Use of commercial protective cultures in portioned sheep milk cheeses to control *Listeria monocytogenes*

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Abstract

The main objective of this study was to innovate soft and semi-cooked sheep milk cheese production processes with the use of a commercial protective culture able to control *Listeria monocytogenes* growth. A freeze-dried commercial culture of *Lactobacillus plantarum* was tested in DS cheese and PS cheese, two types of pasteurized sheep milk, raw-paste cheeses aged for no less than 20 and 30 days respectively. In the first step, *in vitro* tests were conducted to identify the most suitable matrix for the growth of *L. plantarum* in order to create a subculture that could be used at industrial cheese-making plants. During the second phase of the study, *L. plantarum* culture was introduced in the manufacturing process of the cheeses in a production plant. Finally, a challenge test was conducted on portioned DS and PS cheeses in order to evaluate the activity of the protective culture against *L. monocytogenes*: the cheeses were portioned, experimentally contaminated with *L. monocytogenes* strains, vacuum packed and stored at +4°C (correct storage conditions) and at +10°C (thermal abuse). Cheeses were analysed at the end of the shelf-life to evaluate the presence and growth of *L. monocytogenes*, to enumerate lactic acid bacteria and determine chemical-physical features. The results confirmed that protective cultures are a useful technological innovation to control *L. monocytogenes* growth during cheese storage without altering composition, microflora and chemical-physical characteristics of the product. However, the use of protective cultures should be applied as an integration of risk control measures and not as a substitute for preventive actions.

Introduction

Many varieties of soft cheeses produced from sheep milk are manufactured in the region of Sardinia (Italy); DS cheese and PS cheese are two types of soft cheeses made from pasteurized sheep milk, characterized by a semi-cooked paste, aged for no less than 20 and 30 days respectively and with a shelf life of 120 days (DS) and 150 days (PS). Soft cheeses are considered ready-to-eat foods and, in consideration of their pH and a_w values, are defined by Commission Regulation (EC) N. 2073/2005 as permissive for the growth of *Listeria monocytogenes*. Soft cheeses are an important source of listeriosis outbreaks and pose a major concern to the dairy industry and public health (Melo *et al.*, 2015). Contamination of cheeses can come from a variety of sources, such as the milk itself or from the processing environment. Raw sheep milk can be contaminated by *L. monocytogenes* (Gonzales-Barron *et al.*, 2017) and pasteurization, if not properly carried out, could be not enough to eliminate the pathogen (Todd and Notermans, 2011). On the other hand, pasteurized milk cheeses appear more subjected to *L. monocytogenes* growth due to the absence of the typical microbiota of raw milk that can play an antagonistic action on the pathogen (Panebianco *et al.*, 2021). *L. monocytogenes* can persist in processing environments, create biofilms on several materials and surfaces and, therefore, contaminate the products, particularly during post-processing phases, such as cheeses portioning and packaging (Colagiorgi *et al.*, 2017). Additionally, *L. monocytogenes* is able to grow at refrigeration temperatures and until salinity values of 20% (Wiedmann and Sauders, 2007); these features allow the pathogen to survive and grow in contaminated soft cheeses during the entire storage period. Contamination after the production process is a special concern since the finished products are regularly consumed without any further heating or processing to eliminate the pathogen (Falardeau *et al.*, 2021).

Several studies have been conducted to investigate new strategies for the control of *L. monocytogenes* in food (Bahramia *et al.*, 2020) and the use of bioprotective cultures has been gaining interest in the dairy industry (Young and O'Sullivan, 2011). Protective cultures consist of bacteria that are safe for consumption and specifically selected for their ability to inhibit the growth of pathogens or microbiological spoilage agents due to their competition for nutrients and the production of bacteriocins and other antagonistic compounds (Davidson and Techathuvanan, 2015).

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Lactic acid bacteria (LAB) naturally dominate the microbiota of dairy products and are often used as bioprotective culture in the dairy industry to increase microbiological safety, extend shelf life and improve the sensory profile of cheese (Leroy *et al.*, 2004). The qualified presumption of safety status of LABs is granted by the European Food Safety Authority (EFSA, 2007), and the Generally Regarded as Safe status have been approved by the U.S. Food and Drug Agency (EFSA, 2007; Field *et al.*, 2018; Kaya *et al.*, 2019). Among LABs, *Lactobacillus plantarum* is of particular importance. *L. plantarum* is able to produce metabolites with antimicrobial activity capable of inhibiting the development of some pathogenic microorganisms including *L. monocytogenes* (Mills *et al.*, 2011). In particular *L. plantarum* is capable of producing a bacteriocin, known as plantaricin, which is of great interest as bactericidal agent for *L. monocytogenes* and other pathogens as *Staphylococcus aureus, Bacillus cereus* and *Clostridium botulinum* (O'Sullivan *et al.*, 2002; Castellano *et al.*, 2017). This property makes this species an

excellent candidate for use as bioprotective cultures (Sorrentino *et al.*, 2013). To the best of our knowledge, very little studies investigated the use of bioprotective cultures against *L. monocytogenes* in sheep milk soft cheeses in Sardinia. In this framework this study aimed to promote the innovation of soft and semi-cooked sheep milk cheeses production processes with the use of commercial protective cultures capable of producing bacteriocins and control *L. monocytogenes* growth.

Materials and methods

Study set-up

The study was divided into three steps: i) in the first step, the *in vitro* efficacy against *L. monocytogenes* of a *L. plantarum* commercial protective culture was assessed; ii) in the second step, *L. plantarum* protective culture was added in the production process of DS and PS at a cheese making plant in order to obtain experimental and control samples (*i.e.* with and without the use of the protective culture, respectively); iii) in the third step, a challenge test on portioned DS and PS cheeses was conducted to evaluate the effectiveness of the protective culture against *L. monocytogenes*.

