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

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ABSTRACT

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

KEYWORDS

Challenge test, *Listeria monocytogenes*, cheese, growth potential, intra-batch variability, inter-batch variability.

# Introduction

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

Criteria regarding the presence of *L. monocytogenes* in RTE foods are strict. Following Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs, *L. monocytogenes* should not reach a contamination level above 100 cfu/g during shelf-life. Furthermore, before placing the food on the market, the pathogen must remain undetected in 25 g of RTE foods allowing its growth. Based on data available on the growth/no growth of *L. monocytogenes* in food, this regulation also identifies three situations in which one can consider that the growth of *L. monocytogenes* is not permitted. Consequently, pH ≤ 4.4, water activity (aw) ≤ 0.92, or a combination of pH ≤ 5.0 with aw ≤ 0.94 are considered sufficient to prevent growth of the pathogen. When a RTE food is not considered as allowing this growth, a contamination level of 100 cfu/g is also tolerated before placing the food on the market.

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

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

Besides the physicochemical characteristics of cheese, predictive models and comparison with the scientific literature also allow estimation of the fate of *L. monocytogenes* in a given cheese. Nevertheless, traditional and/or artisanal cheeses are sometimes obtained by a particular production process, or present specific characteristics. In Belgium, more than 230 artisanal cheesemakers have been identified during a survey, producing some particular traditional products like Maquée, Boulette, Abbaye and Herve (unpublished results). It is thus difficult to use growth models or the literature to assess if these cheeses could permit the growth of *L. monocytogenes* (Alvarez-Ordonez et al., 2015). Regulation (EC) No 2073/2005 allows cheesemakers to demonstrate, to the satisfaction of the competent authority, that *L. monocytogenes* is not able to grow and exceed a contamination of 100 cfu/g in their products. In this case, contamination up to 100 cfu/g before sales is tolerated (EC, 2005). Several studies can be performed by the producers to reveal the fate of *L. monocytogenes* in cheese, including challenge tests and durability studies. In Europe, various documents are available for food business operators in order to perform challenge tests, namely guidance documents published by the Directorate-General of Health and Consumers (DG SANCO, 2008) and European Union Reference Laboratory for *Listeria monocytogenes* (EURL *Lm*, 2014). In Belgium, the Federal Agency for the Safety of the Food Chain (FASFC, 2016) has also published a scientific opinion related to challenge tests and shelf-life studies for *L. monocytogenes* in cheese.

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

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

# Materials and methods

## *2.1 Sampling and definitions*

Previously, a survey of artisanal cheese producers allowed the identification of the major types of cheese produced in Belgium (unpublished results). A sampling plan was designed in order to select 32 cheeses, representative for the diversity of products found in Belgium, from 32 farmhouses. All batches were collected between July 2018 and March 2019. The classification of cheeses was based on texture and/or ripening, as suggested by the Codex Alimentarius (2006). The study considered (a) unripened acid-curd cheeses, including acidified cheeses consumed without any ripening, (b) mold-ripened soft cheeses, unpressed cheeses with a typical white crust mainly composed of *Penicillium camemberti*, (c) smear-ripened soft cheeses, unpressed cheeses regularly washed with water, brine or smear during ripening and with a typical red crust, and (d) semi-hard cheeses, pressed cheeses with moisture on a fat-free basis (MFFB) higher than 54. Hard cheeses (MFFB < 54) are uncommon in Belgium and were not included in the sampling plan. For each type of cheese, products made from pasteurised as well as from raw milk were analysed.

## *2.2 Determination of the number of batches*

Before collection of whole batches, isolated samples of each cheese were collected in order to measure their pH and aw. The theoretical growth potential (δth) of *L. monocytogenes* in each cheese was predicted using Sym’Previus (Leporq et al., 2005). Selected storage conditions were the same as described in detail in section 2.4 for challenge tests. As advised by EURL *Lm* (2014), it was decided to collect one batch if δth ≤ 0, and three batches if δth > 0. For each batch, at least 12 samples were collected directly after production or after ripening, for unripened and ripened cheeses, respectively.

## *2.3 Cocktail of strains*

In order to avoid bias associated with the use of a unique strain of *L. monocytogenes*, a cocktail of three strains was used to inoculate cheeses. The three selected strains, namely 12MOBO53LM, 12MOBO96LM and 12MOBO98LM, were isolated from dairy products and are suggested by EURL *Lm* (2013) for use during challenge tests. Strains were provided by EURL *Lm* in the form of cryobeads. The latter were suspended separately in 9 ml of brain heart infusion (BHI broth) and incubated at 37 °C for 18 h. One hundred microliters of this culture was diluted into 9.9 ml of BHI broth and incubated at 7 °C for 7 days. Equal quantities of the subculture containing each strain were mixed in a unique tube.

