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

Growth assessment of *Listeria monocytogenes* in butter

El-Hajjaji Soundous[1], Gérard Amaury[1], De Laubier Juliette[1], Di Tanna Sybille[1], Lainé Aurélie[1], Patz Viviane[1], Sindic Marianne[1]

[1] Laboratory of Quality and Safety of Agro-food Products, Gembloux Agro-Bio Tech, University of Liège, Passage des Déportés, 2, 5030 Gembloux, Belgium.

Corresponding author:

El-Hajjaji Soundous (email: soundous.el-hajjaji@student.uliege.be)

University of Liège - Gembloux Agro-Bio Tech

Passage des Déportés, 2

B-5030 Gembloux.

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 an absence 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 (EC, 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" (EURL Lm, 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 1 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.

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 2). 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.

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 3). 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).

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 4. 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/ cm^2) with relatively large size (about 2 mm). An example is shown in Figure 1.

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 5 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.

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 6). 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).

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 7a and 7b). 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 2 and 3).

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

222 reduced the microbial concentration and so, the nutritional competition. The same reason
223 could explain the increase of *L. monocytogenes* in "sweet cream whipped salted butter"
224 reported by Holliday et al. (2003). The product was made from pasteurized cream with
225 absence of preservatives.
226 The size and distribution of water droplets was another characteristic to observe regarding
227 bacterial growth. Bullock and Kenney (1969) found that bacterial counts after the storage
228 period were three to four times higher in the low fat dairy spreads with large serum droplets
229 (> 50 microns), compared to the products with small droplets (3 to 20 microns). Studies have
230 also demonstrated that water droplets size and distribution is a key parameter in preventing
231 the growth of *L. monocytogenes* (Michelon et al., 2016; Voysey et al., 2009). Voysey et al.
232 (2009) observed that *L. monocytogenes* grew easily in coarse butter with large water droplets
233 size. In this study, butter samples had in general large water droplets (about 2 mm), which is
234 favorable for the growth of microorganisms. However, no growth was observed in any of the
235 samples. This could be due to the fact that the initial level of *L. monocytogenes* of the
236 contaminated samples was much lower than that used by Voysey et al. (2009).
237 In this study, the samples showed various microbial profiles in terms of *E. coli*,
238 *Staphylococcus*, *Pseudomonas* spp., total aerobic flora, yeasts and molds. De Reu et al. (2004)
239 noted that the high colony counts of the hygiene indicators coliforms, *E. coli* and
240 *Staphylococcus aureus* are related to the presence of *Listeria* spp. in raw milk butter, although
241 no significant statistical relationship was found. Unlike *E. coli* and *S. aureus*, an increase in
242 yeasts and molds involves a decrease in *L. monocytogenes*. According to a study conducted
243 by Goerges et al. (2006), all tested yeasts had an inhibitory potential on *L. monocytogenes*.
244 The authors related this result to the competition for nutrients. *Pseudomonas* spp. was also
245 reported as an effective competitor of *L. monocytogenes* (Farrag and Marth, 1989). This
246 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.

Acknowledgments

This study was conducted with the financial support of the Service Public of Wallonia (SPW). The authors are thankful to the producers who agreed to give their batches for analysis. They are also thankful to the food laboratory of CdL and LARECO for carrying out the analyses. The authors also thank Valérie Michel for her instructions regarding data interpretation.

References

Advisory Committee on the Microbiological Safety of Food, 2003. Recent trends in listeriosis in the UK.

Ahamad, N., Marth, E.H., 1989. Behavior of Listeria monocytogenes at 7, 13, 21, and 35°C in Tryptose Broth Acidified with Acetic, Citric, or Lactic Acid. J. Food Prot. 52, 688–695. https://doi.org/10.4315/0362-028X-52.10.688

Buchanan, R., Lindqvist, R., Ross, T., Smith, M., Todd, E., Whiting, R., 2004. Risk assessment of <i>Listeria monocytogenes<i/> in ready-to-eat foods, Microbiological risk assessment series. Food and Agriculture Organization of the United Nations ; World Health Organization, Rome : Geneva, Switzerland.