Step one: *in vitro* assessment of the bioprotective culture

A protective commercial culture consisting of freeze-dried *L. plantarum* (LPL, Listeria Dairy Danisco Holdbac) was used for the experimentation. Initially, a test was developed to evaluate the growth of *L. plantarum* in different matrices, in order to define which of them was the most suitable for the realization of a subculture to be used at industrial plants during the cheese-making process. Matrices used were sheep milk whey and *scotta*. *Scotta* is the watery part of whey that remains after the production of *Ricotta*, a dairy product manufactured by heat coagulation of sheep milk whey (Pala *et al.*, 2016). The test was conducted in triplicate for whey and *scotta*. Briefly, 3 liters of each matrix (whey and *scotta*) were equally divided into two sterile bottles and warmed in a thermostated bath at +37°C. Subsequently, 1 g of freeze-dried *L. plantarum* was added for each liter of matrix, in order to have an initial concentration of 10^6 CFU/mL. Before and immediately after inoculation of *L. plantarum* (T_0) and at intervals of 10-12 hours after inoculation, 1 mL of the matrix was used to perform serial dilutions in 0,85% sterile NaCl solution. 1 mL of each serial dilution was inoculated in a Petri dish with De Man, Rogosa and Sharpe agar with Tween® 80 (MRS agar, Biolife Srl, Milan, Italy) for the enumeration of mesophilic LAB according to ISO 15214:1998. Plates were incubated in anaerobiosis at +37°C for 48-72 hours. The pH value and the mesophilic LAB enumeration were evaluated at T_0 and after 8 hours from the inoculum with a time interval of 1 hour until the 12th hour (T_8, T_9, T_{10}, T_{11} and T_{12}). The obtained data were used to draw the growth curves of mesophilic LAB and acidification curves for each matrix analysed.

The Well Diffusion Assay method was used to evaluate the ability of LPL protective culture to inhibit *L. monocytogenes* growth, using the protocol defined by Cosentino *et al.* (2012), with some modifications. Briefly, 1% (v/v) aliquot of an overnight culture of a *L. monocytogenes* reference strain (National Collection of Type Cultures, NCTC 10887) was inoculated into 20 mL of Brain Heart Infusion agar medium (BHI agar, Biolife Srl, Milan, Italy) and poured into Petri dishes. After cooling, wells (6 mm diameter) were cut into the agar and filled with 100 μL of matrix (whey or *scotta*) with LPL protective culture. Plates were incubated at +37°C for 24 hours. The antimicrobial activity was expressed as the diameter of the inhibition zones around the wells.

Scotta showed the best *in vitro* results and it was chosen for the preparation of the LPL subculture to be used at the producing plants for the manufacturing of the experimental cheese batches.

Step two: LPL subculture preparation and cheese production

LPL subculture was prepared with the use of *scotta* as follows: for each litre of *scotta* (S), 1 gram of freeze-dried *L. plantarum* was added; then, the LPL subculture (S+LPL) was stored in a fermenter at +37°C for 12 hours. In an industrial producing plant located in Sardinia, 3 batches of DS and PS cheese were produced and for each kind of cheese two types of samples were produced: DS and PS control samples without LPL (negative control samples: CPS-C and CDS-C), and DS and PS samples added with the *L. plantarum* protective culture (experimental samples: CPS+LPL and CDS+LPL). The experimentation was carried out in triplicate for both kind of cheese, during three different processing days. The cheese production process was conducted following the manufacturing process normally applied at the cheese making plant, and included the following phases: i) milk pasteurization (71°C for 18-19"); ii) cooling of the milk to 38°C and addition of microbial starters (*Streptococcus thermophilus* and *Lactobacillus lactis*) both in the experimental and control batches; iii) addition of the LPL protective culture only in the experimental batches; iv) addition of microbial liquid rennet produced by the fermentation of *Rhizomucor miehei*; v) breaking of the curd at the end of the setting time (about 10' after the addition of the rennet) in grains of about 10 mm (PS) or 20 mm (DS); vi) moulding process and stewing of the curd (50-55°C for 80-120'); vii) 24 hours after moulding, insertion in brine (density 24°Bè) for 48 hours at about 10°C; viii) ripening in the cell at 11-12°C and 80-85% humidity. The production process of the two cheeses is summarized in Figure 1.

Both control samples and experimental

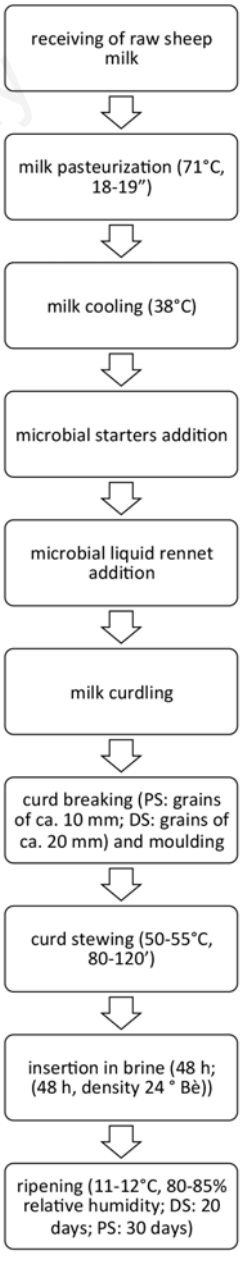

Figure 1. Process flow diagram of PS and DS cheeses.

samples were collected and analysed at the following stages of the cheese making process: i) samples of *scotta* with the addition of LPL protective culture after 12 hours of fermentation; ii) samples of curd immediately after breaking; iii) samples of curd after acidification and before brine. A total of 72 samples were analysed for this step of the experiment.

