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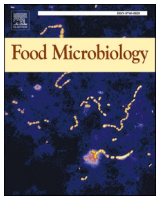

Listeria monocytogenes survives better at lower storage temperatures in regular and low-salt soft and cured cheeses

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ABSTRACT

The behaviour of *Listeria monocytogenes* was investigated in soft pasteurized milk cheese elaborated with different salt concentrations (1.17 and 0.30% w/w) and in cured raw sheep milk cheese over storage up to 189 days at different isothermal conditions. Commercial 25-g cheese samples were inoculated with a 4-strain cocktail of *L. monocytogenes* (serovars 4b, 1/2a, 1/2b and 1/2c) at approximately 10^4 CFU/g. The inoculated samples were stored at 4 and 22 °C and withdrawn at proper intervals for *L. monocytogenes* enumeration. The prevalence of the different serovar strains of *L. monocytogenes* was characterized on soft cheese samples over storage at 4 °C using multiplex PCR. Salt reduction did not affect the survival of *L. monocytogenes* in soft cheeses and a maximum of 1-log reduction was observed in both regular and low-salt cheeses after 189 days of storage at 4 °C. The pathogen showed greater survival capacity in both soft and cured cheeses during storage at 4 °C compared to the storage at 22 °C, where more than 2.5 log reductions were computed. The fate of *L. monocytogenes* was described through a Weibull model fitted to survival data. The time required for a first tenfold reduction of the *L. monocytogenes* population (δ) at 4 °C is around 150 days in soft and 72 days in cured cheeses. At 22 °C, the estimated δ values are at least 60% lower in both cheese types. Among the four *L. monocytogenes* serovars present in the inoculated cocktail, the serovar 4b strain was the most sensitive to refrigerated storage, while the prevalence of serovar 1/2c strain increased over time in soft cheeses. Overall, the data obtained in this study help to deepen knowledge into factors affecting *L. monocytogenes* behaviour on cheeses and evidenced the variability between serovars in terms of survival capacity, which may be considered when performing microbial risk assessments.

1. Introduction

Two thirds of all whole milk available to dairies in the 27 member countries of the European Union (EU-27) is used to produce cheese and butter, and cheese is the second most produced dairy product in EU-27 (EUROSTAT, 2021). In addition, cheese represents 22.2% of the dairy products consumed in Spanish households in 2020, with a per capita consumption of 8.80 kg and an inter-annual increase in consumption of 14% in 2019–2020 (MAPAMA, 2021).

As a ready-to-eat food, cheese can support the survival and growth of *Listeria monocytogenes*. According to the European Rapid Alert System for Food and Feed (RASFF), 46 alert notifications concerning the presence of *L. monocytogenes* in cheeses produced and distributed in the EU countries have been recorded from 2016 to 2021, which represents 82% of the alert notifications related to the presence of pathogenic microorganisms on cheeses in the last five years in the EU. Listeriosis is one of the most serious food-borne diseases in the EU, with an increasing case fatality reported in the last years (from 13.6% to 17.6% from 2017 to 2019) (EFSA, 2021). *L. monocytogenes* can enter cheese processing environments through raw milk and adhere to food contact surfaces and form biofilms, which are hard to eliminate and can result in cheese cross-contamination (Chow et al., 2021; Rodriguez et al., 2021). As a

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result, many listeriosis outbreaks have been linked to the consumption of both raw-milk cheeses (CDC, 2017; FIOD, 2010) and pasteurized milk cheeses (Amato et al., 2017; Anonymous, 2020; FIOD, 2012; Magalhães et al., 2015) worldwide. The *L. monocytogenes* serovars most frequently involved in human listeriosis and isolated from contaminated foods are the 1/2a, 1/2b, 1/2c and 4b (Doumith et al., 2004; Lotfollahi et al., 2017).

Refrigeration together with other strategies such as a_w reduction through salt addition, generally sodium chloride, are considered standard practices for controlling microbial proliferation in cheeses (Luzzi et al., 2021). Despite the notorious role of salt for increasing food safety, the World Health Organization (WHO) has set a global target of 30% reduction in the mean consumption of salt/sodium by 2025, since its excessive consumption represents a risk factor of cardiovascular disease (He and MacGregor, 2018; WHO, 2021). To attain such target reduction, salt reduction by the food and beverage industry is encouraged, as well as changes in lifestyle (WHO, 2021). Thus, the increasing trend of low-salt cheese varieties are answering to these consumers' demands but food safety could be compromised if the shelf-life is not fully demonstrated by food operators.

According to the European Regulation (EC) 2073/2005, the microbiological criterion for *L. monocytogenes* in those ready-to-eat foods that cannot support its growth during their shelf life is a maximum of 100 CFU/g (European Commission, 2005). Durability studies, challenge tests and predictive microbiology models can be applied by food manufacturers to demonstrate to the competent authority that the levels of *L. monocytogenes* in ready-to-eat foods do not exceed the established microbiological criterion (European Commission, 2005).

Predictive microbiology models describing the growth/death kinetics of *L. monocytogenes* in soft and cured cheeses have been extensively documented in literature (Campagnollo et al., 2018; Dalzini et al., 2017; Rosshaug et al., 2012; Tiwari et al., 2014). However, the variability between cheese types in terms of structure, physicochemical characteristics, technological processing and other factors influencing microbial behaviour, *e.g.*, levels of lactic acid bacteria (LAB), hinders the application of a predictive model developed with data obtained in a specific cheese type to predict microbial behaviour in other cheese varieties (Possas et al., 2021). Thus, product-oriented approaches are recommended for the development of more precise and reliable predictive models. On the other hand, for obtaining more representative and accurate results predictive models should be developed using *L. monocytogenes* isolates from dairy companies as these strains would be better adapted to the cheesemaking conditions.

