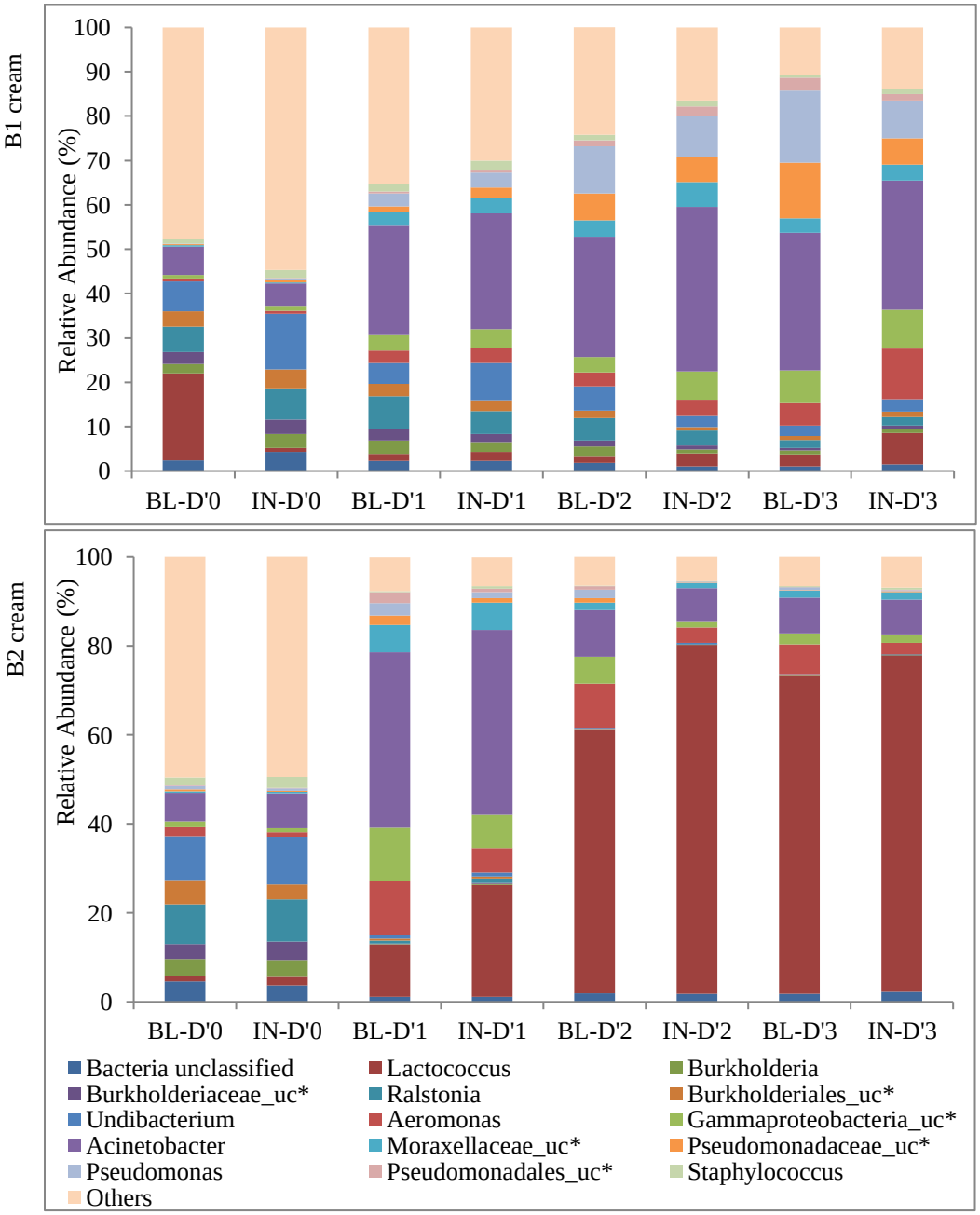


Figure 9: Bacterial microbiota distribution of the two batches of cream samples (B1: cream maturation at 4 °C, B2: cream maturation at 14 °C) depending on day of sampling (D'0, D'1, D'2 and D'3) and the presence or not of *L. monocytogenes* (BL: blanc samples, IN: inoculated samples)

* uc: unclassified bacteria; *Burkholderiaceae* (family): unclassified genera of the family *Burkholderiaceae; Burkholderiales* (order): unclassified family (other than *Burkholderiaceae*) belonging to *Burkholderiales; Moraxellaceae* (family): unclassified genera of the family *Moraxellaceae; Pseudomonadaceae* (family): unclassified genera of the family *Pseudomonadaceae; Pseudomonadales* (order): unclassified family (other than *Moraxellaceae* and *Pseudomonadaceae*) belonging to *Pseudomonadales; Gammaproteobacteria* (class): unclassified order (other than *Pseudomonadales*) of the class *Gammaproteobacteria*

3.5.*Comparison of the bacterial community of samples*

As shown in Figure 11, dissimilarity test based on Yue & Clayton theta distance revealed that the community difference between B1 and B2 butter samples was significant (AMOVA, p value < 0.001). Analyzed results revealed that this significant difference could be owed to the abundance of *Lactococcus* in B2 samples. In contrast, *Acinetobacter* and *Pseudomonas* were more abundant in B1 samples. Dissimilarity test also showed a difference within B1 butter samples linked to the day of analysis except between D7 and D14 (p value 0.247). This difference could be due to the increase in abundance of *Pseudomonas* during storage.

4. Discussion

The objective of this work was to study the bacterial flora of raw milk cream and butter during production, depending on cream maturation temperature and on the presence or not of *L. monocytogenes*. Metagenetics results showed that cream and butter microbiota varied significantly between the two batches made from creams matured at 4 °C (B1) and 14 °C (B2), respectively. The first batch (B1) was mainly characterized by the presence of *Pseudomonas* and *Acinetobacter*, with an increase of their relative abundances during cream maturation at 4 °C and butter storage at 9 °C. As psychrotrophic microorganisms, these bacteria grow well even at 4 °C (Hébraud and Potier, 1999; Oliveira et al., 2015; Perin, 2012). In a study conducted by Raats et al. (2011), the abundance of these two genera in milk samples from dairy plant tank, where it was stored at 4 °C for 54 h at time of sampling, was higher than in those from farm bulk tank (stored at 4 °C for 22 h). The dominance of these Gram negative bacteria in dairy tank milk was also observed by Fricker et al. (2011). Contrary to *Pseudomonas* and *Acinetobacter*, the relative abundance of *Lactococcus* in B1 samples decreased during storage. Refrigeration had an effect on the representation of *Lactococcus* (Lafarge et al., 2004). *Lactococcus* is a mesophilic bacterium with a minimum growth temperature of 5 to 10 °C, hence its representation was low in B1 samples (Anonymous, 2003).