All samples were transported at +4°C to the laboratories of the Department of Veterinary Medicine of the University of Sassari.

pH analysis

On each sample, pH was determined by using the potentiometric measurement with 10 g of sample in 1:1 ratio with sterile distilled water using a pH-meter GLP 22 (Crison Instruments SA, Barcelona, Spain).

Enumeration of mesophilic and thermophilic lactic acid bacteria

From each sample, 25 g were collected and placed inside a sterile stomacher bag (BagFilter® 400P), 225 mL of Buffered Peptone Water (BPW, Biolife Srl, Milan, Italy) were added and the sample homogenized using a stomacher. Then, decimal serial dilutions in 0,85% sterile NaCl solution were prepared in a 1:9 ratio. The dilutions were included in Petri dishes with MRS Agar with Tween® 80. Plates with *scotta* scalar dilutions were incubated in anaerobiosis at +30°C for 72 hours. Plates with curd scalar dilutions were inoculated in duplicate and incubated in anaerobiosis at +30°C and +45°C for 72 hours and 48 hours respectively.

Step three: challenge test

Experimental inoculum of Listeria monocytogenes

An experimental inoculum was set up consisting of a mixture of 3 *L. monocytogenes* strains: 2 wild type strains (previoulsy isolated from the cheese making plant environment) and 1 reference strain (National Collection of Type Cultures, NCTC 10887). *S*trains were stored at -80°C and revitalized after incubation for 18-24 hours at +30°C in Brain Heart Infusion Agar (BHI Agar, Biolife, Milan, Italy). A colony of *L. monocytogenes* was taken from each plate of BHI agar and transferred into a sterile tube containing 10 mL of Brain Heart infusion Broth (BHI Broth, Biolife Srl, Milan Italy). The tubes were incubated under shaking at +30°C for 18 hours, until reaching the stationary growth phase of *L. monocytogenes*, corresponding to the concentration of 10^9 CFU/mL. To allow adaptation to refrigeration temperatures, the tubes containing *L. monocytogenes* strains were stored at +4°C for 7 days, according to EURL Lm Technical guidance document on challenge tests and durability studies for assessing shelf-life of ready-to-eat foods related to *L. monocytogenes* (version 4 July 2021). Then, decimal serial dilutions were prepared for each *L. monocytogenes* strain, until a concentration of 10^2 CFU/mL was obtained. Finally, the 3 strains of *L. monocytogenes* were mixed in equal parts to obtain the final inoculum.

Challenge test

At the end of the ripening period, DS and PS cheese wheels were vacuum-packed at the cheese making plant, transported to the laboratories of the Department of Veterinary Medicine of the University of Sassari and stored at +4°C.

The cheese wheels of DS had a diameter of 16 cm, a height of 8 cm and a weight of about 2.0 kg. The cheese wheels of PS had a diameter of 21 cm, a height of 12 cm and a weight of about 4.0 Kg.

The definition of the protocol for the challenge test was conducted according to the EURL Lm Technical guidance document on challenge tests and durability studies for assessing shelf-life of ready-to-eat foods related to *L. monocytogenes* (version 4 July 2021). For each of the three batches of DS and PS, 3 experimental wheels (containing LPL protective culture, 9 wheels overall) and 3 control wheels (without LPL protective culture, 9 wheels overall) were chosen. From vacuum-packed PS and DS wheels (experimental and control) 4 cheese wedges of equal size of DS (for a total of 36 experimental wedges and 36 control wedges overall) and 6 cheese wedges of equal size of PS (for a total of 54 experimental wedges and 54 control wedges overall) were cut. Afterwards, the surface of each wedge was contaminated with the inoculum of *L. monocytogenes* previously prepared, in order to have a concentration of 10 CFU/cm^2. The experimental contamination was carried out using a sterile spatula, to mimic a contamination, possibly caused by the cutting blade of the portioning machine. After contamination, each cheese wedge was re-packed under vacuum and divided into two groups: one group was stored at +4°C, to simulate ideal storage conditions, and the other group was stored at +10°C, to simulate thermal abuse.

The cheese wedges were analyzed in triplicate at the following analysis times: T_0 (the day after contamination with *L. monocytogenes*) and T_{120} (120 days after contamination with *L. monocytogenes;* 120 days accounts as the shelf life of the product indicated by the manufacturer). Moreover, PS wedges were also analysed at T_{150} (150 days after contamination with *L. monocytogenes,* which accounts as the shelf life of the product indicated by the manufacturer). From each sample, 1 cm of surface depth from the whole wedge was grated, then 25 g were collected and placed inside a sterile stomacher bag (BagFilter® 400P) and analysed.

A total of 180 samples were analyzed for the challenge test.

Quantitative and qualitative detection of Listeria monocytogenes

Quantitative and qualitative detection of *L. monocytogenes* (UNI EN ISO 11290-1/2:2017) was evaluated for each cheese wedge at each time point.

Mesophilic lactic acid bacteria profile

Mesophilic LAB enumeration was determined for each cheese wedge according to ISO 15214:1998, as previously described.

Physico-chemical analysis

For all samples, pH was determined as previously described. Moreover, water activity (a_w) analysis was conducted at +25°C, using an Aqualab CX3 (Decagon, Pullman, Washington, USA).

Statistical analysis

Differences among average microbiological group counts ($\log_{10}$ cfu/g), pH and a_w, over time and among treatments were compared using Fisher's least significant difference test. Statistical analyses were performed with Statgraphics Centurion XIX software (Stat Point Technologies, Warrenton, VA, USA).