Buchanan, R.L., Gorris, L.G.M., Hayman, M.M., Jackson, T.C., Whiting, R.C., 2017. A review of *Listeria monocytogenes*: An update on outbreaks, virulence, dose-response, ecology, and risk assessments. Food Control 75, 1–13. https://doi.org/10.1016/j.foodcont.2016.12.016

Bullock, D.H., Kenney, A.R., 1969. Effect of emulsion characteristics of a low-fat dairy spread on bacterial growth. J. Dairy Sci. 52, 625–628.

Chen, H., Hoover, D.G., 2003. Bacteriocins and their Food Applications. Compr. Rev. Food Sci. Food Saf. 2, 82–100. https://doi.org/10.1111/j.1541-4337.2003.tb00016.x

Cheng, C.-M., Doyle, M.P., Luchansky, J.B., 1995. Identification of *Pseudomonas fluorescens* strains isolated from raw pork and chicken that produce siderophores antagonistic towards foodborne pathogens. J. Food Prot. 58, 1340–1344.

Cole, M.B., Jones, M.V., Holyoak, C., 1990. The effect of pH, salt concentration and temperature on the survival and growth of Listeria monocytogenes. J. Appl. Microbiol. 69, 63–72.

De Reu, K., Grijspeerdt, K., Herman, L., 2004. A Belgian survey of hygiene indicator bacteria and pathogenic bacteria in raw milk and direct marketing of raw milk farm products. J. Food Saf. 24, 17–36.

Dortu, C., Thonart, P., 2009. Les bactériocines des bactéries lactiques: caractéristiques et intérêts pour la bioconservation des produits alimentaires/Bacteriocins from lactic acid bacteria: interest for food products biopreservation. Biotechnol. Agron. Société Environ. 13, 143.

EC, 2005. Commission Regulation (EC) N° 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs.

EURL Lm, 2014. Technical Guidance Document for conducting shelf-life studies on *Listeria monocytogenes* in ready-to-eat foods.

European Food Safety Authority, European Centre for Disease Prevention and Control, 2017. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2016. EFSA J. 15. https://doi.org/10.2903/j.efsa.2017.5077

Farrag, S.A., Marth, E.H., 1989. Behavior of *Listeria monocytogenes* when incubated together with *Pseudomonas* species in tryptose broth at 7 and 13 C. J. Food Prot. 52, 536–539.

Freedman, D.J., Kondo, J.K., Willrett, D.L., 1989. Antagonism of foodborne bacteria by *Pseudomonas spp*.: a possible role for iron. J. Food Prot. 52, 484–489.

Gillespie, I.A., Mook, P., Little, C.L., Grant, K., Adak, G.K., 2010. *Listeria monocytogenes* infection in the Over-60s in England between 2005 and 2008: A retrospective case–control study utilizing market research panel data. Foodborne Pathog. Dis. 7, 1373–1379.