Unlike B1 samples, *Lactococcus* was highly abundant in B2 samples (70% of the total reads). *Lactococcus* belongs to LAB, a group of Gram positive bacteria involved in food fermentation by converting glucose to lactic acid (Stiles and Holzapfel, 1997). LAB is a dominant population in raw milk (Montel et al., 2014; Quigley et al., 2013). Besides *Lactococcus*, the most common LAB genera found in milk are *Lactobacillus*, *Streptococcus*, *Leuconostoc* and *Enterococcus*. These bacteria are also observed in

dairy products (Cogan et al., 1997; Delcenserie et al., 2014; Jayashree et al., 2013; Liu et al., 2015; Yu et al., 2011). However, their representation differs depending on products, production environments and processes. In our study, low relative abundance was detected in cream and butter samples for LAB other than *Lactococcus*. A similar result was reported by Yu et al. (2018) who found that 77.73% of the total reads corresponded to *Lactococcus*, which was thus the most dominant genera in butter samples. In another study conducted by Guessas et al. (2012) on traditional butter (Dhan) made from unpasteurized fermented milk, *Lactobacillus* (46.05%) was the most dominant genera, followed by *Enterococcus* (26.32%), *Lactococcus* (17.11%) and *Leuconostoc* (10.53%). The dominance of species of *Lactobacillus* in butter samples, made from pasteurized milk cream, was also described by Syromyatnikov et al. (2020).

Besides of the dominant genera, other bacteria with relative abundance ≥ 1% were detected. *Undibacterium*, which was never observed in butter, was identified in the two batches. *Undibacterium* are Gram negative bacteria that are often isolated from water (Kämpfer et al., 2007; Kim et al., 2014), which can explain their occurrence in butter. Species of *Undibacterium* were also isolated from soil and feces of cattle (Kim and Wells, 2016; Kim et al., 2014). In fact, water, soil and feces, among other environments, are rich sources of microorganisms and a direct or indirect transfer of cells to milk and dairy products is frequent (Montel et al., 2014; Perin et al., 2019; Quigley et al., 2013). A species of *Undibacterium* was detected in pasteurized milk (Garofalo et al., 2017).

Ralstonia is another uncommon genus which was detected in this study. Like *Undibacterium*, this genus presented high relative abundances in B1 than in B2 butter samples. *Ralstonia* are plant-associated bacteria that are known as important phytopathogens (Gnanamanickam, 2007). However their presence in raw milk and cheese has already been observed (Delbes et al., 2007; Fricker et al., 2011; Kuehn et al., 2013; Salazar et al., 2018). Species of *Ralstonia* were also detected in buttermilk (Jayashree et al., 2013).

Burkholderia, other bacteria that occur in plants, were found in B1 butter. They were formerly classified in the genus *Pseudomonas* (Gnanamanickam, 2007). Species of the genus *Burkholderia* occupy diverse ecological niches including the rhizosphere of plants, water and soil (Coenye and Vandamme, 2003), and can thus be introduced into raw milk (Moore et al., 2001; Saad and Amin, 2012). The presence of *Ralstonia* and *Burkholderia* among other bacteria found in soil and water could also be due to the contamination of DNA during extraction by the kit reagents (Salter et al., 2014). PCR reagents are another source of DNA contamination (Corless et al., 2000; Grahn et al., 2003; Salter et al., 2014). PCR can also lead to other errors which may affect sequencing results (Potapov and Ong, 2017). In this study, the error rate due to PCR amplification and sequencing was not assessed.

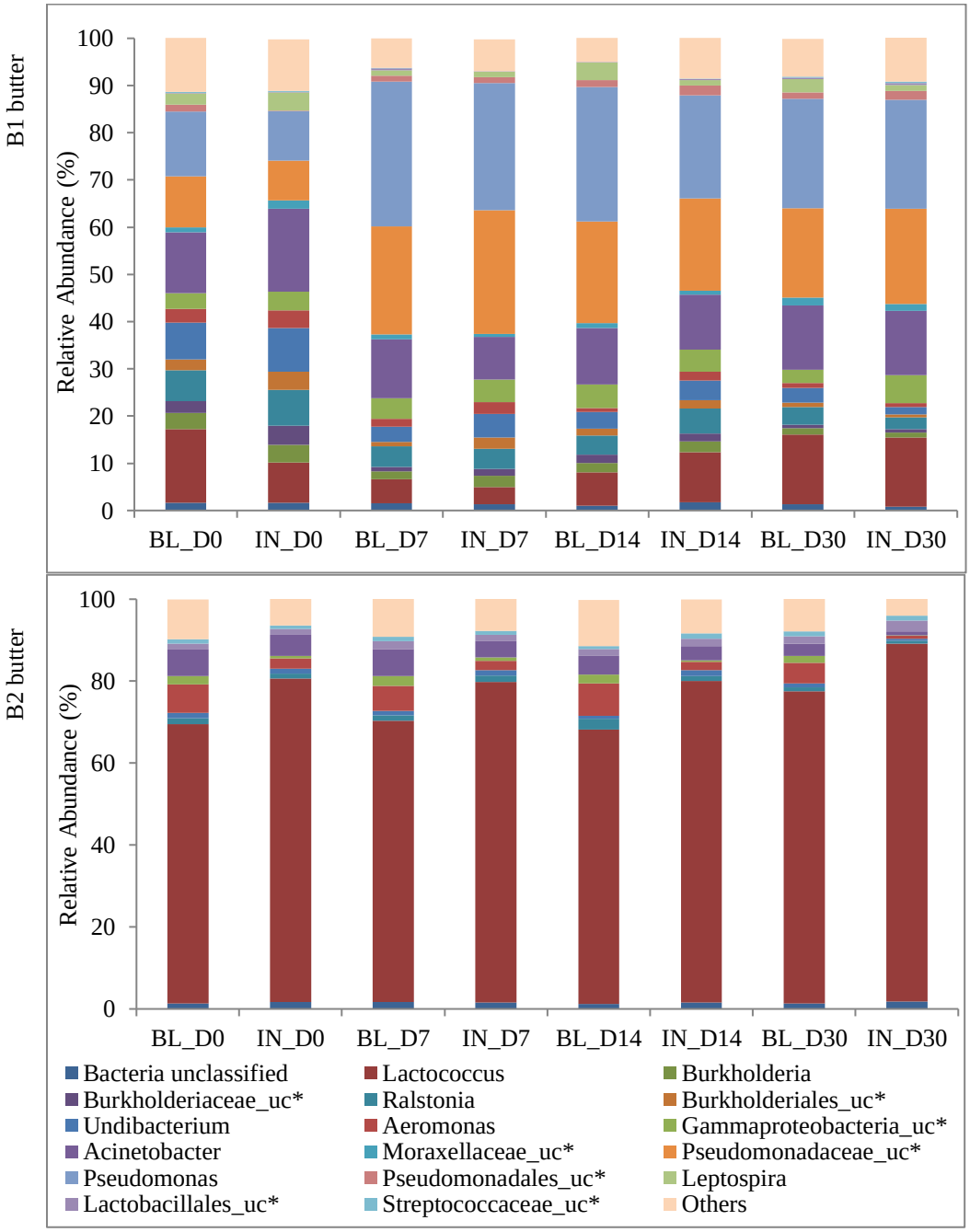


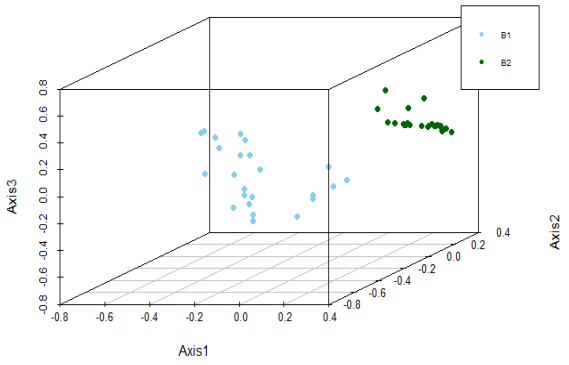
Figure 10: Bacterial microbiota distribution of the two batches of butter samples (B1: cream maturation at 4 °C, B2: cream maturation at 14 °C) depending on storage period (D0, D7, D14 and D30) and the presence or not of *L. monocytogenes* (BL: blanc samples, IN: inoculated samples)