Results

First step: *in vitro* assessment of the bioprotective culture

Experimental tests performed on whey and *scotta* allowed to identify the most suitable matrix for the realization of a *L. plantarum* subculture to be used in the cheese making processing. The test was conducted in triplicate for whey and *scotta*. The pH decreased from 6.39±0.21 (T_0) to 4.72±0.54 (T_{12}) in whey and from 6.08±0.05 (T_0) to 4.70±0.19 (T_{12}) in scotta. As regard mesophilic LAB mean counts ($\log_{10}$ CFU/g; $\bar{x}$ ± standard deviation (SD), an increase of *ca.* 1.1 log was detected in whey samples (6.05±0.06 $\log_{10}$ CFU/g at T_0 and 7.20±0.12 $\log_{10}$ UFC/g at T_{12}), while *scotta* LAB mean levels showed an increase of more than 2 log (5.96±0.10 $\log_{10}$ CFU/g at T_0 and 8.12±0.06 $\log_{10}$ UFC/g at T_{12}). Furthermore, the acidification curve after addition of LPL

protective culture showed better linearity in *scotta* samples in comparison to whey. For these reasons, *scotta* was the matrix of choice for the preparation of the LPL subculture to be used for the subsequent steps.

As regards the Well Diffusion Assay, *L. monocytogenes* growth inhibition halos were observed in all wells inoculated with LPL protective culture. However, inhibition halos were hidden by the growth of bacterial colonies outside the wells, and it was not possible to measure their diameter. Colonies inside the halos were identified by a specific PCR for identification of *L. plantarum* according to the protocol by Oneca *et al.* (2003). Colonies inside the halos were confirmed not to be *L. plantarum*. BHA sterility was confirmed by control plates that were incubated within the inoculated ones.

Second step: experimental and control cheese production

Table 1 shows pH ($\bar{x}$±SD) and mesophilic and thermophilic LAB mean levels (log_{10} CFU/g; $\bar{x}$±SD) detected in samples of LPL subculture and samples collected during cheese making process of DS and PS.

LPL subculture showed pH levels of 5.1±0.72 and mesophilic LAB mean counts of 7.88±1.37 for the three batches. pH in samples of curd collected after breaking was 6.5 with minor differences between experimental (added with LPL) and control samples (without LPL) of PS and DS. Also, in curd samples collected after acidification and before brine, pH showed similar mean levels in both cheeses experimental and control samples (5.4 and 5.2 respectively in PS in DS).

As far as mesophilic LAB enumeration is concerned, *L. plantarum* subculture on *scotta* had mean levels of 7.88±1.37. In samples of curd after breaking and curd after acidification, LAB mean levels were very variable in both kind of cheeses and in both experimental and control samples.

The methods used did not allow to differentiate *L. plantarum* from the other LAB present in the samples analysed.

Third step: challenge test

PS cheese

Table 2 shows pH and a_w mean levels (±SD), mesophilic LAB and L. *monocytogenes* counts (log_{10} CFU/g; $\bar{x}$±SD), and *L. monocytogenes* detection results (positive samples/total) in experimental and control PS samples analysed during the challenge test.

Ph mean levels ($\bar{x}$±SD) showed a decrease during storage both in experimental and control samples, but no significant differences were detected at any analysis times. A_w ($\bar{x}$±SD) levels were comparable between experimental and control samples, with a decrease during storage that was significant (P<0.01) between T_0 and T_{120}. As far as the microbial profile is concerned, mesophilic LAB (log_{10} CFU/g; $\bar{x}$±SD) showed a tendency to increase during storage both in experimental and control samples. In particular, in samples stored at +4°C, LAB mean counts increased significantly during storage (P<0.01) between T_0 and T_{120} (*ca.* 2 log) and at the end of the experiment (1 log in experimental and 0.5 log in control samples). In samples stored at +10°C mean LAB levels showed the same trend in experimental and control samples with a significant increase (P<0.05) of *ca.* 1 log between T_0 and $T_{120,}$ while remained stable until T_{150}.

The use of the protective culture showed a tendency to reduce *L. monocytogenes* growth during storage both at +4°C and +10°C. In fact, *L. monocytogenes* enumeration (log_{10} CFU/g; ±SD) showed higher levels in control samples compared to experimental samples stored at +4°C at all analysis times, although without significant difference (P>0.05). Reduction of *L. monocytogenes* growth during storage with the use of the protective culture was confirmed with the qualitative method, particularly at +4°C. In fact, in samples with protective culture stored at +4°C, the pathogen was detected in 6/9 (66.7%) and 5/9 (55.6%) samples stored at T_{120} and T_{150} respectively. In samples stored at +10°C, the trend was more irregular with 2/9 (22.3%) *L. monocytogenes* positive samples at T_{120} and 4/9 (44.5%) at T_{150}.

DS cheese

Table 3 shows pH and a_w mean levels ($\bar{x}$±SD), mesophilic LAB and L. *monocytogenes* counts (log_{10} CFU/g; $\bar{x}$±SD), and *L. monocytogenes* detection results (positive samples/total) in experimental and control DS samples analysed during the challenge test.

pH was significantly lower (P<0.05) in

Table 1. pH, temperature, mesophilic Lactic acid bacteria (30°C) and thermophilic Lactic acid bacteria (45°C) mean values (log_{10} CFU/g; x ±SD) in samples of curd after breaking and curd after acidification and before brine. Values between brackets indicate the number of positive batches/totals.