## *2.4 Inoculation*

Among the 12 samples of each batch, six were inoculated with the cocktail of strains. This moment was considered as day-0 (D0). Remaining samples were used as controls. The targeted inoculum level was 100 cfu/g of cheese, as advised by FASFC (2016). The inoculation procedure varied between types of cheese. White cheese was homogenised directly after inoculation. Other unripened acid-curd cheeses were more solid but had no crust and were considered as homogeneous. The cocktail of strains was thus only inoculated in the core, with a single injection. Crusts of smear- and mould-ripened soft cheeses are generally eaten by consumers. It was decided to inoculate both core and surface for these types of cheese. *L. monocytogenes* was only inoculated in the core of semi-hard cheeses. Some semi-hard cheeses have an artificial and inedible coating on their surface, and discerning the difference between artificial and natural crusts is quite difficult for consumers. The volume of inoculum did not exceed 1 % of the cheese mass (EURL *Lm*, 2014). Depending on the samples and on the concentration of the mixed cultures, proper dilutions of the latter were thus required. Cheeses were cut into pieces of at least 50 g. Cores were inoculated with a single injection. For inoculation on the surface, the volume was divided into small droplets on the surface and spread with a sterile spreader. Inoculation was judged as satisfactory when the standard deviation of triplicate counts of *L. monocytogenes* for inoculated samples at D0 was lower than 0.5.

## *2.5 Storage*

Three inoculated samples and three controls were directly analysed at D0 (see sections 2.6 and 2.7). White cheese was stored in its original container. All other types of cheese were wrapped in polyethylene film. Given that the term ‘cheese’ includes a huge variety of products, it was not possible to use the same storage scheme during all challenge tests. As an example, unripened acid-curd cheeses can generally not be stored for more than 14 days, while ripened soft and semi-hard cheeses can be kept for at least 30 days at refrigeration temperature. During the challenge tests, storage duration followed the recommendations provided by each producer. As advised by EURL *Lm* (2014) and FASFC (2016), samples with a shelf-life ≤ 21 days were always stored at 7 °C for two-thirds of the shelf-life, before being stored at 9 °C for the remaining third of shelf-life. When the shelf-life was > 21 days, samples were stored at 7 °C for the first half of the shelf-life, and at 9 °C for the second one. At the use-by date, all remaining inoculated and control samples were analysed.

## *2.6 Physicochemical analyses*

At both D0 and use-by date, physicochemical characteristics of cheese samples were studied. In cheese cores, pH and aw were measured with an InLab Surface Pro ISM electrode (Mettler Toledo, Columbus, OH, USA) and an Aqualab 4TE water activity meter (Decagon Devices Inc., Pullman, WA, USA). ISO method 5534 was used to determine dry matter content (International Organization for Standardization, 2004b). Salt and fat contents were only tested at D0, since they always stay the same relative to the dry matter content. A potentiometric titration of chloride ions with a 0.1 M silver nitrate solution was used to determine salt content (International Organization for Standardization, 2006). Fat was treated with hydrochloric acid and extracted with petroleum ether and diethyl ether (International Organization for Standardization, 2004a).

## *2.7 Microbiological analyses*

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

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

## *2.8 Challenge test interpretation*

For each batch, two methods were compared to calculate δ. The first method is based on EURL *Lm* (2014) guidelines. δ was considered as the difference between the median contamination at use-by date and the median contamination at D0, expressed as log10 cfu/g. Otherwise, δ was calculated as the difference between the highest contamination at the use-by date and the lowest value at D0 (FASFC, 2019). The latter method is more stringent and allows intra-batch variability to be taken into account, as suggested by Lahou and Uyttendaele (2017). For both calculation methods, the highest δ of the three batches was used to conclude the fate of *L. monocytogenes*, in order to consider the worst case. Results were compared with δth and considered by type of cheese. When δ was higher than 0.5 log10 cfu/g, the product was considered as potentially suitable for the growth of *L. monocytogenes*. For the opposite, food was recognised as not suitable for the pathogen (EURL *Lm*, 2014).

## *2.9. Statistical* *analyses*

All statistical treatments were performed using Minitab 18 (State College, PA, USA). Provided that the number of cheese samples varied between cheese types, generalized linear models were built to look for potential significant differences for each physicochemical or microbial factor. Tukey’s HSD test was used to perform multiple comparisons. Kruskal-Wallis test was performed when variance homogeneity or data normality were not fulfilled, and Dunn’s test was used for multiple comparisons.