The objectives of this study were to quantify and model the kinetic behaviour of a multi-strain cocktail of persistent *L. monocytogenes* strains belonging to the four most representative genetic serovars (1/2a, 1/2b, 1/2c and 4b) on soft cheese with different salt concentrations and on cured raw sheep milk cheese during storage at different isothermal conditions (4 and 22 °C). Further, the *L. monocytogenes* serovar variability was characterized over storage of soft cheese under refrigeration.

2. Material and methods

2.1. Cheese samples

Two different types of cheeses were used in this study: soft cheese elaborated with pasteurized milk from different animal species and cured raw sheep milk cheese. Cheese samples were provided vacuum packaged by a local cheesemaker (Valladolid, Spain).

The soft cheese used in this study contained a moderate concentration of fat and proteins (approximately 30 and 21%, respectively). The main ingredients of the soft cheese are pasteurized milk from three animal species: cow (min 50%), sheep (min 20%) and goat (min 15%), salt (sodium chloride), rennet, and starter cultures (*i.e.*, *Lactococcus lactis*, *Lactococcus lactis cremoris* and *Streptococcus thermophilus* at ca. 10^6 log CFU/g). This product was elaborated using two different salt concentrations: 1.17% (regular) and 0.30% (low-salt), respectively, and was salted by immersion in a brine solution. The final concentration of salt in the product depended on the immersion time and brine temperature and was measured directly in the product. The soft cheese used in this study is subjected to a ripening process for 7 days at 4 °C and 77% relative humidity (RH) before packaging. According to the manufacturer, the market shelf-life of this type of cheese is increased due to its short ripening period and is usually set between 3 and 5 months (90–150 days).

The cured raw sheep milk cheese contained a high concentration of fat (approximately 37%) and more than 24% of proteins, and 1.51% of salt (salting also by immersion in brine solution). The main ingredients of the cured cheese are raw sheep milk, salt, rennet, starter cultures (*i.e.*, *Lactococcus lactis*, *Lactococcus lactis cremoris* and *Streptococcus thermophilus* at ca. 10^6 log CFU/g), and some additives like potassium nitrate and lysozyme. The ripening process took place for 45 days at 5.5 °C and 85% RH. According to the manufacturer, the shelf-life of this type of cheese is usually set between 3 and 6 months (90–180 days).

2.2. Bacterial strains and inoculum preparation

Four strains of *L. monocytogenes* were used to prepare cocktails for samples inoculation: LBMM1009 (serogroup 4b, sequence type ST6), LBMM 1111 (serogroup 1/2a, ST 204), LBMM 1104 (serogroup 1/2b, ST5) and LBMM 1109 (serogroup 1/2c, ST9). The selected strains were isolated from the dairy company providing the cheese samples. These strains were chosen based on previous evidence that they can survive the stressful environment of cheesemaking better than other strains, as they were repetitively isolated from food contact surfaces in cheese processing environments, more specifically in the cheese salting area (LBMM 1009, LBMM1109, LBMM1104) and in the grating areas (LBMM 1111) (Melero et al., 2019). All strains were maintained at −80 °C in cryovial containing beads and cryopreservatives (Oxoid TP15731 Maintenance Freeze medium, Oxoid, Hampshire, UK).

Prior to the experiments, a bead of each strain was transferred to individual sterile tubes containing 10 mL of Brain Heart Infusion (BHI, Beckton, Dickinson and Co.) broth with the aid of sterile tweezers and incubated at 37 °C for 24 h. The individual grown cultures were surface plated onto individual BHI agar Petri dishes and incubated at 37 °C for 24 h. Then, a loopful of one colony of each strain was transferred aseptically into individual 10-mL BHI broth tubes and incubated overnight at 37 °C.

The overnight cultures of each strain were serially diluted in 0.1% peptone water (Oxoid, UK). Then, 1-mL aliquots of the diluted cultures of each strain were transferred to a sterile tube and shaken to obtain a cocktail with ~2.5×10^6 CFU/mL. To ensure that the same concentration of each strain was present in the mixed cocktail, individual cultures were plated onto BHI agar plates, and their initial concentration was determined, after incubation at 37 °C for 24 h. To determine the initial concentration of each working multi-strain cocktail, an aliquot was serially diluted in 0.1% peptone water and surface plated onto BHI agar plates. After incubation at 37 °C for 24 h, colonies were enumerated.

2.3. Artificial contamination of cheese samples and storage

Cheese was aseptically removed from the commercial bags and transferred into sterile co-extruded polyamide/polyethylene packing bags (Industrias Pargón, Salamanca, Spain). Cheese samples of 25 g were inoculated with 100 μL of the *L. monocytogenes* cocktail with the aid of a pipette (Valero et al., 2018). To enable a homogeneous distribution of the inoculum, 10 μL aliquots of the cocktail were evenly distributed in ten different surface areas (for a final concentration of ~1.0×10^4 CFU/g). Then, inoculated samples were air-dried in a biosafety cabinet for 30 min and subsequently vacuum packaged. Different storage conditions according to the type of cheese have been set. Regarding the low-salt cheese, assuming that it would allow a better

L. monocytogenes survival at low temperatures, two different storage scenarios were set at 4 and at 22 °C. On the contrary, the regular soft cheese type was stored at the recommended conditions by the manufacturer, *i.e.*, 4 °C. Finally, for the cured cheese, storage at 4 and at 22 °C was performed since it can be stored at both conditions. Experiments were performed in triplicate.