Samples	Parameters		
	pH	LAB 30°C	LAB 45°C
Curd after breaking			
CPS-C	6.5±0.06	5.23±0.13 (2/3)	5.87±1.22 (3/3)
CPS+LPL	6.5±0.10	6.06±1.14 (3/3)	6.72±1.04 (3/3)
CDS-C	6.5±0.06	6.14±0.48 (2/3)	7.36±0.51 (3/3)
CDS+LPL	6.5±0.06	7.25±0.56 (3/3)	7.78±0.38 (3/3)
Curd after acidification and before brine			
CPS-C	5.4±0.21	6.14±0.41 (3/3)	6.82±0.50 (3/3)
CPS+LPL	5.4±0.20	6.88±0.86 (3/3)	7.27±0.21 (3/3)
CDS-C	5.2±0.06	6.12±1.47 (2/3)	7.61±0.45 (3/3)
CDS+LPL	5.2±0.06	5.61±1.03 (3/3)	6.75±1.56 (3/3)

SD, standard deviation; LAB, Lactic acid bacteria; CPS-C and CDS-C: samples of PS and DS cheeses without protective culture (control samples); CPS+LPL and CDS+LPL: samples of PS and DS cheeses with protective culture (experimental samples); LAB 30°C, mesophilic lactic acid bacteria; nd, not determined.

samples with the addition of protective cultures rather than in control samples at both analysis times. No significant difference (P>0.05) was detected between analysis times. In terms of a_w results, a significant decrease (P<0.05) during storage was detected for both experimental and control samples, but the addition of the *L. plantarum* protective culture did not show any significant influence (P>0.05).

As observed for PS, mesophilic LAB showed a variable growth at both temperatures, in experimental and control samples. In fact, the initial contamination level at T_0 in samples stored at +4°C were of 7.35±0.77 and 6.56±0.46, respectively in experimental and control samples with a significant (P<0.05) increase at T_{120} of *ca.* 0.7 log in experimental samples; a comparable trend was registered in samples stored at +10°C with levels of 7.54±0.59 and 6.59±0.50 respectively in experimental and control samples at T_0, followed by a significant rise (P<0.05) of the mean levels at T_{120}. In terms of *L. monocytogenes* enumeration, a general reduction was observed during storage in samples produced with the protective culture. In fact, in samples with protective cultures at +4°C a significant reduction (P<0.01) of mean levels was detected between T_0 (1.51±0.35) and T_{120} (1.36±0.10). Control samples also showed a reduction, although not significant, of mean counts between T_0 (1.41±0.32) and T_{120} (1.30±0.30). In experimental samples stored at +10°C a similar trend was observed, with a significant reduction (P<0.01) of *L. monocytogenes* between T_0 (1.36±0.31) and T_{120} (1.00), in which the pathogen was detected only in 1/9 (11.2%) samples. In control samples stored at +10°C a not significant increase of *L. monocytogenes* mean levels was recorded between T_0 (1.53±0.30) and T_{120} (2.06±1.29).

The tendency to a reduction of *L. monocytogenes* with the use of the protective culture during storage was confirmed with the qualitative method: at T_0 *L. monocytogenes* was detected from 9/9 (100%) experimental samples both at +4°C and +10°C, while at T_{120}, the pathogen was detected from 6/9 (66.7%) and 3/9 (33.4%) experimental samples at +4°C and +10°C respectively. On the other hand, at T_{120} *L. monocytogenes* was detected from 9/9 (100%) and 8/9 (88.9%) control samples stored respectively at +4°C and at +10°C.

Discussion

The occurrence of *L. monocytogenes* in soft cheeses represents a risk of listeriosis and emphasizes the need for adequate control measures throughout the production chains (Falardeau *et al.*, 2021). The use of a bioprotective culture in the production of soft cheeses in Sardinia represents a significant innovation compared to the standard technologies adopted during production. The aim of this work was to test *L. plantarum* as a protective culture applied in the manufacturing process of soft sheep milk cheese in order to prevent *L. monocytogenes* post-production contamination.

The food matrix can significantly impact on the efficacy of a bioprotective cultures in inactivating the foodborne pathogens (Muñoz *et al.*, 2007; Silva *et al.*, 2018). Therefore, in the first step of the study, the growth of *L. plantarum* in sheep's milk whey and *scotta* was evaluated, in order to determine which matrix was most suitable for the creation of a subculture to be used in cheese-making production. After the addition of LPL protective culture, LAB growth showed higher levels and pH decrease showed a better linearity in scotta samples in comparison to whey. In terms of the Well Diffusion Assay, *L. monocytogenes* growth inhibition halos were observed in all wells inoculated with LPL protective culture and were greater in *scotta* samples. The reduced anti-*listeria* activity observed in the cheese whey may be a result of bacteriocins and/or other antimicrobial compounds inactivation because of the adhesion to the surface of milk proteins or cells (Coelho *et al.*, 2014). Similar findings were made by other authors who were unable to detect bacteriocin activity on cheese whey (Rodríguez *et al.*, 2005; Sarantinopoulos *et al.*, 2002). Due to these factors, *scotta* was chosen as the most suitable matrix for preparing the LPL subculture that would be utilized in the following steps.

The addition of the *L. plantarum* culture in the production process of PS and DS cheeses did not cause changes in the chemical-physical (pH and a_w) characteristics during production, without any statistically significant difference between the control and experimental samples. The additional culture did not exhibit a further acidifica-

Table 2. pH, a_w, mesophilic Lactic acid bacteria (30°C) *Listeria monocytogenes* enumeration (log_{10} CFU/g; $\bar{x}$ ±SD) and *Listeria monocytogenes* detection in samples of PS after ripening. Values between brackets indicate the number of positive batches/totals.