# Results and discussion

## *3.1 Characterization of cheeses*

Table 1 summarizes the physicochemical parameters measured for all cheeses. Statistical differences between types of cheeses are also presented. Globally, at D0, for all types of cheese, the variability in pH was limited. Regarding unripened acid-curd cheeses, average pH was just above the threshold value of 4.4 provided by Regulation (EC) No 2073/2005. Other types of cheeses had less acidic pH. All pH measurements were performed in cheese pastes. Values for ripened cheeses, in the case of natural crusts, would have been higher if pH was measured on the surface, due to the metabolic activity of the ripening microflora (Mounier et al., 2005). The variability in aw values was limited, but averages were significantly different for all categories, except between mold-ripened and smear-ripened soft cheeses (p-value < 0.001). However, no samples had a sufficiently low aw to theoretically prevent the growth of *L. monocytogenes*, i.e. aw ≤ 0.92. Globally, pH and aw values of ripened cheeses were similar to those found in the literature (Gérard et al., 2018). Variations were more important regarding dry matter and salt and fat content. Average fat content of unripened acid-curd cheese was much lower because four out of 12 samples were made from skimmed milk. Unripened acid-curd cheeses were not salted during their production, but an average salt content of 0.4 ± 0.4 % was observed. No significant differences in dry matter content were observed between D0 and the end of shelf-life (all p-values > 0.220). During storage, aw did not vary significantly (all p-values > 0.690). Regarding pH, a significant increase was observed for all types of cheese. In soft cheeses, average pH increased by more than one unit.

Total microflora, LAB, *E. coli* and yeasts and moulds were enumerated at D0 and at the end of shelf-life. Enumerations and statistical differences are presented in Table 2. *E. coli* is an indicator of hygiene during cheese production. For all types of cheese, average *E. coli* loads at D0 were between 1.9 and 2.5 log10 cfu/g. These levels are lower than those observed by Lahou and Uyttendaele (2017) for Belgian artisanal cheeses. In 38 % of the samples, *E. coli* levels did not exceed 1 log10 cfu/g. Average *E. coli* counts decreased significantly during shelf-life of unripened acid-curd cheeses (p-value = 0.045); however, that was not the case in ripened soft cheeses and semi-hard cheeses.

Given that cheese is a fermented product, total microbial load was generally very high, reaching 8.3 log10 cfu/g in some samples. Comparable levels were observed by Lahou and Uyttendaele (2017) in soft and semi-hard cheeses. Total microflora remained at the same level during shelf-life (all p-values > 0.050). Standard deviations were limited, meaning that microbial load was comparable between cheeses made from pasteurised milk and from raw milk. This is in accordance with the observations of Delcenserie et al. (2014). LAB represent the majority of the total microflora, whether coming from starters or not (Gobbetti et al., 2018). At D0, yeasts and moulds counts were lower in unripened acid-curd cheeses and semi-hard cheeses (p-value < 0.001), in comparison with both types of soft cheese. At the end of the shelf-life, yeasts and moulds counts increased by 2 log10 cfu/g in unripened and semi-hard samples (p-value < 0.001, while they remained at the same level in soft cheese (p-value > 0.700).

## *3.2 Study of the growth potential of L. monocytogenes*

Table 3 summarizes the results of the challenge tests, and the growth potential of *L. monocytogenes* following the two methods of calculation. All initial contaminations ranged from 30 to 300 cfu/g and were thus satisfactory regarding available guidelines (FASFC, 2016). Globally, real δ was always lower than δth, except for challenge test SH10 with the most stringent calculation method. This is not surprising given that current models are only based on data obtained *in vitro* (Kapetanakou et al., 2017). Growth models on cheese matrices remain unavailable on the major online modelling platforms, including Sym’Previus and ComBase (Baranyi and Tamplin, 2004; Leporq et al., 2005). Aside from pH and aw, some intrinsic factors of the cheese matrix are not taken into account by current models, including cheese microbiological characteristics. As a consequence, growth models often overestimate the growth of *L. monocytogenes*, and this enlightens the importance of performing challenge tests in order to obtain more realistic growth data.