2.4. Microbial analysis

Non-inoculated samples (25 g) were tested to investigate the presence of *L. monocytogenes* using the ISO 11290-1 detection method (ISO, 2017a). Microbiological studies on the presence and enumeration of *L. monocytogenes* at each sampling time were conducted using three independent inoculated cheese samples according to ISO 11290-1 (ISO, 2017a) and ISO 11290-2 (ISO, 2017b), respectively. Enumeration of LAB was also conducted in each sampling time according to ISO 15214 (ISO, 1998). For the soft cheeses, the analysis period covered 189 and 133 days at 4 °C and at 22 °C, respectively. Cured cheeses stored at 4 °C and 22 °C were microbiologically analysed for 198 days and 84 days, respectively (Beaufort et al., 2014).

Samples of 25 g were transferred to sterile stomacher bags, which were filled with peptone water (0.1%) (1:10) and homogenized in Stomacher (IUL, Spain) for 1.5 min. The homogenate was serially diluted in saline solution (0.85% w/v) and aliquots of 0.1 mL were plated onto ALOA® (Oxoid, UK) and MRS (De Man, Rogosa and Sharpe, Oxoid, UK) agar plates for enumeration of *L. monocytogenes* and LAB, respectively. ALOA plates were incubated for 24 h at 37 °C and the MRS plates were incubated under 10% CO_2 for 48 h at 33 °C and the number of colonies were determined. The quantification limit was 10 CFU/g and the counts from the triplicate samples were expressed as log CFU/g. In case cheese samples yielded three successive negative results over storage using the standard detection method, samples were not further analysed.

2.5. Physicochemical analysis

The pH and a_w of cheese samples stored at different temperatures were monitored every week along the storage (Valero et al., 2014). The pH was measured after blending 10 g of cheese in 90 mL of distilled and deionized water using a pH meter Crison Basic 20+ equipped with an electrode pH 0–14 (Crison Instruments, S.A., Barcelona, Spain). The a_w was measured at 22 °C using an Aqualab a_w meter (Aqualab model Series 4 Decagon Devices Inc., Pullman, WA).

2.6. PCR-serogrouping of L. monocytogenes

The prevalence of each *L. monocytogenes* serovar strain in the inoculated cocktail, *i.e.*, 4b, 1/2a, 1/2b, 1/2c, was evaluated in inoculated soft cheeses during 189 days of storage at 4 °C. The prevalence was evaluated in soft cheeses, since this cheese type is more frequently associated with listeriosis outbreaks than cured cheeses (Kousta et al., 2010; Lundén et al., 2004). Twenty presumptive colonies of *L. monocytogenes* grown in the ALOA agar plates were assayed in each time point for each cheese sample to determine the percentage of each *L. monocytogenes* serogroup. *L. monocytogenes* serogrouping was performed using a multiplex PCR targeting the specific target genes lmo0737, lmo1118, ORF2819, ORF2110 and *Listeria* spp. specific prs published by Doumith et al. (2004) and amended by Leclercq et al. (2011) for PCR IVb-VI.

2.7. Data analysis and modelling

Data analysis and modelling were performed by using R (R Core Team, 2020). Microbial reductions in soft cheeses expressed as the difference between the initial *L. monocytogenes* concentration (N_0, log CFU/g) in samples and its concentrations at the different time points (N, log CFU/g), *i.e.*, log N_0/N, were compared by ANOVA ($p \leq 0.05$). The prevalence of the strains of *L. monocytogenes* of different serovars during storage of soft cheeses were also compared by ANOVA.

The Weibull model (Eq. (1)) was fitted to the *L. monocytogenes* survival data observed alongside time on soft and cured cheeses, by using the nonlinear least square (*nls*) function, using a Gauss-Newton algorithm in R.

$$\log\ N = \log N_0\ - (t/\delta)^{\beta} \quad 1$$

where N is the number of surviving cells in cheese samples during storage (CFU/g); N_0 is the initial number of cells in cheese samples (CFU/g); t is the storage time (days); δ is the model scale parameter, representing the time required to have a first tenfold reduction of the *L. monocytogenes* population (days); and β is the shape parameter (dimensionless) of the Weibull model. The curve is concave upward when $\beta < 1$, concave downward if $\beta > 1$ and the model is linear when $\beta = 1$.

The prediction intervals were estimated by forward uncertainty propagation using Monte Carlo simulations based on the parameter estimates (Akkermans et al., 2018; Garre et al., 2017). The number of Monte Carlo iterations was increased until the prediction intervals converged, requiring 1000 iterations. The Root Mean Square Error (RMSE) was determined to evaluate the goodness-of-fit of the models.

3. Results and discussion

3.1. Effect of salt concentration on Listeria monocytogenes behaviour in soft cheeses stored at 4 °C

The behaviour of *L. monocytogenes* on soft cheeses elaborated with low-salt concentration, *i.e.*, 0.30%, during storage at 4 °C was compared with the behaviour of the pathogen on regular soft cheese, *i.e.*, 1.17%. *L. monocytogenes* concentrations decreased over time on samples regardless of salt concentration. The survival kinetics in low-salt soft cheese is shown in Fig. 1A. No significant differences were found between the reductions observed in low-salt and regular cheeses at the different sampling times at 4 °C ($p > 0.05$) (Table 1). *L. monocytogenes* reductions after 189 days of storage were 1.02 ± 0.10 and 1.02 ± 0.02 log CFU/g in low-salt and regular cheeses, respectively.

LAB concentrations ranged from 6.35 to 6.84 log CFU/g in low-salt cheeses during storage at 4 °C (Table 2) while in regular cheeses their levels oscillated within the range 6.92–7.58 log CFU/g (Table 2). The mean a_w of samples over storage was 0.98 ± 0.005 for low-salt cheeses and 0.97 ± 0.004 for regular cheeses. Regarding pH, mean values over refrigerated storage were 5.59 ± 0.029 and 5.56 ± 0.023 for low-salt and regular soft cheeses, respectively (Table 2).