Parameters	Samples	T°C	Analysis times		
			T0	T120	T150
pH	CPS+LPL	4°C	5.51±0.25 (9/9) ^{a1}	5.38±0.16 (9/9) ^{a1}	5.30±0.12 (9/9) ^{a1}
	CPS-C		5.65±0.06 (9/9) ^{b1}	5.50±0.15 (9/9) a^{b1}	5.33±0.15 (9/9) ^{a1}
a_w	CPS+LPL	4°C	0.974±0.003 (9/9) ^{a1}	0.961±0.001 (9/9) ^{b1}	0.962±0.002 (9/9) ^{b1}
	CPS-C		0.976±0.004 (9/9) ^{a1}	0.960±0.004 (9/9) ^{b1}	0.960±0.003 (9/9) ^{b1}
LAB 30°C (log_{10} CFU/g; $\bar{x}$ ±SD)	CPS+LPL	4°C	7.46±1.16 (9/9) c1	9.44±0.79 (9/9) ^{a1}	8.39±0.49 (9/9) ^{b1}
	CPS-C		7.10±0.72 (9/9) ^{b1}	9.32±0.75 (9/9) ^{a1}	8.82±0.59 (9/9) ^{a1}
	CPS+LPL	10°C	7.54±1.14 (9/9) ^{b1}	8.65±0.64 (9/9) ^{a1}	8.69±0.59 (9/9) ^{a1}
	CPS-C		7.41±0.82 (9/9) ^{b1}	9.75±0.76 (9/9) ^{a2}	9.25±0.22 (9/9) ^{a2}
L. monocytogenes enumeration (log_{10} CFU/g; $\bar{x}$ ±SD)	CPS+LPL	4°C	1.69±0.30 (8/9) ^{a1}	1.22±0.21 (5/9) ^{a1}	1.25±0.33 (4/9) ^{a1}
	CPS-C		1.80±0.31 (8/9) ^{a1}	1.53±0.23 (6/9) ^{a1}	1.31±0.26 (6/9) ^{a1}
	CPS+LPL	10°C	1.65±0.40 (8/9) ^{a1}	3.17 (1/9) ^{a1}	1.39±0.12 (2/9) ^{b1}
	CPS-C		1.96±0.24 (8/9) ^{a1}	1.41±0.35 (5/9) ^{a1}	1.78 (1/9) ^{a1}
L. monocytogenes detection (positive samples/total)	CPS+LPL	4°C	9/9	6/9	5/9
	CPS-C		9/9	7/9	7/9
	CPS+LPL	10°C	9/9	2/9	4/9
	CPS-C		8/9	7/9	4/9

SD, standard deviation; LAB, Lactic acid bacteria; T0, day 0 of inoculum; T120 and T150, respectively 120 and 150 days of storage after the inoculum; CPS+LPL, samples of PS cheeses with protective culture (experimental samples); CPS-C, samples of PS cheeses without protective culture (control samples); LAB 30°C, mesophilic lactic acid bacteria. Means in the same row with different superscript letter were significantly different (P<0.05); means in the same column among treatments with different superscript number were significantly different (P<0.05).

tion other than that induced by the microbial starter culture; it is indeed essential to avoid an over-acidity of the curd, which would have negative consequences on the cheese's physico-chemical properties and sensory acceptability (Gobbetti *et al.*, 2015). As far as the challenge test is concerned, pH levels showed a decrease during storage both in DS and PS cheese, but significant differences were only detected in DS samples, with greater acidification ($P<0.05$) in samples with the addition of the protective culture. However, acidification was contained and no samples showed values lower than 5.2. No other significant modification was observed in physico-chemical or microbiological characteristics between the experimental and control samples.

The experimental post-process surface contamination showed the ability of *L. plantarum* protective culture to promote a reduction, although moderate, of the initial levels of *L. monocytogenes*. A general reduction in *L. monocytogens* mean levels was observed during storage in samples produced with the protective culture, despite a statistically significant difference was observed only in DS samples ($P<0.01$); in particular, *L. monocytogenes* decrease was more regular in the samples stored at +4°C, while in thermal abuse conditions (+10°C) the reduction showed an irregular trend. The antimicrobial activity observed during storage could be attributed to a reduction in pH values due to an increased production of lactic acid by LAB strains, but not solely, as demonstrated by the fact that in most samples pH values were identical to the control and there was little pH drop during cheese storage. Authors have shown the ability of LAB to compete with *L. monocytogenes* avoiding its growth (Arena *et al.*, 2016; Campagnollo *et al.*, 2018; García *et al.,* 2020; Scatassa *et al.*, 2017) and, given that dairy-associated strains of *L. plantarum* are able to produce bacteriocins active against *L. monocytogenes* (Hernandez *et al.*, 2005; Panebianco *et al.,* 2021), the synthesis of these compounds by LAB is the most likely explanation for the observed *L. monocytogenes* reduction (Coelho *et al.,* 2014). Despite the fact that the kinetics of bacteriocin synthesis was not assessed in the current study, several authors report that bacteriocin production increases during the exponential phase of LAB growth and stabilizes during the stationary phase (Benkerroum *et al.*, 2002; Han *et al.*, 2013; Martínez *et al.*, 2013). Similar studies concluded that *L. plantarum* and bacteriocins activity led to a reduction of *L. monocytogenes* count during soft cheese shelf-life and the bioavailable bacteriocin activity has proven to be the most likely determining factor for the in situ antibacterial action observed (Martin *et al.*, 2022; Panebianco *et al.*, 2021).

Moreover, *L. plantarum* bioprotective culture in scotta has proven to be simple and quick to use in the cheese-making plant, and it doesn't require any changes to the standard production process procedures; as a result, the financial commitment for the production facilities is reasonable and meets the needs of the Food Business Operators. An interesting data is that the cheeses object of our study resulted not permissive to *L. monocytogenes* growth, despite their chemical-physical features (*i.e.* pH and a_w levels) in relation to what was established by Reg. CE n.2073/2005. This data should be considered by the Competent Authority when adopting measures in case of non-compliance as regards *L. monocytogenes* in these products (although <100 CFU/g).

It has to be taken into consideration that the use of protective cultures must be understood as an integration of risk control measures associated with *L. monocytogenes* contamination and not a substitute for other preventive actions. Therefore, it is important that, in association with the use of protective cultures, good manufacturing practices (GMP) and good hygiene practices (GHP) are respected, to reduce the risk of cheese contamination during and after the production process.