Results were contrasted between types of cheese. In unripened acid-curd cheeses, the pathogen was never able to grow, regardless of the method of calculation. In 20 out of 36 samples analysed at the end of shelf-life, *L. monocytogenes* levels dropped under the limit of detection (i.e. 10 cfu/g). No samples had a contamination above 100 cfu/g at the end of shelf-life. With the most stringent method of calculation, all δ were between 0.00 and −1.45 log10 cfu/g. δ were comparable between unripened acid-curd cheeses produced from raw milk and from pasteurised milk. Belgian unripened cheeses are produced by extended lactic acidification, before shaping or not, and cannot be compared with Hispanic-style unripened cheeses, including Queso Fresco which is mainly obtained by adding rennet to milk, and which has been extensively studied (Ibarra-Sanchez et al., 2017). Queso Fresco has a high aw, a salt content of approximately 1.0 % and a nearly neutral pH. This RTE food is thus favourable for the growth of *L. monocytogenes* (Ibarra-Sanchez et al., 2017).Whey cheeses and buttermilk cheeses are also considered as unripened cheeses, but cannot be compared with unripened lactic-curd cheeses studied in this paper. Unripened acid-curd cheeses analysed during this study had an aw higher than 0.99, and a low salt content (0.4 % on average) but had a much more acidic pH, slightly higher than the threshold value for no growth of *L monocytogenes* (i.e. 4.4). For Galotyri, a product more comparable to Belgian unripened acid-curd cheeses, a similar decrease of *L. monocytogenes* levels was observed, although the inoculum levels were higher, i.e. 3 to 7 log10 cfu/g (Rogga et al., 2005). In contrast, the pathogen remained at 2 log10 cfu/g during 7 days of storage at 4 °C of an Irish unripened acid-curd cheese with pH 4.3 (Schoder et al., 2003). Similarly, in a cottage cheese with pH 5.03, an aw of 0.99 and 1.0 % salt, levels of the pathogen remained constant during the whole storage period at 7 °C (Kapetanakou et al., 2017).

Contrary to unripened acid-curd cheeses, mould- and smear-ripened soft cheeses are suitable for the growth of *L. monocytogenes*. For this type of product, three batches were always studied, since δth was always > 0. Growth potentials up to 4.7 log10 cfu/g have been observed, even with the EURL *Lm* (2014) calculation based on median enumerations. During the storage of a similar cheese at 7 °C for 14 days, Lahou and Uyttendaele observed δ of up to 1.92 log10 cfu/g. These δ are lower than those found in the present study, but the shelf-life was longer during the latter, and *L. monocytogenes* thus had more time to grow. This type of product has to be considered as dangerous for food safety, even in cases of low initial contamination with the bacterium. During this study, the cocktail of *L. monocytogenes* strains was distributed between the core and the crust. It is well known that the surface of mould- and smear-ripened cheeses represents a highly favourable medium for growth of the pathogen (Dalzini et al., 2017). For instance, Back et al. (1993) observed that *L. monocytogenes* did not grow in the core of Camembert during 40 days of refrigerated storage, but its levels increased by 2 log10 cfu/g on the rind. Furthermore, yeasts can favour the growth of *L. monocytogenes* (Corsetti et al., 2001). Surprisingly, for challenge test SRSC1, all batches had a negative δ. This product thus has to be considered unsuitable for the growth of *L. monocytogenes* (EURL *Lm*, 2014). By investigating this cheese in more detail, it was observed that it did not differ significantly from other smear-ripened soft cheeses in terms of pH, aw, dry matter, salt content, fat content and microbial counts. A potential hypothesis would be that the microflora of this cheese includes particular LAB able to act against *L. monocytogenes*. In cheeses contaminated with 100 cfu/g of *L. monocytogenes*, Morandi *et al.* (2019) observed an inhibitive action of some LAB species, including *Carnobacterium* spp., *Lactobacillus sakei* and some strains of *Lactococcus lactis*. This hypothesis should be confirmed using metagenetics.

Regarding semi-hard cheeses, contrasting results were observed. *L. monocytogenes* levels decreased during the storage of all batches of semi-hard cheeses made from pasteurised milk, following EURL *Lm* (2014) calculation. This was not the case for all samples made from raw milk. For semi-hard cheeses made from raw milk, huge intra- and inter-batch variability was observed. Four out of nine cheeses showed at least one positive δ among the three batches studied, with the EURL *Lm* (2014) method of calculation. During challenge tests SH4, SH6, SH8 and SH11, opposite tendencies were observed between batches regarding growth of the pathogen (Table 3). For instance, during challenge test SH8, a decrease of approximately 1 log10 cfu/g was observed in the first batch; *L. monocytogenes* remained at a level close to the inoculum in a second batch, while an increase of 1 log10 cfu/g was observed in the last batch. No significant inter-batch differences were identified regarding pH and aw. These differences could be associated with bias introduced by inoculation of the pathogen directly into cheese cores, including variation of the inoculum’s dispersion in cheese.