Competition between *L. monocytogenes* and LAB is expected in chilled foods with extended shelf-lives (Cornu et al., 2011). The inactivation of *L. monocytogenes* in soft cheeses, which have growth-permissive a_w and pH values, has been mainly attributed to the production of organic acids by LAB and pH reduction along storage (da Costa et al., 2018). In this study, the mechanism of the interaction between both microorganisms in soft cheese at 4 °C is not associated with the decrease in pH, since this physicochemical parameter remained almost constant along storage. Rather, the survival behaviour observed in this study for *L. monocytogenes* may be induced by the production of metabolites with antilisterial activity by LAB, such as bacteriocins (de Niederhäusern et al., 2020) or by the exhaustion of nutrients in cheese due to the consumption of carbohydrates by starter cultures during fermentation, *i.e.*, early stages of cheese production (Dalzini et al., 2017).

In the present study, salt reduction did not affect the a_w and pH of soft cheeses, which could partially explain the similar levels of *L. monocytogenes* inactivation observed during refrigerated storage of low-salt and regular soft cheeses. Consistent with our findings, salt

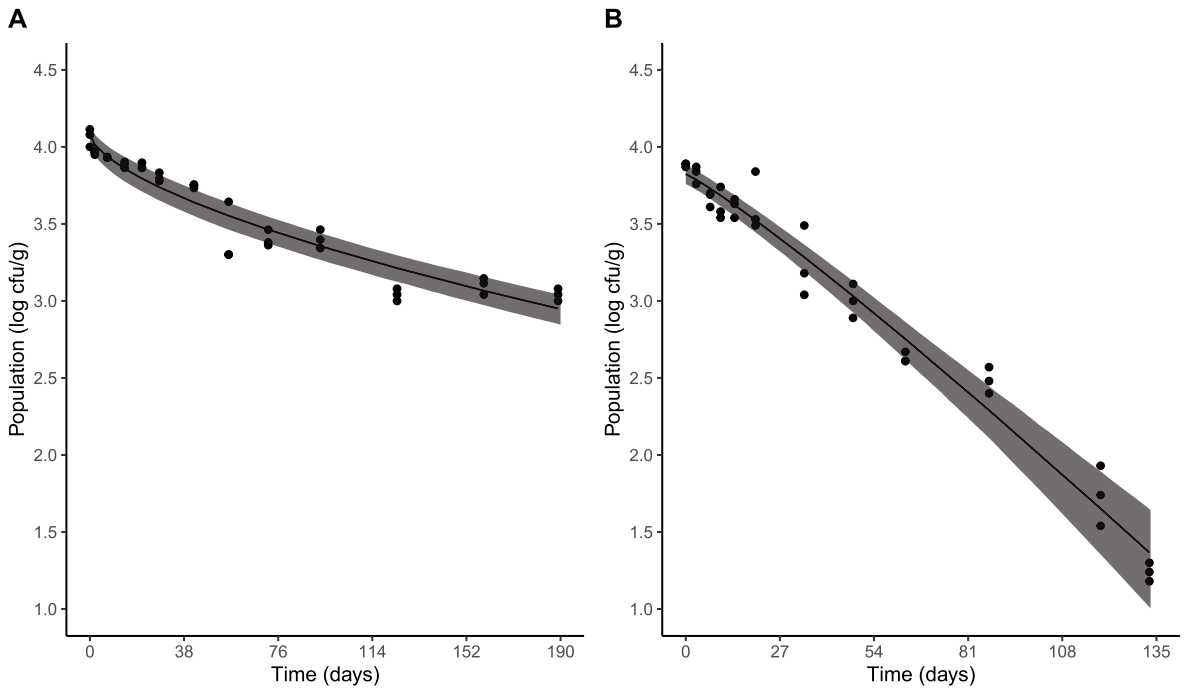

Fig. 1. Survival kinetics of *Listeria monocytogenes* in low-salt soft cheese at (A) 4 °C and (B) 22 °C. The grey zones represent the confidence intervals of the model predictions (95% CL).

Table 1
Listeria monocytogenes reductions (log N_0/N) on soft cheeses during storage at 4 °C.

Time (days)	Low-salt cheese	Regular cheese
0	0.00 ± 0.00	0.00 ± 0.00
2	0.10 ± 0.06	0.06 ± 0.02
7	0.13 ± 0.06	0.14 ± 0.02
14	0.18 ± 0.07	0.21 ± 0.02
21	0.19 ± 0.08	0.19 ± 0.04
28	0.26 ± 0.05	0.24 ± 0.03
42	0.32 ± 0.06	0.29 ± 0.05
56	0.65 ± 0.16	0.50 ± 0.10
72	0.66 ± 0.11	0.57 ± 0.07
93	0.66 ± 0.12	0.63 ± 0.13
124	1.02 ± 0.08	1.05 ± 0.04
159	0.96 ± 0.08	1.19 ± 0.02
189	1.02 ± 0.10	1.02 ± 0.02

Mean ± Standard deviations are provided.

Table 2
Lactic acid bacteria (LAB) concentration and pH of cheese samples stored at different temperatures.