* uc: unclassified bacteria; *Burkholderiaceae* (family): unclassified genera of the family *Burkholderiaceae; Burkholderiales* (order): unclassified family (other than *Burkholderiaceae*) belonging to *Burkholderiales; Moraxellaceae* (family): unclassified genera of the family *Moraxellaceae; Pseudomonadaceae* (family): unclassified genera of the family *Pseudomonadaceae; Pseudomonadales* (order): unclassified family (other than *Moraxellaceae* and *Pseudomonadaceae*) belonging to *Pseudomonadales; Gammaproteobacteria* (class): unclassified order (other than *Pseudomonadales*) of the class *Gammaproteobacteria; Streptococcaceae* (family): unclassified genera of *Streptococcaceae; Lactobacillales* (order): unclassified family (other than *Streptococcaceae*) of *Lactobacillales*

Raw milk microbiota may also contain *Aeromonas* (Benner, 2014; Quigley et al., 2013), which was detected in the studied butters. This genus was also observed in other dairy products including fermented milk, buttermilk, yoghurt and cheese (ElBalat et al., 2014; Jayashree et al., 2013; Liu et al., 2015).

Microorganisms can play either a positive or a negative role in food. LAB are widely recognized as food preservatives. Their production of lactic acid results in pH reduction (Caplice, 1999; Widyastuti et al., 2014). In the current study, pH of the second batch of butter (4.75 ± 0.04) was significantly lower than pH of the first batch (6.81 ± 0.01). The former had LAB counts higher than the latter (Table 15). pH is an important factor for the growth of microorganisms. The growth of *L. monocytogenes* is possible at pH values between 4.4 and 9.6 (Magalhães et al., 2014). Based on this, the growth of *L. monocytogenes* was supposed possible in the two batches of butter studied in this paper. However, the results showed that the bacterium did not grow in butter samples from the second batch (δ = - 1.72 log cfu/g). This finding was in accordance with a previous study where no growth of *L. monocytogenes* was observed in naturally contaminated raw milk butter samples, presenting an average pH value of 5.12 ± 0.47 at the beginning of storage (El-Hajjaji et al., 2020a). The second batch in the present study was characterized by a dominance of *Lactococcus*, a genus of LAB. The abundance of *Lactococcus* was even higher in samples containing *L. monocytogenes* compared to control samples. Besides reducing pH, lactic acid has an inhibitory effect on the growth of microbial pathogens, including *L. monocytogenes* (Anang et al., 2007; Ariyapitipun et al., 2000; Lin et al., 2002; Wang et al., 2015). LAB also produce bacteriocins, substances possessing antimicrobial activities (Dortu and Thonart, 2009; Soomro et al., 2002).

A)



B)

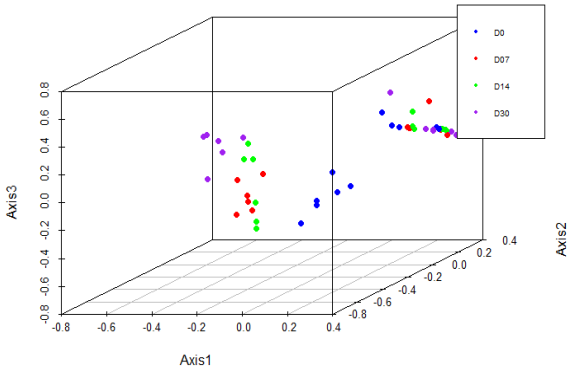


Figure 11: NMDS plot of butter samples generated via Yue & Clayton distance matrix, depending on cream maturation (A) and storage period (B).

B1: cream maturation at 4 °C, B2: cream maturation at 14 °C, D0: butter before storage, D7: butter after 7 days of storage, D14: butter after 14 days of storage, D30: butter after 30 days of storage

5. Conclusion

This study was conducted to analyze bacterial flora of raw milk butter depending on cream maturation temperature. The two batches studied showed a different bacterial profile with a much more diversity in butter made from refrigerated matured cream. This butter was characterized by an abundance of psychrotrophic bacteria mainly *Pseudomonas* while butter made from acidic cream was dominated by *Lactococcus* bacteria. Besides, the growth of *L. monocytogenes* was not observed in this batch. It was also observed that the relative abundance of *Lactococcus* was even higher in the second batch samples containing *L. monocytogenes* compared to control samples. The temperature of cream maturation has a strong influence on raw milk butter subdominant microbiota, which can affect the growth of pathogenic bacteria like *L. monocytogenes*.

As this study was conducted on one batch as a first experiment to draw hypotheses, it would be interesting to work on other batches to confirm the results regarding the growth of *L. monocytogenes* following the two conditions of cream maturation.

Metagenetic analysis was a first approach to explain the different behavior of *L. monocytogenes* in the two batches. Further studies should be performed in order to assess the real difference in community composition between the samples. It would be interesting to conduct a co-sequencing of mock communities to assess the error rate due to the biases introduced in PCR amplification and sequencing.

VI

General discussion, conclusion, and perspectives

Currently, *L. monocytogenes* is considered one of the most important foodborne pathogens. It has been isolated from various foods especially RTE foods including dairy products. Butter, as a RTE food, has also been found contaminated with *L. monocytogenes*. Available information in the scientific literature concerning the occurrence and behavior of *L. monocytogenes* in butter is limited and is often linked to specific parameter or formulation. Nevertheless, highest occurrences were observed for butters made from raw milk at a small scale (El-Hajjaji et al., 2020). In a study conducted by "Cellule Qualité Produits Fermiers" from 2007 to 2010 on the microbiological quality of artisanal dairy products in Wallonia, the principal producer of raw milk butter in Belgium, 59.5% of the 333 analyzed butters were satisfactory regarding *L. monocytogenes* (absence), 40% presented levels < 100 cfu/g and only 0.5% exceeded the limit of 100 cfu/g (Sanchez-Alcaraz et al., 2011).

In accordance with Regulation (EC) N°2073/2005 microbiological criteria for foodstuffs, producers have the obligation to guarantee non-detection of the pathogen in 25 g of raw milk butter before the sale. In case of unsatisfactory results, financial and social impacts can be serious: mandatory notification to FASFC, cessation of production and sale, foreclosure, inventory destruction and loss of customers. These also generate moral impacts namely stress and frustration which can sometimes lead to a halt of farm diversification. In fact, from the 278 producers listed during our survey, 67 were no longer active of which 34% stopped their activity due to FASFC and/or the difficulty to respect the strict standards.

The aim of this project was to study the behavior of *L. monocytogenes* in raw milk butter through different approaches and determine butter's ability to support survival or growth of *L. monocytogenes*. After data collection on raw milk butter and its manufacturing, two experiments (durability studies and challenge tests) were conducted, presenting real and simulated cases.