Considering the EURL *Lm* (2014) method of δ calculation, 30 out of 32 batches of semi-hard cheeses did not show substantial growth (i.e. δ ≤ 0.5 log10 cfu/g), meaning that these products would not represent a threat to food safety in cases of low contamination, i.e. < 10 cfu/g, at D0. Regarding the two remaining batches, with δ > 0.5 log10 cfu/g, the absence of *L. monocytogenes* in 25 g must remain compulsory. Positive δ have already been reported in Belgian semi-hard cheeses stored at 7 °C for 14 days (Lahou and Uyttendaele, 2017). In contrast, inoculation studies on Edam and Gouda contaminated after ripening did not show any growth of *L. monocytogenes* during storage (Kapetanakou et al., 2017).

As a reminder, the goal of a challenge test is to classify an RTE food as suitable or not for the growth of *L. monocytogenes*, depending on whether δ is lower or higher than 0.5 log10 cfu/g. Nevertheless, looking at absolute contamination levels in semi-hard cheeses, five extra batches have to be considered as potentially allowing growth of the pathogens. Indeed, contamination of up to more than 4.0 log10 cfu/g was observed (challenge test SS12). These high levels are totally ignored when δ is calculated from median values, remaining lower than 0.5 log10 cfu/g. While this method of calculation had no influence on the results of challenge tests for unripened acid-curd cheeses and mould- and smear-ripened soft cheeses, it led to underestimated growth in semi-hard cheeses. According to the chosen approach, food safety considerations were thus totally changed. The issue of intra-batch variability has already been pointed out by Lahou and Uyttendaele (2017) and FASFC (2019), for semi-hard cheese and butter, respectively. A hypothesis could be that the method of inoculation in cheese cores could introduce bias responsible for this intra-batch variability. In the case of Lahou and Uyttendaele’s (2017) study, using extreme values would not have changed the conclusion regarding the potential growth of *L. monocytogenes* in the cheese samples concerned. The only effect would have been an increased extent of growth. In contrast, in the present survey, giving more attention to absolute contamination levels sometimes changed the conclusions on potential growth of the pathogen.

# Conclusion

The number of cases of listeriosis has increased during the last decade, as well as pressure has increased on artisanal producers, who are supposed to guarantee the absence of *L. monocytogenes* in 25 g of cheese. It thus remains important to identify RTE food allowing or not the growth of this bacterium. As a first approach, growth models remain an interesting solution, but they present extensively described drawbacks. Comparison with the literature is an alternative. Nevertheless, due to high variability between studies regarding inoculation level (1 to 6 log10 cfu/g), temperature of storage (from refrigeration to room temperature) or shelf-life duration, it is often difficult to make a proper comparison. Appropriate advice for producers would be to perform challenge tests for their products, with a standardised protocol, allowing them to make a more accurate comparison and to make a decision on the potential growth of *L. monocytogenes*. Indeed, as shown by the present study, each cheese has its own characteristics, and two products with similar pH, aw, dry matter and microbial counts can lead to opposite behaviours of the pathogen. A surprising example is the smear-ripened soft cheese from the present study, which combined all conditions favourable for the growth of the bacterium, as did all cheeses of the same type, but which did not allow its growth during challenge tests. Challenge tests on semi-hard cheeses allow indication of the issue of inter- and intra-batch variability, as well as the eventual bias linked to the choice of inoculation method. A growth potential calculated with median values does not guarantee that *L. monocytogenes* will not be able to reach levels > 100 cfu/g. Due to these phenomena, it seems logical to consider these cheeses as at-risk products. Nevertheless, a global conclusion seems possible for unripened acid-curd cheeses, obtained by lactic acid production by LAB or by direct acidification. None of the samples studied allowed the growth of *L. monocytogenes*. FASFC was invited to revise the current classification of these cheeses following Regulation (EC) No 2073/2005. Notwithstanding this, the presence of *L. monocytogenes* in RTE food should always been avoided, and a good cleaning and disinfection protocol, as well as Hazard Analysis and Critical Control Points, must be implemented. Similarly, in cases of contamination, proper investigations must be implemented to identify its origin.

Although the goal of challenge tests is to assess the growth potential of *L.*monocytogenes during RTE foods storage, it is important to note that the conclusions of this study could be improved by monitoring the evolution of the contamination during shelf-life. In further experiments, microbiological analyses, including *L. monocytogenes* enumeration, could be performed daily or weekly in order to identify an eventual early growth of the pathogens in some cheese varieties. Alternatively, a most realistic way to predict the growth of *L. monocytogenes* in cheese would be to inoculate the pathogen in milk, and to produce cheese with this raw material. However, this method has a lot of drawbacks which make it difficult to implement, including the necessity of performing preliminary studies to determine the cheese-specific inoculum to reach 100 cfu/g at the end of the ripening process. Another tricky point is to be able to mimic ripening conditions found in artisanal cheese factories at a laboratory scale.