Cheese Type	Storage temperature (°C)	LAB concentration (log CFU/g)	pH
Regular soft	4	7.36 ± 0.19	5.56 ± 0.023
Low-salt soft	4	6.67 ± 0.14	5.59 ± 0.029
	22	7.57 ± 0.35	5.51 ± 0.056
Cured	4	6.92 ± 0.27	5.47 ± 0.161
	22	6.89 ± 0.38	5.43 ± 0.093

Mean ± Standard deviations are provided.

reduction from 1.8% to 0.7% did not affect the survival of *L. monocytogenes* in low-salt aged Cheddar cheese with pH ranging from 5.1 to 5.8, stored at 4 °C for up to 90 days (Shrestha et al., 2011). Moreover, Luzzi et al. (2021) found that the reduction in salt content from 1.5 to 2.0 g/100 g to levels lower than 1.0 g/100 g did not affect the bacterial composition of Edam cheeses, including spoilage and potential hazards such as *L. monocytogenes*, during a 6-week ripening period. Therefore, salt reduction in the ranges considered in our study and in the abovementioned studies did not compromise the safety of the evaluated cheeses with respect to the presence of *L. monocytogenes*, while contributing to the reduction of salt/sodium target intake set by WHO (WHO, 2021).

3.2. *Effect of storage temperature on Listeria monocytogenes behaviour in low-salt soft cheeses stored at 4 and 22°C*

The levels of *L. monocytogenes* also decreased over time in low-salt soft cheeses stored at 22 °C (Fig. 1B). While just 1-log reduction of *L. monocytogenes* was observed in low-salt cheeses stored at 4 °C for 189 days (Fig. 1A), maximum reductions of 2.64 ± 0.06 log CFU/g were observed after 133 days of storage in low-salt cheeses stored at 22 °C. LAB concentrations in low-salt cheeses stored at 22 °C were higher than the concentration observed in low-salt cheeses stored at 4 °C, ranging between 7.0 and 8.0 log CFU/g (Table 2). The pH of samples stored at 22 °C varied within the range 5.42 and 5.57 (Table 2), while the a_w ranged within 0.97 and 0.98.

Results of previous investigations indicate that the survival capacity of *L. monocytogenes* in cheeses decreases with the increase of storage temperature (Angelidis et al., 2010, 2013; Mataragas et al., 2008; Panagou, 2008). At acid environments, the consumption of cells resources is intensified by the acceleration of their metabolism, as an attempt to overcome the acidic stress and keep their vital processes (Mataragas et al., 2008). The consumption of these resources occurs more quickly at temperatures closer to their optimum temperature for growth (Mataragas et al., 2008; Possas et al., 2021). Furthermore, LAB can grow faster and reach higher concentrations in soft cheeses stored at 22 °C than in soft cheeses stored at 4 °C, which may result in the presence of higher

level of metabolites, *i.e.*, organic acids and bacteriocins, that contribute to *L. monocytogenes* inactivation (Possas et al., 2021).

3.3. Effect of storage temperature on Listeria monocytogenes behaviour in cured raw sheep milk cheeses stored at 4 and 22 °C

A gradual reduction of *L. monocytogenes* occurred during storage of raw sheep milk cured cheese at both temperatures evaluated (Fig. 2). The pathogen could be detected in cured cheeses up to 185 days of storage at 4 °C and up to 76 days of storage at 22 °C. Counts were lower the quantification limit from the storage day 162 at 4 °C and by storage day 53 at 22 °C. The a_w of cured cheese samples oscillated between 0.92 and 0.94 during 198 days of storage at 4 °C (0.93 ± 0.006). In cured cheese samples stored at 22 °C, a reduction trend on a_w levels from 0.94 ± 0.005 to 0.89 ± 0.006 over 84 days of storage was noted.

LAB did not grow on cured cheese samples during storage and their concentrations in samples stored at different temperatures are shown in Table 2. The pH of cured cheese samples stored at 4 °C varied within the range 5.12–5.81 (Table 2), while for the samples stored at 22 °C the pH values varied from 5.26 to 5.59 (Table 2). The survival capacity of *L. monocytogenes* was lower in cured cheese in comparison with soft cheese, probably due to the lower a_w of the former.

Higher survival capacity of *L. monocytogenes* in cured cheeses stored under refrigeration compared to cheeses stored at room temperatures has been previously reported (Gonzales-Barron et al., 2020; Valero et al., 2014). Besides the faster consumption of cell resources during storage at room temperature in comparison with refrigerated storage mentioned for soft cheeses, the lower survival capacity of *L. monocytogenes* in cured cheeses stored at 22 °C could be attributed to the reduction of a_w noted on samples, which was not observed in samples stored at 4 °C.

On the other hand, the use of antimicrobials such as potassium nitrate and lysozyme in the cured raw sheep milk cheese could have been related to the survival patterns of *L. monocytogenes* during storage. Specifically, lysozyme (E-1105) is authorized in ripened cheeses according to the Regulation (EU) 1129/2011 to avoid the late blowing defect attributed to *Clostridium tyrobutyricum* when the concentration of spores is low (European Commision, 2011). However, this enzyme hydrolyses the peptidoglycan of the cellular wall of Gram-positive bacteria such as *L. monocytogenes*, thus producing growth inhibition (Brändle et al., 2016). Some studies indicated that lysozyme combined with other antimicrobials such as ethylenediaminetetraacetate or nisin is effective against *L. monocytogenes* in matured cheeses (Ko et al., 2008; Sozbilen and Yemenicioğlu, 2021). Its application in active packaging films is also reported to prevent *L. monocytogenes* growth in cheese at refrigeration temperatures (Ünalan et al., 2013). Therefore, growth inhibition of *L. monocytogenes* in cured raw sheep milk cheese may have been attributed to the joint action of the abovementioned hurdles during its elaboration and subsequent storage.