Maturation conditions and cream acidification, key factors

In Chapter III, we describe raw milk butter at both technological and physicochemical level. Our study showed that Walloon artisanal butter presents various characteristics especially in term of pH, which is known for its effect on the growth of *L. monocytogenes*. To cover this variability, durability studies (Chapter IV) were conducted on naturally contaminated batches of raw milk butter. Through these studies, it was found that the contamination of raw milk butter with *L. monocytogenes* is a sporadic phenomenon. Besides, the levels of *L. monocytogenes* in most of the contaminated samples of raw milk butter were low (< 10 cfu/g). The same result was reported by Kozak et al. (1996), Lewis et al. (2006) and N'Guessan et al. (2015).

The analyzed samples presented pH values that ranged from 4.47 to 6.15 with a mean value of 5.12 ± 0.47 and have undergone different manufacturing process especially in the maturation step (Table 18).

Table 18: Summary of the characteristics of butter batches at “day 0

ID	Sequence of maturation	Starter culture	Storage temperature (°C)	pH at “day 0” (mean + SD)	*L. monocytogenes* at “day 0” (detection in 25g)	*L. monocytogenes* at "day 0" (mean ± SD in log cfu/g)	*E. coli* at "day 0" (mean ± SD in log cfu/g)	*Staphylococci* at "day 0" (mean ± SD in log cfu/g)
EV_04	Workshop - Fridge	No	12	5.49 ± 0.22	12	-0.43 ± 1.21	0.95 ± 0.00	1.93 ± 0.22
EV_16			7 - 12	5.20 ± 0.09	30	2.57 ± 0.08	4.48 ± 0.00	0.95 ± 0.00
EV_02		Yes	7 - 12	4.72 ± 0.06	25	0.76 ± 1.01	NA	NA
EV_07	Workshop	No	7 - 12	4.60 ± 0.09	21	0.25 ± 1.10	3.91 ± 0.13	1.03 ± 0.13
EV_13			7 - 12	4.54 ± 0.05	12	-0.46 ± 1.17	1.26 ± 0.24	0.95 ± 0.00
EV_14			12	5.40 ± 0.06	23	0.58 ± 1.12	4.48 ± 0.00	3.53 ± 0.08
EV_20			7 - 12	4.67 ± 0.10	22	0.37 ± 1.09	4.70 ± 0.00	3.74 ± 0.13
EV_01		Yes	12	5.02 ± 0.06	4	-1.08 ± 0.81	NA	NA
EV_03			7 - 12	4.62 ± 0.13	1	-1.32 ± 0.43	0.95 ± 0.00	0.95 ± 0.00
EV_17			12	5.20 ± 0.19	15	-0.16 ± 1.26	0.95 ± 0.00	0.95 ± 0.00
EV_09	Fridge - Workshop	No	7 - 12	4.60 ± 0.08	22	0.33 ± 1.06	3.26 ± 0.07	0.95 ± 0.00
EV_08		Yes	7 - 12	4.72 ± 0.14	24	0.48 ± 0.96	3.17 ± 0.15	1.68 ± 0.59
EV_06	Fridge	No	12	6.12 ± 0.04	28	0.80 ± 0.60	3.88 ± 0.10	0.95 ± 0.00
EV_10			12	5.42 ± 0.04	19	0.09 ± 1.15	4.48 ± 0.00	3.65 ± 0.06
EV_11			12	5.42 ± 0.27	29	2.51 ± 0.74	2.51 ± 0.04	4.70 ± 0.00
EV_19			12	5.85 ± 0.10	30	0.95 ± 0.00	3.26 ± 0.04	2.82 ± 0.19
EV_05	/	No	7 - 12	5.40 ± 0.17	23	0.41 ± 1.01	3.21 ± 0.02	3.03 ± 0.05
EV_15			12	4.88 ± 0.08	7	-0.85 ± 1.01	2.33 ± 0.08	0.95 ± 0.00
EV_18			7 - 12	5.50 ± 0.15	30	1.48 ± 0.32	1.88 ± 0.09	0.95 ± 0.00
EV_21			12	4.83 ± 0.07	21	0.26 ± 1.10	4.48 ± 0.00	0.95 ± 0.00

As shown in Chapter III, pH is strongly linked to maturation conditions. The first important parameter in maturation is temperature. When maturation is performed at refrigeration temperature, cream acidification is very slow compared to maturation in room temperature. LAB involved in maturation include mesophilic and thermophilic species with optimum growth rates at 30 and 42 °C, respectively (Kassas, 2017). The minimum growth temperature ranges from 5 to 10 °C (Anonymous, 2003). However cream maturation is not only about temperature, but maturation period is also an important factor. The longer is the maturation time, the lower is the pH obtained. If a favorable maturation temperature is provided, 72 hours are the minimum required to obtain a sufficiently acidic cream (< 4.8) (Table 19). However, this may also lead to the growth of undesirable bacteria and toxin production by some pathogens which may result in an unsafe product. In Advice 11-2019 of the Scientific Committee established at the FASFC on the growth potential of *L. monocytogenes* in raw milk butter, a cream pH below 5.2 after 10 hours of the maturation was recommended (FASFC, 2019). To accelerate the acidification process and limit the growth of undesirable bacteria like *E. coli* and *Staphylococcus*, the use of starter cultures is therefore highly recommended. As shown in Table 18, the two batches (EV_03 and EV_17) that presented satisfactory results (< 10 cfu/g) regarding both *E. coli* and *Staphylococcus* were made from cultured creams matured at room temperature. This could be due to the high concentration of LAB in these samples. No LAB analyses were conducted during durability studies. However, given the importance of this parameter regarding the behavior of *L. monocytogenes*, LAB analyzes were planned during the challenge tests. LAB involved in cream maturation can originate from raw milk or be intentionally added as starter cultures. In this study, it was noted that the adjunction of starter cultures was not commonly adopted in Wallonia (68%).

Table 19: Summary of maturation conditions and pH results for creams with pH < 5.2 at the end of maturation. Data originate from pH evolution measurement during on-site monitoring

Farm	Starter cultures	Temperature of maturation	Total days of maturation	Number of hours to reach pH 5,2	Cream pH beginning	Cream pH end
C_01	No	4°C (12h), 18°C	6	36	6.54	3.99
C_02	No	14-15°C	6	31	6.68	4.35
C_03	No	18°C (24h), 13°C	5	46	6.55	4.66
C_04	Yes	4°C (20h), 15-16°C	4	31	6.56	4.45
C_06	No	16°C	4	32	6.65	4.29
C_08	No	20°C	4	35	6.64	4.56
C_10	No	24°C (48h), 7°C	6	19	6.63	4.17
C_11	Yes	13°C (60h), 5°C	3	25	6.62	4.18
C_13	Yes	20-25°C (48h), 4°C	5	19	6.70	4.27
C_15	Yes	22°C	5	12	6.60	4.44
C_16	No	20°C (12h), 7°C	4	70	6.58	4.67
C_18	Yes	18°C	3	12	6.61	4.25
C_20	No	10°C	3	27	6.61	4.41

Temperature also affects the growth of other bacteria including *L. monocytogenes*. Although *L. monocytogenes* is a psychrotrophic bacterium which can grow and survive at refrigeration temperatures, the growth remains to be faster in ambient than chill temperatures (Chan and Wiedmann, 2008; Lanciotti et al., 1992; Rosset, 2001). The durability studies did not make it possible to highlight the effect of temperature on the behavior of the pathogen.