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**Table 1**
Physicochemical characteristics of the four types of cheese at D0 and at the end of shelf-life (averages and standard deviations).

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Type of cheese | pH |  |  | aw |  |  | Dry matter (%) |  | Fat (%) D0 | Salt (%) D0 |
| D0 | ESL | Statistical letters | D0 | ESL | Statistical letters | D0 | ESL | Statistical letters |
| Unripened acid-curd | 4.4 ± 0.1a | 4.7 ± 0.4a | H1,72=16.3***p<0.001*** | 0.99 ± 0.01a | 0.99 ± 0.01a | F1,72=0.0p=0.938 | 25.9 ± 9.7a | 23.8 ± 7.8a | F1,23=0.3p=0.577 | 10.5 ± 8.4a | 0.4 ± 0.4a |
| Mold-ripened soft | 5.8 ± 0.6b,c | 7.0 ± 0.7b | H1,90=44.9***p<0.001*** | 0.97 ± 0.02b | 0.98 ± 0.02b | H1,90=0.0p=0.958 | 50.1 ± 3.0b | 49.9 ± 8.5b | F1,24=0.0p=0.942 | 26.3 ± 2.0b | 1.6 ± 0.8b |
| Smear-ripened soft  | 5.7 ± 0.3c | 7.0 ± 0.7b | H1,54=37.1***p<0.001*** | 0.97 ± 0.01b | 0.97 ± 0.01b | F1,54=0.1p=0.748 | 49.9 ± 4.1b | 47.8 ± 3.8b | F1,22=1.6p=0.227 | 24.6 ± 3.4b | 2.0 ± 0.4c |
| Semi-hard  | 5.8 ± 0.2b | 6.0 ± 0.3c | H1,186=30.5***p<0.001*** | 0.96 ± 0.01c | 0.96 ± 0.02c | H1,186=0.2p=0.696 | 59.7 ± 4.8c | 61.4 ± 5.7c | H1,61=0.8p=0.382 | 31.1 ± 2.7c | 1.6 ± 0.5b |
| Statistical analyses | H3,204=99.4 | H3,198=135.4 |  | H3,204=110.3 | H3,198=102.0 |  | H3,71=53.12 | F3,65=98.7 |  | H3,71=51.9 | H3,70=31.5 |
| P-values | ***<0.001*** | ***<0.001*** |  | ***<0.001*** | ***<0.001*** |  | ***<0.001*** | ***<0.001*** |  | ***<0.001*** | ***<0.001*** |

Legend : D0, first day of storage; ELS, end of shelf-life; aw, water activity; p-values written in italic bold are statistically significant; superscript letters allow identification of significantly different groups.

**Table 2**
Microbial counts at D0 and at the end of shelf-life for all types of cheese (averages and standard deviations).

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Types of cheese | Total microflora | LAB | *E. coli* | Yeasts and molds |
| D0 | ESL | Statistical letters | D0 | ESL | Statistical letters | D0 | ESL | Statistical letters | D0 | ESL | Statistical letters |
| Unripened acid-curd | 8.2 ± 0.5a,b | 7.7 ± 0.5a | H1,23=3.1p=0.079 | 8.1 ± 0.4 | 7.9 ± 0.5 | F1,23=0.9p=0.347 | 2.5 ± 1.6 | 1.4 ± 0.6 | H1,23=4.0***p=0.045*** | 3.2 ± 1.6a | 5.8 ± 0.4a | F1,23=12.3***p<0.001*** |
| Mold-ripened soft  | 7.9 ± 0.5a,b | 7.9 ± 0.6a,b | H1,24=0.2p=0.686 | 7.6 ± 0.8 | 7.6 ± 0.8 | F1,24=0.0p=0.961 | 2.3 ± 1.3 | 1.9 ± 1.5 | F1,24=0.7p=0.402 | 5.8 ± 0.5b | 5.8 ± 0.4a | H1,24=0.1p=0.729 |
| Smear-ripened soft  | 8.1 ± 0.2a | 8.2 ± 0.1b | F1,24=4.0p=0.059 | 7.8 ± 0.2 | 7.7 ± 0.6 | H1,24=0.3p=0.583 | 2.1 ± 1.3 | 2.2 ± 1.3 | F1,24=0.0p=0.861 | 6.0 ± 0.0b | 6.0 ± 0.0a | H1,34=0.2p=0.707 |
| Semi-hard  | 7.6 ± 0.8b | 7.7 ± 0.5a | H1,62=0.2p=0.678 | 7.6 ± 0.7 | 7.5 ± 0.7 | F1,62=0.5p=0.486 | 1.9 ± 1.3 | 1.5 ± 0.9 | H1.62=1.5p=0.223 | 3.3 ± 1.5a | 5.1 ± 1.3b | H1,62=17.9***p<0.001*** |
| Statistical analyses | H3,67=4.9 | H3,66=12.8 |  | F3,67=2.2 | H3,66=5.2 |  | H3,67=2.5 | H3,66=2.2 |  | H*3,67*=34.1 | H3,66=10.4 |  |
| P-value | 0.176 | ***0.005*** |  | 0.099 | 0.159 |  | 0.468 | 0.542 |  | ***<0.001*** | ***0.016*** |  |