Although in this study a reduction on *L. monocytogenes* levels rather than growth was observed in both cheese types evaluated, the pathogen could survive for long storage periods at room temperature and under refrigeration. This is a matter of concern for dairy processors, since the exposure to sub-lethal environmental stresses may induce adaptative responses in *L. monocytogenes*, resulting in cells with increased resistance to subsequent severe stresses (Melo et al., 2015). Exposure of cells to the pH of cheeses, which usually ranges from 5.0 to 6.0, can induce an acid tolerance response (ATR), which can protect *L. monocytogenes* from subsequent lethal pH, such as the pH of disinfectants used in the dairy industry (Dhowlaghar et al., 2019) and the pH encountered in the human gastrointestinal tract (Sue et al., 2004). The mechanisms and systems of ATR include activation of stress-responsive sigma factors (Alessandria et al., 2013), the glutamate decarboxylase system (Ryan et al., 2009), among others (Melo et al., 2015). The high resistance to acid environments is a crucial facet which makes *L. monocytogenes* a potential hazard that is frequently associated with listeriosis outbreaks linked to the consumption of cheeses.

3.4. Modelling Listeria monocytogenes survival kinetics in soft and cured cheeses stored at 4 and 22°C

The Weibull model was fitted to the survival data due to its versatility to describe the non-linearity on microbial survival kinetics, reflected by the presence of shoulders or tails on curves (Vose, 2008). This model has been frequently applied to describe microbial

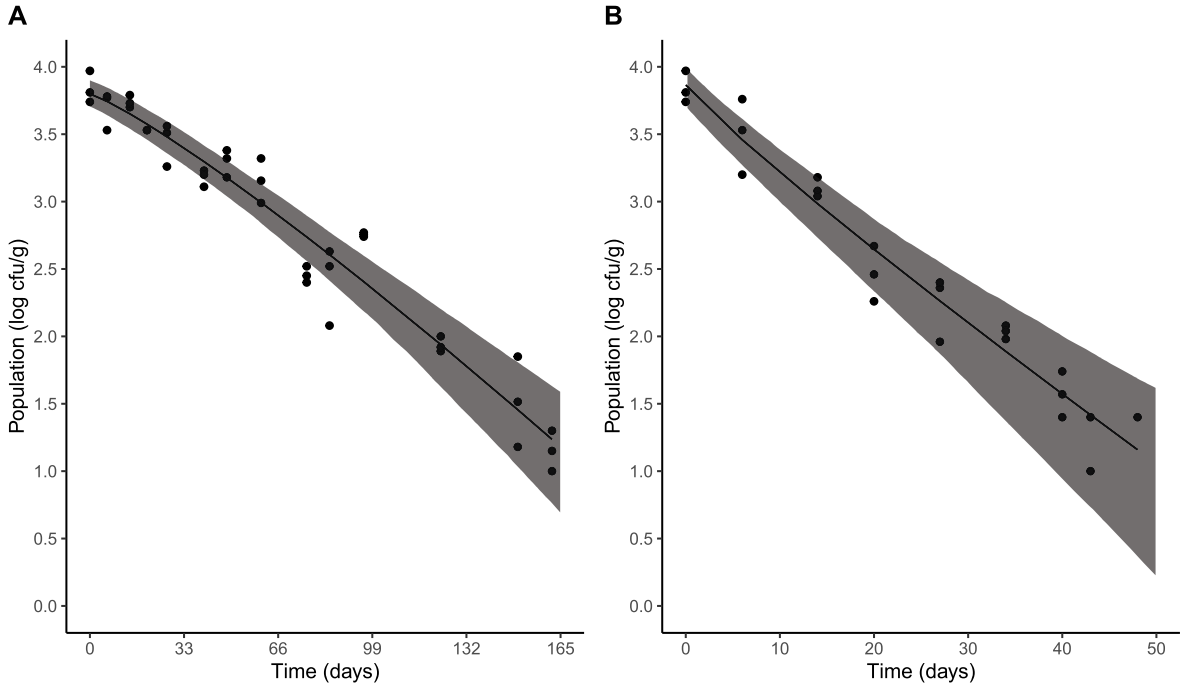

Fig. 2. Survival kinetics of *Listeria monocytogenes* in cured raw sheep milk cheese during storage at (A) 4 °C and (B) 22 °C. The grey zones represent the confidence intervals of the model predictions (95% CL).

survival/inactivation on cheeses during the production processes and storage (Angelidis et al., 2010, 2013; Lobacz et al., 2020).

Regarding the survival curves obtained for soft cheeses, an upward concavity pattern was observed. The time required for a first tenfold reduction of the *L. monocytogenes* population (δ) at 4 °C is estimated as 149.82 ± 10.20 and 155.76 ± 13.42 days in regular and low-salt soft cheeses, respectively, reflecting the high survival capacity of *L. monocytogenes* during soft cheese refrigerated storage (Table 3, Fig. 1A). The same pattern was observed by Sibanda and Buys (2019) in soft cheeses, obtaining much lower δ values during storage at 4 °C for 15 days (0.03–10.81 days). Such differences may be attributed to the lower pH of their cheeses (4.17) compared to the one studied here. During storage of low-salt soft cheese at 22 °C, the estimated δ was approximately 60% lower than the value estimated for the survival data obtained during refrigerated storage (Table 3). The same trend was confirmed by Mataragas et al. (2008) for the Greek soft cheese Katiki, where a biphasic behaviour of *L. monocytogenes* was observed during storage from 5 to 20 °C. The inactivation rate of the sensitive subpopulation at 5 and at 20 °C (k = 0.68 and 1.73 d^{-1}) was in the same proportion to the ratio observed in our study for the δ values at 4 and at 22 °C.