Even though durability studies may be considered more realistic, their implementation is limited in case of low contamination. In this study, 61% of the contaminated samples had a contamination level of less than 1.0 log cfu/g (the enumeration limit). The shortcoming of this study was the absence of exact enumeration for these samples. Although an enumeration value of 9 cfu/g was fixed in this case for calculation purposes, uncertainties were raised especially regarding the estimation of growth potential. The assumption that <10 cfu/g is equal to 9 cfu/g may lead to an overestimation of growth potential (FASFC, 2016).

In addition, with two points of analyses (the beginning and the end of storage period), the behavior of *L. monocytogenes* during butter storage was not covered. Noted also that the beginning of analyses (beginning of storage) was different from the beginning of shelf life, so the studied samples presented different ages at the beginning of analyses. In fact, bacterial growth goes through different phases. These include the lag phase, the exponential or log phase, the stationary phase, and the death phase (Maier and Pepper, 2015). With two points of analyses, we cannot be sure that no growth occurred during storage. Challenge tests were thus conducted to assess the growth potential of *L. monocytogenes* and thus the possibility of considering the raw milk butter as a product not allowing the growth of *L. monocytogenes*. Given the importance of maturation conditions, two production processes were simulated, and analyses were conducted during cream maturation and butter storage (Figures 12 and 13). These challenge tests allowed to distinguish between two types of raw milk butter regarding the behavior of *L. monocytogenes*.

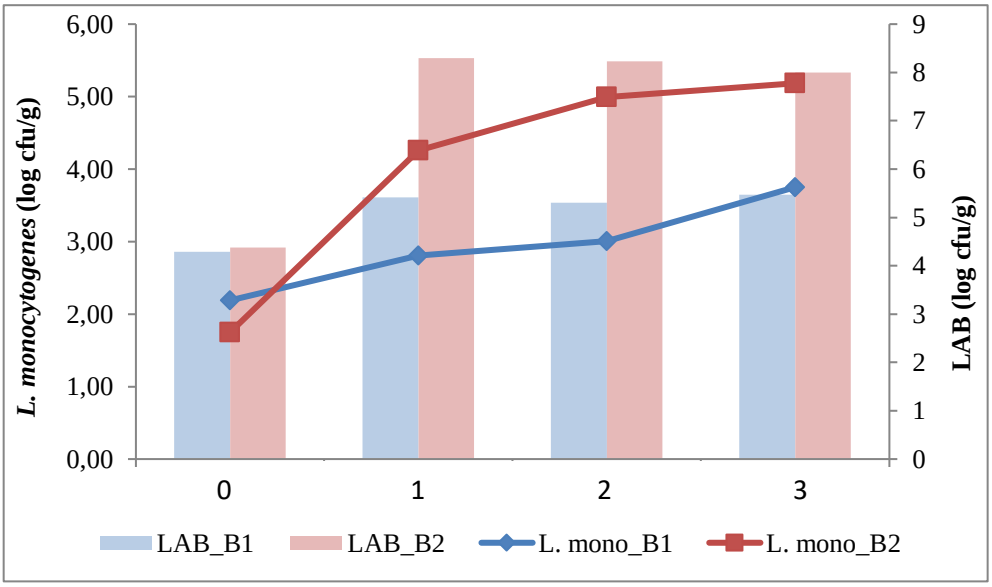


Figure 12: Evolution of *L. monocytogenes* (lines) and LAB (sticks) in cream during maturation

B1: maturation at 4°C, B2: maturation at 14°C

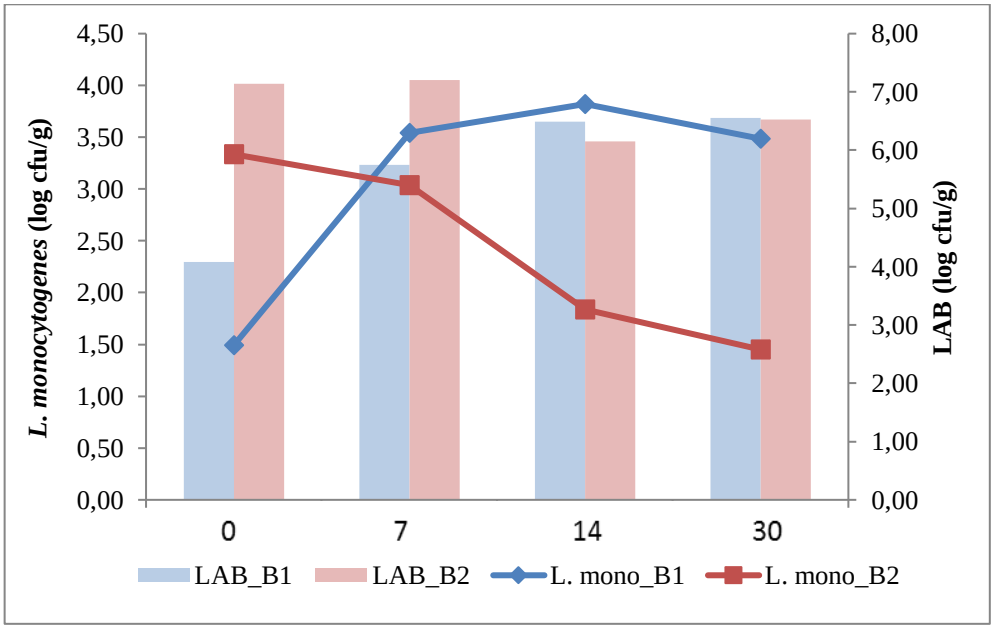


Figure 13: Evolution of *L. monocytogenes* (lines) and LAB (sticks) in butter during storage

B1: butter made from cream maturated at 4°C
B2: butter made from cream maturated at 14°C

Acidic butter, a product not allowing the growth of *L. monocytogenes*

Results showed that butter made from cream matured at 14°C (B2) presented a δ ≤ 0.5 log cfu/g. The same results were obtained for three other batches of raw milk butter produced later under the same conditions (maturation at 14 °C for 3 days and storage at 9 °C for 30 days) as presented in Table 20. These were realized in accordance with ISO 20976 (ISO, 2019).