Legend : D0, first day of storage ; ESL, end of shelf-life; LAB, lactic acid bacteria; p-values written in italic bold are statistically significant; superscript letters allow identification of significantly different groups (by column).

**Table 3**
Results of the challenge-tests performed on artisanal cheeses artificially contaminated with *L. monocytogenes*.

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  |  |  |  |  |  |  |  | **Following EURL *Lm* (2014)a** |  | **Following FASFC (2019)b** |  |  |
| **Type of cheese** | **ID** | **Type of milk** | **Number of batches** | **pH**  | **aw**  | **Storage (days)** | **δth** **(log cfu/g)** | **δB1** **(log cfu/g)** | **δB2** **(log cfu/g)** | **δB3** **(log cfu/g)** | **Growth (Yes/No)** | **δB1** **(log cfu/g)** | **δB2** **(log cfu/g)** | **δB3** **(log cfu/g)** | **Growth (Yes/No)** | **Range of final contamination (log cfu/g)** |
| Unripened acid-curd  | UC1 | R | 1 | 4.5 | 0.99-1.00 | 19 | ≤0 | **-1.04** | / | / | No | **-0.42** | / | / | No | 0.95-1.48 |
| UC2 | R | 1 | 4.4 | 0.98-1.00 | 7 | ≤0 | **-1.43** | / | / | No | **-0.92** | / | / | No | 0.95-1.48 |
| UC3 | R | 1 | 4.5 | 0.98 | 10 | ≤0 | **-1.16** | / | / | No | **-0.30** | / | / | No | 0.95-1.60 |
| UC4 | R | 1 | 4.4 | 0.99 | 10 | ≤0 | **-0.68** | / | / | No | **-0.63** | / | / | No | 1.00-1.60 |
| UC5 | R | 1 | 4.4-4.5 | 0.99 | 10 | ≤0 | **-0.48** | / | / | No | **0.00** | / | / | No | 1.00-1.48 |
| UC6 | R | 1 | 4.5 | 0.99-1.00 | 14 | ≤0 | **-0.95** | / | / | No | **-0.60** | / | / | No | 0.95-1.00 |
| UC7 | P | 1 | 4.4 | 0.99 | 16 | ≤0 | **-0.53** | / | / | No | **-0.53** | / | / | No | 0.95 |
| UC8 | R | 1 | 4.5 | 0.98-1.00 | 12 | ≤0 | **-1.59** | / | / | No | **-1.45** | / | / | No | 0.95 |
| UC9 | P | 1 | 4.4-4.9 | 0.97-0.98 | 10 | ≤0 | **-1.04** | / | / | No | **-0.42** | / | / | No | 0.95-1.48 |
| UC10 | R | 1 | 4.4 | 0.99 | 15 | ≤0 | **-0.95** | / | / | No | **-0.55** | / | / | No | 0.95-1.30 |
| UC11 | R | 1 | 4.3-4.4 | 0.99-1.00 | 8 | ≤0 | **-1.19** | / | / | No | **-1.08** | / | / | No | 0.95-1.00 |
| UC12 | R | 1 | 4.4 | 0.97-0.99 | 14 | ≤0 | **-1.05** | / | / | No | **-0.95** | / | / | No | 0.95 |
| Mold-ripened soft  | MRSC1 | P | 3 | 5.6-7.1 | 0.97-0.99 | 30 | 8.0 | **4.70** | 3.35 | 2.20 | Yes | 4.98 | 3.54 | **5.31** | Yes | 1.78-6.79 |
| MRSC2 | R | 3 | 5.6-6.7 | 0.97-0.99 | 30 | 8.0 | 4.44 | **4.45** | 3.84 | Yes | 4.53 | **5.36** | 4.27 | Yes | 5.38-7.26 |
| MRSC3 | R | 3 | 4.7-7.0 | 0.93-0.98 | 28 | 8.0 | **0.93** | -0.68 | -0.20 | Yes | **1.05** | 1.03 | 0.83 | Yes | 1.48-3.23 |
| MRSC4 | R | 3 | 5.5-6.1 | 0.97-0.99 | 30 | 5.8 | **1.53** | 1.33 | 0.70 | Yes | **1.63** | 1.53 | 1.19 | Yes | 1.70-3.23 |