Regarding the modelling results for cured raw sheep milk cheeses, the survival curves of *L. monocytogenes* at 4 and 22 °C were nearly linear, *i.e.*, β close to 1 in both cases (Table 3). The δ parameter estimates confirm that cured raw sheep milk cheese is less suitable for *L. monocytogenes* survival than the soft cheese studied here at the different temperatures evaluated, *i.e.*, lower values estimated for the former (Table 3). In accordance with the results obtained for soft cheese, the δ value estimated at 4 °C (72.20 days) in cured raw sheep milk cheese is higher than the value estimated at 22 °C (16.09 days) (Table 2). Similar values were obtained by Valero et al. (2014) in raw sheep milk cured cheese at 4 °C (73.07 days), but the estimated δ value was higher at 22 °C (45.25 days). However, in the mentioned study microbial populations presented an initial resistance to decay, showing a downward concavity behaviour, probably attributed to differences in the strains evaluated and storage conditions among studies.

The 95% confidence intervals of the model predictions were determined to assess model accuracy (Figs. 1 and 2). Determining these intervals are relevant when building predictive models for microbial risk assessments, due to the variation observed on experimental data (Akkermans et al., 2018; Garre et al., 2017). The confidence intervals estimated for the fitted models in this study are wider at the late stages of cheese storage, reflecting the propagation of uncertainty on parameter estimates (Figs. 1 and 2). This could be associated with the fact that variability between cells increases in small microbial populations, compared to high populations (Aspridou and Koutsoumanis, 2015).

In addition, wider confidence intervals were estimated for the models fitted to the data obtained for the cured cheese (Fig. 2) and at the highest storage temperature evaluated (*i.e.*, 22 °C) (Figs. 1B and 2B). Environmental conditions such as low pH and low a_w in cured cheese could have triggered acid and osmotolerance stress-response mechanisms and systems in *L. monocytogenes* cells, affecting their viability (Melo et al., 2015). Moreover, it has been reported that natural *L. monocytogenes* isolates vary in response to these stresses so that higher temperatures could induce an increased resistance to pH and salt concentration (Alessandria et al., 2013; Faleiro et al., 2003; Melo et al., 2015).

Table 3
Parameters and goodness-of-fit of the Weibull model fitted to the survival data observed in cheese samples.

Cheese Type	Storage temperature (°C)	N_0 (log CFU/g)	β	δ (days)	RMSE
Regular soft	4	4.09 ± 0.04	0.76 ± 0.08	149.82 ± 10.20	0.10
Low-salt soft	4	4.08 ± 0.05	0.63 ± 0.07	155.76 ± 13.42	0.11
	22	3.82 ± 0.05	1.11 ± 0.08	59.24 ± 3.95	0.14
Cured	4	3.80 ± 0.07	1.16 ± 0.11	72.20 ± 6.31	0.20
	22	3.86 ± 0.11	0.91 ± 0.11	16.09 ± 2.39	0.21

Mean ± Standard errors of the estimates are provided.
N_0 is the initial concentration of *L. monocytogenes* on cheese samples.
β and δ are Weibull model parameters.
RMSE = Root Mean Square Error.

3.5. Prevalence of different serovars of Listeria monocytogenes in soft cheeses stored at 4 °C

The prevalence of the four different serovars of *L. monocytogenes* present in the inoculated cocktail were evaluated during refrigerated storage of soft cheeses. No differences were detected between the prevalence of the four serovars at the time 0, *i.e.*, immediately after inoculation ($p > 0.05$). These results were expected since the inoculum was adjusted with approximately the same concentration of each serovar evaluated.

All the four *L. monocytogenes* serovars present in the inoculated cocktail could be detected in soft cheese samples over 189 days of storage (Fig. 3). After 189 days of storage, the prevalence of *L. monocytogenes* serovar 1/2c strain on low-salt soft cheeses stored at 4 °C was statistically higher, *i.e.,* 55% ($p \leq 0.05$), than the prevalence of the other three serovars tested, which ranged from 7 to 20% (Fig. 3A). In line with these results, the serovar 1/2c strain was the most prevalent one in regular soft cheese after 189 days of storage, *i.e.,* 58%, compared to the other serovars which were present at 12–17%. These results indicate that survival behaviour of the multi-strain cocktail was dominated by the serovar 1/2c strain. On the other hand, the prevalence of the serovar 4b strain decreased on soft cheeses over time, being the most sensitive to the refrigerated storage and the least prevalent after 189 days.

The findings of this study are in line with the findings of Valero et al. (2018), which evaluated the fate and persistence of the four *L. monocytogenes* strains evaluated in this study in processed grated cheese, inoculated as individual cultures and as a cocktail. The serovar 1/2c strain was the most prevalent one by the end of cheese storage at 4 °C, exhibiting lower inactivation rate than the other serovars when inoculated individually. On the other hand, the serovar 4b strain exhibited higher inactivation rate at 4 °C, compared to strains of serovars 1/2a, 1/2b and 1/2c (Valero et al., 2018).

The *L. monocytogenes* strains differ in their ability to cause disease and therefore, in their epidemic potential (Alía et al., 2020), which highlights the relevance of characterizing the serovar variability of strains within dairy plants and on cheeses during their shelf-life. Despite being less prevalent than the serovar 1/2c by the end of soft cheese storage, the serovars 4b and 1/2a have been the leading serovars responsible for foodborne listeriosis cases (Amato et al., 2017). For instance, an outbreak associated with the consumption of cheese in Switzerland was traced back to a persistent *L. monocytogenes* serovar 4b strain in 2018, found in the cheese production environment (Nüesch-Inderbinen et al., 2021). Moreover, a serovar 4b strain caused a cheese-related outbreak in Portugal in 2009–2012, with a high fatality rate (36.7%) (Ferreira et al., 2018). This serovar was also found in cheese and processing facilities.