Table 20: *L. monocytogenes* evolution in raw milk acidic butter produced during additional challenge tests

	pH butter	***L. monocytogenes* (log cfu/g)**					***δ (log cfu/g)****
		D0	**D7**	**D14**	**D21**	**D30**	
Batch1	4.60 ± 0.00	3.26 ± 0.32	2.19 ± 0.26	1.19 ± 0.36	0.95 ± 0.00	0.95 ± 0.00	0.00
Batch2	4.67 ± 0.06	4.33 ± 0.28	4.04 ± 0.04	2.58 ± 0.19	1.72 ± 0.36	1.00 ± 0.00	0.00
Batch3	4.70 ± 0.00	4.58 ± 0.02	4.48 ± 0.00	3.20 ± 0.19	2.36 ± 0.04	1.30 ± 0.30	0.00

*δ (log cfu/g) = max (log cfu/g during the test with intermediate measures) – level at day 0 (log cfu/g)

Metagenetic analysis showed that this butter is characterized by an abundance of *Lactococcus* spp which belong to LAB. This can explain the non-growth of *L. monocytogenes* in this butter. As presented in Figure 13, levels of LAB were higher in acidic butter (B2) than in sweet butter (B1), especially the first 7 days. It has also been observed that the abundance of *Lactococcus* spp was different between blank and contaminated samples. By combining the counts of the total flora and the proportions obtained in metagenetics, the concentration of *Lactococcus* spp was estimated. As shown in Table 21, the latter was higher in inoculated samples than in blank samples especially in the first two weeks. The inhibitory effect of LAB against *L. monocytogenes* was largely covered in literature. All genera of LAB are able to produce a broad spectrum of bacteriocins which are known for their antimicrobial activity (Tumbarski et al., 2018). Nisin, the best-known LAB bacteriocin produced by *Lactococcus lactis*, has a large antimicrobial spectrum, against Gram-positive and - negative bacteria (Ahmad et al., 2017). LAB also produce lactic acid known for its inhibitory effect on the growth of microbial pathogens (Wang et al., 2015).

Table 21: Evolution of the concentration of *Lactococcus* in blank (BL) and inoculated (IN) samples of butter (B2) during storage

	***Lactococcus* (log cfu/g)**			
	J0	**J7**	**J14**	**J30**
BL_butter	4.98	5.00	4.67	4.73
IN_butter	5.50	5.51	4.90	3.83

- Revision of food safety criterion for acidic butter

Despite the high level of contamination in acidic butter at the beginning of storage (3.34 ± 0.64 log cfu/g), levels of *L. monocytogenes* decreased during butter storage and a growth potential ≤ 0.5 log cfu/g was obtained. According to EURL-Lm technical guidance document for conducting shelf-life studies on *L. monocytogenes* in RTE foods (Beaufort et al., 2014), this butter can thus be considered not allowing the growth of *L. monocytogenes*. The batch presented a pH value of 4.75 ± 0.04 and an a_w value of 0.98, which are considered favorable for the growth of the pathogen. A revision of food safety criterion for this product has been requested to the FASFC. If allowed, acidic butter can be 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 therefore benefiting from a limit of 100 cfu/g before marketing. The demand is still in progress, and it concerns raw milk butter with pH < 4.8 regardless of the cream acidification conditions. During the survey, 40% of the samples collected from the Walloon market presented a pH < 4.8. In Advice 11-2019 of the Scientific Committee established at the FASFC on the growth potential of *L. monocytogenes* in raw milk butter, it was stated that the risk of growth of the pathogen is low if the cream pH drops below 5.2 after the first 10 hours of the maturation (FASFC, 2019). Control of cream acidification makes it indeed possible to "modulate" the development of *L. monocytogenes*. However, the results show (Figure 13) that even if the cream pH does not drop below 5.2 after 10 hours (slow acidification), the growth of *L. monocytogenes* does not occur in butter, provided that pH butter is < 4.8. Moreover, the results of the laboratory tests and the production monitoring conducted in the first experimental study (Chapter III) show that the limit of 5.2 cannot be reached in 10 hours even in the presence of starter cultures. The limit of 5.2 in 10 hours is unattainable. Based on the results obtained in this study, an alternative would be a cream pH limit of 5.2 at the end of maturation, provided that the maximum maturation period is 4 days.

- Use of starter cultures and pH recording

Although acidic butter (pH < 4.8) was found not allowing the growth of *L. monocytogenes* regardless of the rate of acidification, cream acidification must nevertheless be controlled. Not only can the development of *L. monocytogenes* be modulated by controlling cream acidification, but also that of other microorganisms. The first check point is pH. It is one of the factors to limit the growth of microorganisms, based on which, food safety criteria are defined. Monitoring of this parameter is thus essential. This would require from producers some investments, whether in terms of equipment or time. In fact, producers need to possess a pH-meter with a precision of at least 0.1 pH unit. They also need to learn how to adequately use it (calibration, maintenance, and cleaning) and perform an accurate measurement. This task can appear dreadful for the producers. However, it would help them to have a better control on their production process and allow them to act rapidly in case of an anomaly. Good manufacturing practices and HACCP remains good ways to limit products contamination. The least is to record pH butter for each production.

Getting a low pH is important, but so is the rate at which the latter is obtained. By increasing the acidification rate, the fermentation degree of cream is increased and thus the concentration of lactic acid produced by LAB. To accelerate the cream acidification, the use of starter cultures is recommended. Starter culture addition also affect butter's composition and structure by influencing the crystallization of milk fat. It favors the formation of more and smaller initial crystals (Herrera and Hartel, 2000a, 2000b). Milk fat crystallization which determines the spreadability, hardness and appearance of butter, is also influenced by the temperature regimes of ripening (Ceylan and Ozcan, 2020; Herrera and Hartel, 2000a, 2000b). The texture of butter is firmer when using a faster cooling compared to slow cooling (Wiking et al., 2009).

Sweet butter, a product at risk regarding the growth of *L. monocytogenes*

Contrary to acidic butter (pH < 4.8), an increase of *L. monocytogenes* was observed in butter made from cream kept at 4 °C during maturation phase (Figure 14). A growth potential of 2.33 log cfu/g was obtained using ISO calculation method ($\log_{max}$ - $\log_i$). This butter presented a high pH value (> 6.2, the maximum value observed during durability studies). The growth of *L. monocytogenes* is thus possible for butter made under the same conditions (maturation under refrigerated temperatures) and presenting thus a high pH value. During the survey, this practice was found used by 26% of the producers. For these latter, some options can be provided. One option would be milk pasteurization. The latter can provide a reasonable margin of safety given the effect of heat treatment on the inactivation of *L. monocytogenes* (Casadei et al., 1998; Doyle et al., 2001; Farber et al., 1992). Nevertheless, pasteurized milk butter can still be contaminated due to post-pasteurization and post-processing steps.

Another option would be to demonstrate that the product will not exceed the limit of 100 cfu/g throughout the shelf-life using predictive microbiology.

- Predictive microbiology, a useful tool to develop

Traditionally, the study of food safety has been addressed through a microbiological approach based on end-products and in-process samples testing. However, in the last few decades, preventive approaches were incorporated into food safety namely Hazard Analysis Critical Control Points (HACCP) and risk assessment (Griffith, 2006). To help the decision making of Critical Control Points (CCP) in HACCP and risk assessment, predictive microbiology is a powerful tool (Fakruddin et al., 2012). Predictive microbiology or quantitative microbial ecology consists of a simulation of the behavior of microorganisms under a variety of conditions summarized as equations or mathematical models (McMeekin, 1997; Rosso, 2009). The literature provides various models based on microbial culture media for the growth of *L. monocytogenes*; however, they may deviate from the actual behavior of bacteria in food. And, to our knowledge, there is no model available for the growth of *L. monocytogenes* in butter.