Conclusions

The use of *L. plantarum* as a bioprotective culture within the standard production process of soft and semi-cooked sheep milk soft cheese represents a significant technological innovation for the producing plants and it was proved to be a valid aid to control the growth of *L. monocytogenes* during storage. Inoculation of the protective culture did not require any modification and/or implementation of the standard production process of the cheeses and its application is

Table 3. pH, a_w, mesophilic Lactic acid bacteria (30°C) *Listeria monocytogenes* enumeration (log10 CFU/g; $\bar{x}$ ±SD) and *Listeria monocytogenes* detection in samples of DS after ripening. Values between brackets indicate the number of positive batches/totals.

Parameters	Samples	T°C	Analysis times	
			T0	T120
pH	CDS+LPL	4°C	5.35±0.10 (9/9) ^{a1}	5.27±0.24 (9/9) ^{a1}
	CDS-C		5.59±0.08 (9/9) ^{a2}	5.56±0.05 (9/9) ^{a2}
a_w	CDS+LPL	4°C	0.976±0.00 (9/9) ^{a1}	0.970±0.00 (9/9) ^{a2}
	CDS-C		0.977±0.00 (9/9) ^{a1}	0.972±0.00 (9/9) ^{a2}
LAB 30°C (log_{10} CFU/g; $\bar{x}$ ±SD)	CDS+LPL	4°C	7.35±0.77 (9/9) ^{b1}	8.08±0.39 (9/9) ^{a1}
	CDS-C		6.56±0.46 (9/9) ^{b2}	8.74±0.40 (9/9) ^{a2}
	CDS+LPL	10°C	7.54±0.59 (9/9) ^{a1}	8.12±0.57 (9/9) ^{a1}
	CDS-C		6.59±0.50 (9/9) ^{b2}	8.98±0.39 (9/9) ^{a2}
L. monocytogenes enumeration (log_{10} CFU/g; $\bar{x}$ ±SD)	CDS+LPL	4°C	1.51±0.35 (9/9) ^{a1}	1.36±0.10 (3/9) ^{b1}
	CDS-C		1.41±0.32 (5/9) ^{a2}	1.30±0.30 (5/9) ^{a2}
	CDS+LPL	10°C	1.36±0.31 (9/9) ^{a1}	1.00 (1/9) b1
	CDS-C		1.53±0.30 (7/9) ^{a1}	2.06±1.29 (4/9) ^{a1}
L. monocytogenes detection (positive batches/total)	CDS+LPL	4°C	9/9	6/9
	CDS-C		9/9	9/9
	CDS+LPL	10°C	9/9	3/9
	CDS-C		9/9	8/9

SD, standard deviation; T0, day 0 of inoculum; T120, 120 days of storage after the inoculum; CDS+LPL, samples of DS cheeses with protective culture (experimental samples); CDS-C, samples of DS cheeses without protective culture (control samples); LAB 30°C, mesophilic lactic acid bacteria. Means in the same row with different superscript letter were significantly different ($P<0.05$); means in the same column among treatments with different superscript number were significantly different ($P<0.05$).

not expensive for the Food Business Operators; moreover it did not altered the chemical-physical characteristics of the product. It remains confirmed that protective cultures must be used in conjunction with other preventative measures to reduce the risk of *L. monocytogenes* contamination.

References

Arena MP, Silvain A, Normanno G, Grieco F, Drider D, Spano G., Fiocco D, 2016. Use of Lactobacillus plantarum strains as a bio-control strategy against food-borne pathogenic microorganisms. Front Microbiol 7:464.

Bahramia A, Baboli ZM, Schimmel K, Jafari SM, Williams L, 2020. Efficiency of novel processing technologies for the control of Listeria monocytogenes in food products. Trends Food Sci Tech 96:61-78.

Benkerroum N, Ghouati Y, Ghalfi H, Elmejdoub T, Roblain D, Jacques P, Thonart P, 2002. Biocontrol of Listeria monocytogenes in a model cultured milk (Iben) by in situ bacteriocin production from Lactococcus lactis ssp. lactis. Int J Dairy Technol 55:145-51.

Campagnollo F, Margalho L, Kamimura B, Feliciano M, Freire L, Lopes L, Alvarenga V, Cadavez V, Gonzales-Barron U, Schaffner D, Sant'Ana AS, 2018. Selection of indigenous lactic acid bacteria presenting anti-listerial activity, and their role in reducing the maturation period and assuring the safety of traditional Brazilian cheeses. Food Microbiol 73:288–97.

Castellano P, Pérez Ibarreche M, Blanco Massani M, Fontana C, Vignolo G, 2017. Strategies for pathogen biocontrol using lactic acid bacteria and their metabolites: a focus on meat ecosystems and industrial environments. Microorganisms 5:38.

Coelho MC, Silva CCG, Ribeiro SC, Dapkevicius MLNE, Rosa HJD, 2014. Control of Listeria monocytogenes in fresh cheese using protective lactic acid bacteria. Int J Food Microbiol 191:53–9.

Colagiorgi A, Bruini I, Di Ciccio PA, Zanardi E, Ghidini S, Ianieri A, 2017. Listeria monocytogenes biofilms in the wonderland of food industry. Pathogens 6:41.

Cosentino S, Fadda ME, Deplano M, Melis R, Pomata R, Pisano MB, 2012. Antilisterial activity of nisin-like bacteriocin-producing Lactococcus lactis subsp. lactis isolated from fermented Sardinian dairy products. J Biomed Biotechnol 2012:376428.

Davidson PM, Techathuvanan C, 2015. The use of natural antimicrobials in food, chapter in: Taylor TM, Handbook of natural antimicrobials for food safety and quality, Woodhead Publishing, 2015.