317 Goerges, S., Aigner, U., Silakowski, B., Scherer, S., 2006. Inhibition of *Listeria*
318 *monocytogenes* by food-borne yeasts. Appl. Environ. Microbiol. 72, 313–318.
319 https://doi.org/10.1128/AEM.72.1.313-318.2006
320 Goulet, V., Hedberg, C., Le Monnier, A., De Valk, H., 2008. Increasing incidence of
321 listeriosis in France and other European countries. Emerg. Infect. Dis. 14, 734.
322 Gram, L., 1993. Inhibitory effect against pathogenic and spoilage bacteria of *Pseudomonas*
323 strains isolated from spoiled and fresh fish. Appl. Environ. Microbiol. 59, 2197–2203.
324 Hitchins, A.D., Whiting, R.C., 2001. Food-borne Listeria monocytogenes risk assessment.
325 Food Addit. Contam. 18, 1108–1117. https://doi.org/10.1080/02652030110050104
326 Holliday, S.L., Adler, B.B., Beuchat, L.R., 2003. Viability of *Salmonella, Escherichia coli*
327 O157: H7, and *Listeria monocytogenes* in butter, yellow fat spreads, and margarine as
328 affected by temperature and physical abuse. Food Microbiol. 20, 159–168.
329 Jordan, K., Hunt, K., Dalmasso, M., 2016. *Listeria monocytogenes* in milk products, in: Garg,
330 N., Abdel-Aziz, S.M., Aeron, A. (Eds.), Microbes in Food and Health. Springer
331 International Publishing, pp. 289–315. https://doi.org/10.1007/978-3-319-25277-3_15
332 Kozak, J., Balmer, T., Byrne, R., Fisher, K., 1996. Prevalence of *Listeria monocytogenes* in
333 foods: incidence in dairy products. Food Control 7, 215–221.
334 Lewis, H.C., Little, C.L., Elson, R., Greenwood, M., Grant, K.A., McLauchlin, J., 2006.
335 Prevalence of *Listeria monocytogenes* and other *Listeria* species in butter from United
336 Kingdom production, retail, and catering premises. J. Food Prot. 69, 1518–1526.
337 Liu, D., Lawrence, M.L., Ainsworth, A.J., Austin, F.W., 2005. Comparative assessment of
338 acid, alkali and salt tolerance in *Listeria monocytogenes* virulent and avirulent strains.
339 FEMS Microbiol. Lett. 243, 373–378. https://doi.org/10.1016/j.femsle.2004.12.025
340 Lyytikäinen, O., Autio, T., Maijala, R., Ruutu, P., Honkanen-Buzalski, T., Miettinen, M.,
341 Hatakka, M., Mikkola, J., Anttila, V.J., Johansson, T., others, 2000. An outbreak of

342 *Listeria monocytogenes* serotype 3a infections from butter in Finland. J. Infect. Dis.
343 181, 1838–1841.
344 Magalhães, R., Mena, C., Ferreira, V., Silva, J., Almeida, G., Gibbs, P., Teixeira, P., 2014.
345 Bacteria: *Listeria monocytogenes*, in: Encyclopedia of Food Safety. Elsevier, pp. 450–
346 461. https://doi.org/10.1016/B978-0-12-378612-8.00101-3
347 McLauchlin, J., Mitchell, R.T., Smerdon, W.J., Jewell, K., 2004. *Listeria monocytogenes* and
348 listeriosis: a review of hazard characterisation for use in microbiological risk
349 assessment of foods. Int. J. Food Microbiol. 92, 15–33. https://doi.org/10.1016/S0168-
350 1605(03)00326-X
351 Merkus, H.G., 2009. Particle Size Measurements: Fundamentals, Practice, Quality. Springer
352 Science & Business Media.
353 Michelon, D., Leclercq, A., Garric, G., Guillier, L., Beaufort, A., Bergis, H., 2016. Growth
354 Potential Assessment of *Listeria* in milk fat products by challenge testing: Growth
355 potential of *Listeria* in milk fat products. J. Food Saf. 36, 260–270.
356 https://doi.org/10.1111/jfs.12239
357 N'Guessan, E., Godrie, T., De Laubier, J., Di Tanna, S., Ringuet, M., Sindic, M., 2015. A
358 survey of bacteria found in Belgian dairy farm products. Biotechnol. Agron. Société
359 Environ. 19, 346–354.
360 Rueden, C.T., Schindelin, J., Hiner, M.C., DeZonia, B.E., Walter, A.E., Arena, E.T., Eliceiri,
361 K.W., 2017. ImageJ2: ImageJ for the next generation of scientific image data. BMC
362 Bioinformatics 18. https://doi.org/10.1186/s12859-017-1934-z
363 Ryser, E.T., Marth, E.H., 1999. *Listeria*, listeriosis, and food safety. Second Edition. CRC
364 Press.
365 Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T.,
366 Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.-Y., White, D.J.,

Hartenstein, V., Eliceiri, K., Tomancak, P., Cardona, A., 2012. Fiji: an open-source platform for biological-image analysis. Nat. Methods 9, 676.