Predictive microbiology makes it possible, "from available observations on the growth, survival or inactivation of bacteria depending on environmental factors, to predict the responses of the same microorganisms under other conditions, by monitoring environmental factors" (Delhalle et al., 2012). For that, two steps are

required. The first is to describe the evolution of the concentration of microorganisms over time in a given environment (primary modeling). Then, describe the influence of the environmental factors on the primary parameters (secondary modeling) (Augustin, 1999).

Primary modeling

To estimate the primary parameters characterizing the kinetics of bacterial growth, the primary model is to be fitted to the observed growth curves. However, several models have been published and depending on the model used, estimations can be different. The first model is the exponential model. It describes the exponential phase, but does not take into account the lag phase or the stationary phase (Buchanan, 1918). Then, there is Gompertz model based non-linear functions. This model does not show a good ability to fit experimental data and results in an overestimation of the maximum growth rate and lag time (Augustin, 1999; López et al., 2004). In the opposite, Baranyi model, one of the most used models, gives satisfactory results (Lobacz et al., 2013; López et al., 2004; Thomas et al., 2019). This model not only describes the exponential growth phase, but can also characterize the transition between lag phase and exponential phase by accounting for biological factors that affect bacterial growth (Baranyi and Roberts, 1994; Delhalle et al., 2012).

In an attempt to estimate the growth rates of *L. monocytogenes* in sweet butter as a function of time, challenge tests for *L. monocytogenes* were performed as described in a previous study by El-Hajjaji et al. (2021). Three batches of butter were produced from inoculated cream stored for 3 days at 4 °C. Each batch was stored for 30 days at 7 °C, 9 °C or 12 °C. The obtained data was then applied to the Baranyi model using Excel add-in DMFit (Baranyi and Roberts, 1994). Even though the predicted values were a good statistical fit to the observed data, we couldn't go further due to the limited data. First, during these challenge tests, analyses were conducted at: 0h, 2 days (48h), 4 days (96h), 5 days (120h), 7 days (168h), 14 days (336h) and 30 days (720h) of storage. As shown in Figure 14, only two points are included in the exponential phase. Additional analyses are thus needed, especially during the first 48h for a more accurate and presice kinetic parameters. Also, only three temperatures were tested, which is rather few to be able to get a good model.

Another point of attention is that the data used to develop the model presented high levels of contamination. As shown in durability studies, levels of contamination are generally low (< 10 cfu/g). The potential influence of the level of the initial contamination on the subsequent growth has to be tested. The usage of the model in these cases has to be evaluated.

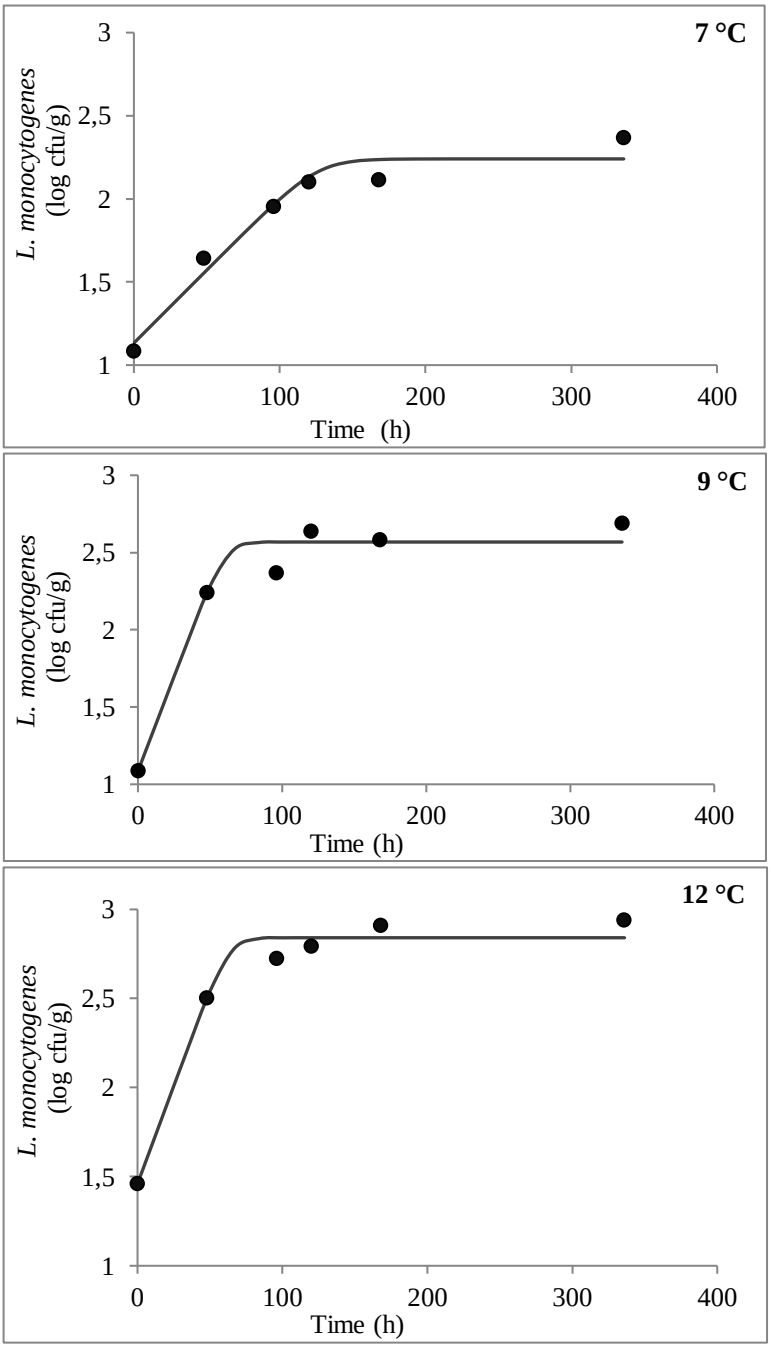


Figure 14: Growth of *L. monocytogenes* in raw milk sweet butter at different storage temperatures

Points correspond to observed *L. monocytogenes* populations at different time intervals, and the curves to the Baranyi models fitted to the *L. monocytogenes* populations at different temperatures.

Secondary modeling

To describe the relation between the factors and the growth parameters, several secondary models can be used. These can be distinguished on two groups based on the approach used (Perez-Rodriguez and Valero, 2013). The first approach studies the effect of environmental factors individually and it is applied in the cardinal model and the Ratkowsky-type model (square root-type) among others (Perez-Rodriguez and Valero, 2013). The two models, as well as the others using the same approach, are characterized by a minimum number of parameters with a biological significance and are robust (Augustin, 1999; Delignette-Muler cited in Le Marc et al., 2002). However, while the cardinal model considers the entire range of temperature allowing growth, the Ratkowsky model considers only the suboptimal range (Ratkowsky et al., 1983; Rosso et al., 1995). In accordance with the storage temperatures used for butter, the use of the Ratkowsky model is more appropriate. The second approach allows the description of the simultaneous effect of several environmental factors through a polynomial function (Perez-Rodriguez and Valero, 2013).