EFSA, 2007. Introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA. EFSA J 587:1–16.

Falardeau J, Trmčić A, Wang S, 2021. The occurrence, growth, and biocontrol of Listeria monocytogenes in fresh and surface-ripened soft and semisoft cheeses. Compr Rev Food Sci Food Saf 20:4019–48.

Field D, Ross RP, Hill C, 2018. Developing bacteriocins of lactic acid bacteria into next generation biopreservatives. Curr Opin Food Sci 20:1–6.

García MJ, Ruíz F, Asurmendi P, Pascual L, Barberis L, 2020. Searching potential candidates for development of protective cultures: evaluation of two lactobacillus strains to reduce Listeria monocytogenes in artificially contaminated milk. J Food Saf 40:e12723.

Gobbetti M, De Angelis M, Di Cagno R, Mancini L, Fox PF, 2015. Pros and cons for using non-starter lactic acid bacteria (NSLAB) as secondary/adjunct starters for cheese ripening. Trends Food Sci Technol 45:167–78.

Gonzales-Barron U, Gonçalves-Tenório A, Rodrigues V, Cadavez V, 2017. Foodborne pathogens in raw milk and cheese of sheep and goat origin: A meta-analysis approach. Curr Opin Food Sci 18:7–13. https://doi.org/10.1016/j.cofs.2017.10.002.

Kaya HI, Ozel B, Simsek O, 2019. A natural way of food preservation: bacteriocins and their applications. in health and safety aspects of food processing technologies. Malik A, Erginkaya Z, Erten H (Eds). Berlin/Heidelberg, Germany: Springer; 633–59.

Leroy F., de Vuyst L, 2004. Lactic acid bacteria as functional starter cultures for the food fermentation industry. Trends Food Sci Technol 15:67–78.

Martín I, Rodríguez A, Alía A, Martínez-Blanco M, Lozano-Ojalvo D, Córdoba JJ, 2022. Control of Listeria monocytogenes growth and virulence in a traditional soft cheese model system based on lactic acid bacteria and a whey protein hydrolysate with antimicrobial activity. Int J Food Microbiol 361:109444.

Martínez RCR, Wachsman M, Torres NI, LeBlanc JG, Todorov SD, Franco BDGM, 2013. Biochemical, antimicrobial and molecular characterization of a noncytotoxic bacteriocin produced by Lactobacillus plantarum ST71KS. Food Microbiol 34:376–81.

Melo J, Andrew PW, Faleiro ML, 2015. Listeria monocytogenes in cheese and the dairy environment remains a Food Safety Challenge: the role of stress responses. Int Food Res J 67:75–90.

Mills S, Serrano L, Griffin C, O'Connor PM, Schaad G, Bruining C, Hill C, Ross P, Meijer WC, 2011. Inhibitory activity of Lactobacillus plantarum LMG P-26358 against Listeria innocua when used as an adjunct starter in the manufacture of cheese. Microb Cell Fact 10:S7.

Muñoz A, Ananou S, Gálvez A, Martínez-Bueno M, Rodríguez A, Maqueda M, 2007. Inhibition of Staphylococcus aureus in dairy products by enterocin AS-48 produced in situ and ex situ: bactericidal synergism with heat. Int Dairy J 17:760–9.

Oneca M, Irigoyen A, Ortigosa, M, Torre P, 2003. PCR and RAPD identification of L. plantarum strains isolated from ovine milk and cheese. Geographical distribution of strains. FEMS Microbiol Lett 227:271-7.

Pala C, Scarano C, Venusti M, Sardo D, Casti D, Cossu F, Lamon S, Spanu V, Ibba M, Marras M, Paba A, Spanu C, De Santis EPL, 2016. Shelf life evaluation of Ricotta Fresca sheep cheese in modified atmosphere packaging. Ital J Food Saf 5:5502.

Panebianco F, Giarratana F, Caridi A, Sidari R, De Bruno A, Giuffrida A, 2021. Lactic acid bacteria isolated from traditional Italian dairy products: activity against Listeria monocytogenes and modelling of microbial competition in soft cheese. LWT 137:110446.

Rodríguez E, Calzada J, Arqués JL, Rodríguez JM, Nuñez M, Medina M, 2005. Antimicrobial activity of pediocin-producing Lactococcus lactis on Listeria monocytogenes, Staphylococcus aureus and Escherichia coli O157:H7 in cheese. Int Dairy J 15:51–7.

Scatassa ML, Gaglio R, Cardamone C, Macaluso G, Arcuri L, Todaro M, Mancuso I, 2017. Anti-Listeria activity of lactic acid bacteria in two traditional Sicilian cheeses. Ital J Food Saf 6:6191.

Silva CCG, Silva SPM, Ribeiro SC, 2018. Application of bacteriocins and protective cultures in dairy food preservation. Front Microbiol 9:594.

Sorrentino E, Reale A, Tremonte P, Maiuro

L, Succi M, Tipaldi L, Di Renzo T, Pannella G, Coppola R, 2013. Lactobacillus plantarum inhibits Penicillium spp.. involved in the spoilage of black truffles (Tuber aestivum). J Food Sci 78:M1188-94.

Todd ECD, Notermans S, 2011. Surveillance of listeriosis and its causative pathogen, Listeria monocytogenes. Food Control 22:1484–90.

Wiedmann M, Sauders B, 2007. Ecology of Listeria species and L. monocytogenes in the natural environment. In: Ryser ET, Marth EH (eds). Listeria, listeriosis, and food safety. 3rd ed. Boca Raton, FL: CRC Press. pp. 21–53.

Young NWG, O'Sullivan GR, 2011. The influence of ingredients on products stability and shelf life. In: Kilcast D, Subramaniam P. Food and Beverage Stability and Shelf Life. Sawston, UK: Woodhead Publishing.