A Chilean study on artisanal cheese elaborations found that the most prevalent serovars were associated to 1/2b; 4b and 1/2a (Barría et al., 2020). In European studies, the most commonly reported serovar is 1/2a, followed by 4b (Gnanou Besse et al., 2019) while another study found 4b and 1/2b to be the dominant serovars (Vallim et al., 2015). These results, seem to be contradictory to the higher prevalence of serovar 1/2c found in our study. However, it has been shown that serovar prevalence can vary according to the geographical region,

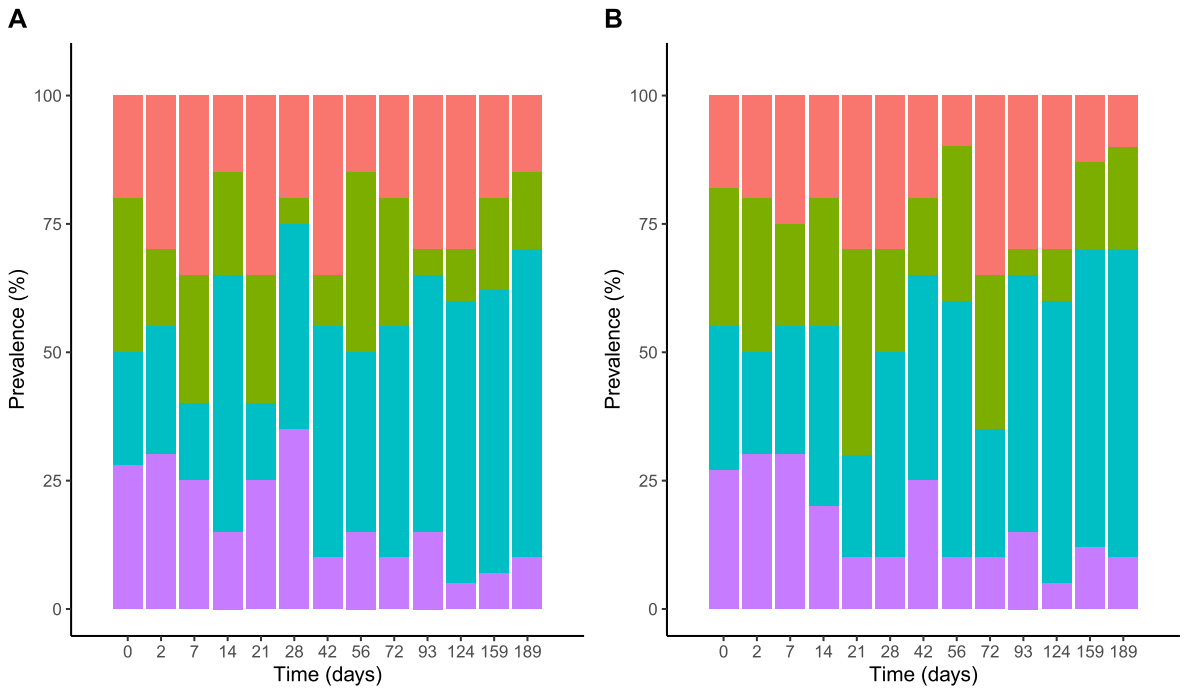

Fig. 3. Prevalence of the different serovars of *Listeria monocytogenes* along 189 days of storage at 4 °C in (A) low-salt soft cheese and (B) regular soft cheese. (■) ½a; (■) ½b; (■) ½c; (■) 4b.

seasonal variations, and industrial processing environment. This is confirmed by previous studies which indicate that certain strains of *L. monocytogenes* can survive better within the food processing environment (Wagner et al., 2006), and their persistence in the processing plant is a matter of concern as they may constitute a recurrent contamination source of the processed products (Sasahara and Zottola, 1993).

Further, *L. monocytogenes* contamination in dairy facilities is often detected in the processing environments rather than in the raw materials. The contamination patterns are related to more persistent than transient *L. monocytogenes* strains that could be found in the final product. Our study where a survivor strain was found at the end of storage corroborates the results found by other authors using a strain cocktail in soft cheese, where one strain out-lived others, remaining as the most prevalent by the end of the storage (Sibanda and Buys, 2019). It could be hypothesized that *L. monocytogenes* 1/2c strain was a natural isolate closely related to the individual factory where cheeses were elaborated. Thus, the higher prevalence of serovar 1/2c strain by the end of storage of soft cheese does not imply a higher risk of listeriosis caused by serovar 1/2c as the relationship between clinical strains and cheese isolates needs to be elucidated (Wagner et al., 2006).

4. Conclusions

The results obtained in this study confirm that *L. monocytogenes* can survive during prolonged storage at refrigeration and room temperatures in soft and cured cheeses, and that survival is favoured at lower temperatures. The adaptation of cells to the acid and osmotic conditions of the evaluated cheeses may result in increased microbial resistance to harsh conditions, which could lead to an increased risk of listeriosis to consumers. Salt reduction did not affect pathogen behaviour in soft cheese under the evaluated conditions. The survival parameters estimated through the Weibull model fitting would support on the evaluation of the safety implications of the storage temperature on *L. monocytogenes* behaviour in soft and cured raw sheep milk cheeses. The *L. monocytogenes* strain belonging to the serogroup 4b was the most sensitive to refrigeration temperatures in soft cheeses with different salt concentrations, while the *L. monocytogenes* serogroup strain 1/2c was the most resistant. The inter-strain variability in terms of resistance/persistence must be considered when selecting strains for challenge tests, particularly when defining best- and worst-case scenarios for microbial risk assessments. Further studies considering different strains belonging to the same serovar are required to investigate whether this resistance is a serovar trait.

Data availability

Data will be made available on request.

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