The behavior of microorganisms in food can be affected by several factors, including the product's characteristics and the storage conditions. In our attempt, only the factor of temperature was included. Temperature is an important environmental factor to control microbial growth. For *L. monocytogenes*, the bacterium can grow at -1.5 to 45 °C with an optimum between 30 and 37 °C (Magalhães et al., 2014). However, other factors are important as well. For butter, these factors include pH, salt, water dispersion and the presence of inhibitors or competitive microflora like LAB (El-Hajjaji et al., 2020b). The effect of pH and LAB was highlighted in this thesis where butter with low pH (high counts of LAB) was found not allowing the growth of *L. monocytogenes*, contrary to high pH butter (low counts of LAB). As for salt, although not tested in the challenge studies, it can reduce the water activity of the substrate and thus the available water for microorganisms (Elias et al., 2020; Man, 2007; Ravishankar and Juneja, 2014). In fact, during durability studies, it was found that salt was negatively correlated with *L. monocytogenes*. No statistical relationship was found though (p value > 0.05). Regarding water distribution, studies have demonstrated that water droplets size and distribution is a key parameter in preventing the growth of *L. monocytogenes* (Michelon et al., 2016; Voysey et al., 2009). These factors need to be included in the model as well. Further challenge tests and repetitions are needed, preferably with various batches (various creams from various farms), to collect enough data for the model.

In this work, we focused on LAB and bacterial composition. However, other microorganisms including yeasts could also contribute to explain the behavior of *L. monocytogenes* in raw milk butter. Goerges et al. (2006) found that all tested yeasts had an inhibitory potential on *L. monocytogenes*. it would be interesting to study eukaryotic microorganisms.

Another point to consider for the model is the interactions between factors. As shown in Table 22, pH values and evolution were different between batches. The same is observed for LAB. The growth of LAB is relative to temperature, and so is pH. With the first approach of secondary modeling, used in Ratkowsky model, interactions

between ecological factors and their effect are neglected (Augustin, 1999). However, model extinsions are available and continue to be developed to include factors' interactions (Augustin and Carlier, 2000; Ross et al., 2003). Another option would be to use the polynomial model. However, these models quickly become difficult to handle because of their large number of parameters (Augustin and Carlier, 2001).

Table 22: pH and LAB (mean ± standard deviation) of contaminated butter samples during storage at different temperatures

Time (h)	pH			LAB (log cfu/g)		
	7 °C	9 °C	12 °C	7 °C	9 °C	12 °C
0	6.63 ± 0.06	6.50 ± 0.00	6.60 ± 0.00	5.18 ± 1.14	4.71 ± 0.40	4.50 ± 0.05
48	6.40 ± 0.00	6.40 ± 0.00	6.33 ± 0.06	4.81 ± 0.23	5.74 ± 0.17	5.80 ± 0.23
96	6.77 ± 0.12	6.37 ± 0.12	6.17 ± 0.06	5.71 ± 1.06	6.50 ± 0.01	7.11 ± 0.11
120	6.40 ± 0.00	6.07 ± 0.06	5.93 ± 0.15	5.10 ± 0.10	7.11 ± 0.06	7.15 ± 0.10
168	6.20 ± 0.00	5.90 ± 0.00	5.83 ± 0.06	4.77 ± 0.35	6.54 ± 0.01	6.95 ± 0.35
336	6.00 ± 0.00	5.70 ± 0.00	5.70 ± 0.00	5.40 ± 0.20	6.74 ± 0.40	6.67 ± 0.24
720	NA	5.50 ± 0.00	5.50 ± 0.00	NA	6.87 ± 0.11	6.64 ± 0.10

L. monocytogenes in other types of butter?

In this thesis, we could decide on the behavior of *L. monocytogenes* in two types of raw milk butter, acidic butter (< 4.8) and sweet butter. However, during the survey, it was found that 75% of the samples presented a pH value < 5.6 while the mean value was 5.12. This range was unfortunately not covered during challenge tests, which would have been interesting.

For these producers, one option is, as mentioned before, addition of starter cultures and pH monitoring. The other option is to fund challenge studies on their products to potentially beneficiate from individual revisions of food safety criteria. In this study, we chose to inoculate in the cream to simulate a contamination at the beginning of the production process. This approach allows the obtention of more realistic and homogeneous contamination. To carry out this approach, a fully equipped plant with biosafety level 2 is required where the production takes place. For the producers, it is a difficult approach to perform. An alternative approach would be to inoculate *L. monocytogenes* in butter after production. The advantage of this method is that no logistic requirements are needed. The drawback is that it does not allow a monitoring of the contamination with *L. monocytogenes* during manufacturing. Another drawback is that the contamination may be concentrated on one part of the product due to inoculation procedure.

As shown through durability studies, contamination of raw milk butter with *L. monocytogenes* is occasionnal and generally with low levels. Good hygiene application could prevent the transmission of the pathogen to the food. It is thus important to identify the sources of contamination. It would have been interesting if *L. monocytogenes* and other microbiological analyses were conducted on samples

(surfaces and products) taken from different stages of the production from milking to packaging.

Conclusion

Globally, this thesis contributed to the general knowledge on Walloon raw milk butter and its artisanal production. The present study also addressed the behavior of *L. monocytogenes* in this product. Raw milk butter is a typical indigenous product made without any heat treatment and without much use of starter cultures. It presents various physico-chemical characteristics mainly pH. This variation is especially due to maturation temperature. Maturation temperature has a strong influence on raw milk butter subdominant microbiota, which affects the product's characteristics and thus the growth of pathogenic bacteria like *L. monocytogenes*. For butter made from cream matured at room temperature (14 °C), no growth of the pathogen was observed. This butter presented low pH values (< 4.8). This work allowed identifying a novel threshold value of 4.8 at the end of manufacture as a limit for the growth of *L. monocytogenes* in raw milk butter, instead of the commonly accepted value of pH 4.4. The role of LAB as inhibitor of *L. monocytogenes* was also supported. Refrigerated maturation leads to high pH butter (≥ 6.5) that allows the growth of *L. monocytogenes* during storage.

It can also be concluded that the use of starter cultures is an efficient way to limit the growth of *L. monocytogenes*. Maintaining good manufacturing practices and HACCP remains good way to limit products contamination. A regular record of pH butter at each production is thus recommended.

The present work was the first research to present a full study on the behavior of *L. monocytogenes* in raw milk butter. However, it cannot pretend having explored all the related aspects including risk factors. In fact, milking and processing environment are source of many contaminations including *L. monocytogenes*. An assessment of the microbiological quality of these environments would be interesting to evaluate the association between the contamination of raw milk butter with *L. monocytogenes* and the different stages of production.

Besides, this thesis didn't allow covering the growth potential of *L. monocytogenes* in raw milk butter with pH from 4.9 to 5.6 (the latter is the highest value observed in 75% of the samples from the Waloon market). Also, although a range of likely factors (i.e., pH, water distribution, salt content, LAB, storage temperature and microbiota) were investigated during this thesis through durability studies and challenge tests, these factors were not all included in both experiments. It would be interesting to combine all these factors in a challenge test, which was proven to be more suited to study the behavior of *L. monocytogenes* in a food product and assess its growth potential. It would also be interesting to investigate other factors like fatty acids or eukaryotic microorganisms.

Furthermore, further experimental studies and repetitions are needed to better characterize the kinetics and the behavior of *L. monocytogenes* and develop a

predictive tool that can provide valuable assistance to producers. Therefore, this study should be a starting point for further risk assessment studies on raw milk butter.

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