| Smear-ripened soft  | SRSC1 | R | 3 | 5.1-5.8 | 0.96-0.97 | 40 | 8.0 | **-0.68** | -0.99 | -1.05 | No | -0.30 | **0.17** | -0.35 | No | 0.95-1.95 |
| SRSC2 | R | 3 | 5.2-5.9 | 0.96-0.97 | 30 | 5.1 | 0.83 | **1.59** | -0.14 | Yes | 1.47 | **2.24** | 2.15 | Yes | 2.11-4.53 |
| SRSC3 | R | 3 | 5.2-5.9 | 0.96-0.98 | 30 | 8.0 | 1.04 | **2.68** | -0.30 | Yes | 1.80 | **2.93** | 0.15 | Yes | 1.60-4.53 |
| SCRC4 | R | 3 | 5.6-6.0 | 0.97-0.98 | 40 | 5.0 | 1.29 | **2.16** | 1.33 | Yes | 1.91 | **2.68** | 1.38 | Yes | 3.15-4.68 |
| Semi-hard  | SH1 | P | 3 | 5.8-6.1 | 0.96-0.97 | 21 | 6.4 | -1.49 | **-0.74** | -0.89 | No | -1.36 | **-0.30** | -0.83 | No | 0.95-1.48 |
| SH2 | P | 3 | 5.5-5.9 | 0.95-0.97 | 30 | 8.0 | -0.12 | -0.38 | **-0.07** | No | 0.30 | **0.77** | 0.04 | Yes | 0.95-3.11 |
| SH3 | P | 3 | 5.8-6.0 | 0.92-0.96 | 21 | 8.0 | -1.13 | -0.60 | **-0.23** | No | -0.63 | -0.07 | **0.04** | No | 1.30-2.08 |
| SH4 | R | 3 | 5.8-6.1 | 0.96-0.97 | 21 | 8.0 | **1.19** | -0.90 | -0.18 | Yes | **1.23** | 0.10 | 0.28 | Yes | 0.95-3.23 |
| SH5 | R | 3 | 5.6-5.8 | 0.94-0.95 | 14 | 4.2 | -0.35 | -0.41 | **-0.08** | No | -0.14 | **0.04** | **0.04** | No | 1.30-2.40 |
| SH6 | R | 3 | 5.6-6.1 | 0.94-0.96 | 14 | 8.0 | -0.52 | **0.12** | -0.41 | No | -0.10 | **0.33** | 0.05 | No | 1.30-2.18 |
| SH7 | R | 1 | 5.4 | 0.95-0.96 | 21 | ≤0 | **-0.48** | / | / | No | **-0.45** | / | / | No | 1.70-1.85 |
| SH8 | R | 3 | 5.8-6.0 | 0.96-0.97 | 14 | 8.0 | 0.04 | -0.94 | **0.93** | Yes | 0.50 | -0.70 | **1.38** | Yes | 0.95-3.08 |
| SH9 | R | 3 | 5.5-5.9 | 0.96-0.98 | 21 | 8.0 | -0.27 | -0.48 | **-0.20** | No | **0.87** | 0.10 | 0.24 | Yes | 1.78-3.23 |
| SH10 | R | 1 | 5.6-5.7 | 0.96-0.97 | 30 | ≤0 | **-0.53** | / | / | No | **0.45** | / | / | No | 1.48-2.60 |
| SH11 | R | 3 | 5.8-6.0 | 0.95-0.96 | 14 | 6.1 | -0.37 | -0.05 | **0.23** | No | -0.22 | 0.28 | **0.70** | Yes | 1.48-2.30 |
| SS12 | R | 1 | 5.0-6.0 | 0.95-0.98 | 21 | 8.0 | **-0.05** | -0.08 | -0.12 | No | 0.36 | **2.75** | 1.18 | Yes | 1.78-4.60 |

Legend : P, pasteurized milk ; R, raw milk ;δth, theoretical growth potential estimated using Sym’Previus ; δB1, growth potential for the first batch; δB2, growth potential for the second batch; δB3, growth potential for the third batch; growth potential in bold represents the highest value obtained for a given cheese; EURL *Lm* (2014), growth potential considered as the difference between medians of the contamination, expressed as log10 cfu/g at end of shelf-life and at D0; FASFC (2019), growth potential considered as the difference between the highest contamination at end of shelf-life and the lowest contamination at D0, both expressed as log10 cfu/g; growth of *L. monocytogenes* is considered as possible if δ > 0.5 log10 cfu/g.