Voysey, P.A., Anslow, P.A., Bridgwater, K.J., Lavender, B., Watson, L., 2009. The effects of butter characteristics on the growth of *Listeria monocytogenes*. Int. J. Dairy Technol. 62, 326–330. https://doi.org/10.1111/j.1471-0307.2009.00505.x

373 **Table 1:** Physico-chemical and microbiological parameters analyzed during each durability
374 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

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

377 **Table 2:** 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

378

379 **Table 3:** 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

380

381 **Table 4:** Results of water dispersion, expressed by scale units, of raw milk butters collected

382 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

383

384 **Table 5:** 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

385

386 **Table 6:** 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

387 **Table 7a:** Physico-chemical and microbiological characteristics of the batches at “day 0”

ID	Starter cultures	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
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
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

388

389 **Table 7b:** 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
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

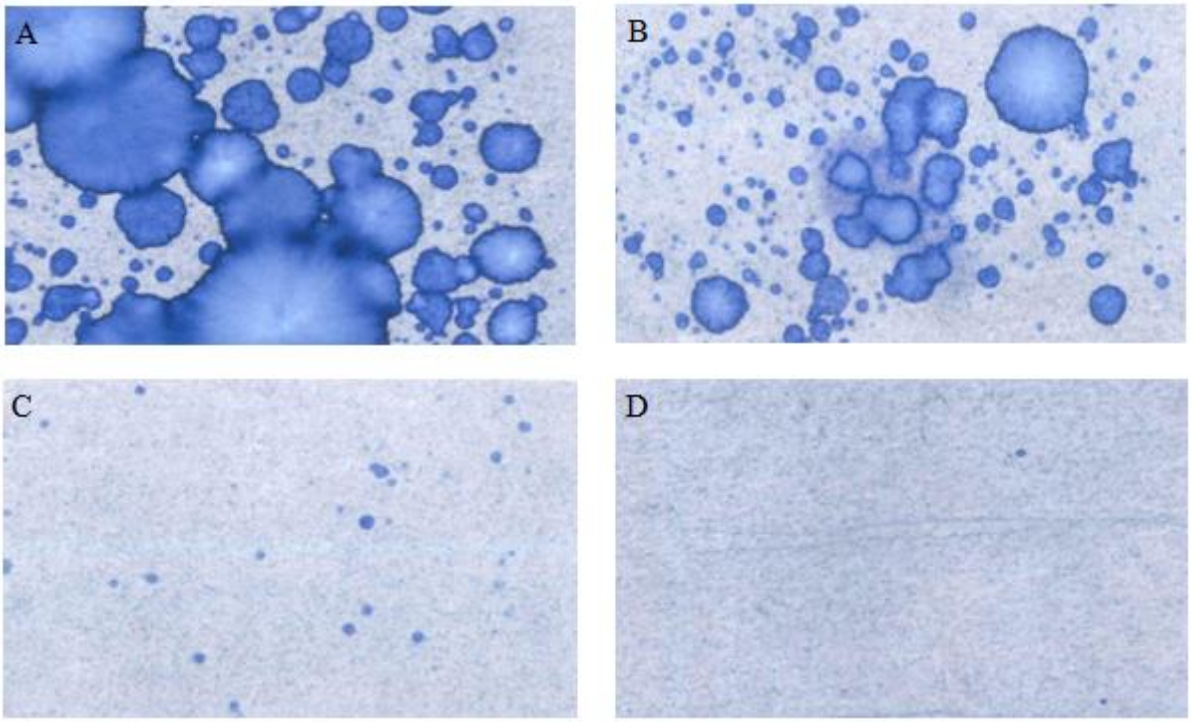

Figure 1: Examples of the spots obtained for water distribution and their classification using the grading scale. A: scale unit 1, B: scale unit 2A, C: scale unit 3B, D: scale unit