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Selected lactic acid bacteria as a hurdle to the microbial spoilage of cheese: Application on a traditional raw ewes' milk cheese

Luca Settanni [a,*], Raimondo Gaglio [a], Rosa Guarcello [a], Nicola Francesca [a], Stefania Carpino [b], Ciro Sannino [a], Massimo Todaro [a]

[a] Department of Agricultural and Forestry Science, University of Palermo, Viale delle Scienze 4, 90128 Palermo, Italy
[b] CoRFiLaC, Regione Siciliana, S.P. 25 Km 5 Ragusa Mare, 97100 Ragusa, Italy

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ABSTRACT

To evaluate the efficacy of lactic acid bacteria (LAB) to improve the hygienic safety of a traditional raw milk cheese, the raw ewes' milk protected denomination of origin (PDO) Pecorino Siciliano cheese was used as a model system. Different Pecorino Siciliano curds and cheeses were used as sources of autochthonous LAB subsequently used as starter and non-starter LAB. These were screened for their acidification capacity and autolysis. Starter LAB showing the best performance were genotypically differentiated and identified: two strains of *Lactococcus lactis* subsp. *lactis* were selected. From the non-starter LAB, *Enterococcus faecalis*, *Lactococcus garvieae* and *Streptococcus macedonicus* strains were selected. The five cultures were used in individual or dual inocula to produce experimental cheeses in a dairy factory for which production was characterised by high numbers of undesirable bacteria. At 5-month of ripening, the experimental cheeses produced with LAB were characterised by undetectable levels of enterobacteria and pseudomonads and the typical sensory attributes.

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1. Introduction

Cheese classification based on raw materials and microbial inocula includes six categories (Mucchetti & Neviani, 2006): pasteurised milk and selected starters; pasteurised milk and natural starters; thermal treated milk and natural starters; raw milk and selected starters; raw milk and natural starters; raw milk without starters. From a hygienic perspective, the latter cheese category is the one that deserves major attention, since final cheeses can become contaminated by pathogenic microorganisms as a result of their presence in raw milk and their subsequent survival during the cheese making process (Donnelly, 2004). Regarding pathogenic bacteria, the factors that mainly contribute to the safety of cheese are milk quality, starter cultures or native lactic acid bacteria (LAB), pH, salt, control of ripening conditions and chemical changes that occur in cheese during ripening (Johnson, Nelson, & Johnson, 1990).

Cheese cannot be made without the action of certain species of LAB (Parente & Cogan, 2004). Thus, cheese production performed with raw milk without starter addition relies on the presence of indigenous LAB in milk and/or those transferred by the equipment used for the processing and from the environment. However, this may also determine a great variability of the final characteristics of the cheese that cannot be easily controlled by the cheese maker (Franciosi, Settanni, Carlin, Cavazza, & Poznanski, 2008).

Considering that the microbiology of the cheeses produced with raw milk without starters can be unpredictable, the addition of selected LAB may drive the fermentation process in an appropriate direction (Caplice & Fitzgerald, 1999). Raw milk is generally used to produce extra-hard cheeses that are ripened for a long period. However, some hygienic issues with respect to the presence of some pathogenic bacteria have been found during the long ripening of traditional cheeses, such as protected denomination of origin (PDO) Pecorino Siciliano, an extra-hard Italian cheese produced with raw ewes' milk (Todaro et al., 2011).

For the reasons mentioned above, the present work was aimed to evaluate the efficacy of autochthonous LAB to improve the hygienic safety of a typical cheese obtained with raw milk. The autochthonous LAB were expected to be adapted to the technology, as well as to the cheese typology. The PDO Pecorino Siciliano cheese was used as a model cheese to convert the production process from a production performed with raw milk without starters to a production carried out with raw milk and natural starters. The specific objectives for this study were: to isolate and select starter LAB (SLAB) from acidified PDO Pecorino Siciliano curds; to select

* Corresponding author. Tel.: +39 091 23896043.
E-mail address: luca.settanni@unipa.it (L. Settanni).

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non-starter LAB (NSLAB); to produce experimental cheeses with different inocula of SLAB alone or in combination with NSLAB autochthonous for PDO Pecorino Siciliano cheese; to evaluate the improvement of the hygienic conditions of the final products and the preservation of their typical properties by sensory analysis.

2. Materials and methods

2.1. Isolation and grouping of starter lactic acid bacteria from curd samples

Curd samples were provided by three dairy factories producing PDO Pecorino Siciliano cheese following the traditional production protocol that excludes the addition of starter LAB (GURI, 1955). The dairy factories were located within Trapani and Agrigento provinces/districts (Sicily, Italy). Five curd samples were collected from each factory in two consecutive weeks 24 h after adding rennet. The acidified curds were transferred into sterile plastic bags and transported for approximately 90 min in a portable fridge at 8 °C. Once in laboratory, 10 g of each sample were homogenised into 90 mL of sodium citrate (2%, w/v) solution using a Stomacher (BagMixer® 400, Interscience, Saint Nom, France) and serially diluted in Ringer's solution (Sigma–Aldrich, Milan, Italy). Presumptive rod LAB were grown on de Man–Rogosa–Sharpe (MRS) agar (Oxoid, Milan, Italy), acidified to pH 5.4 with lactic acid (5 mol L^{-1}), while presumptive coccus LAB were grown on M17 agar (Oxoid). Both agars were incubated anaerobically at 30 °C for 48 h. After growth, the presumptive LAB colonies were picked up, purified and phenotypically characterised as reported by Settanni et al. (2012).

2.2. Acidification and autolysis of starter and non-starter lactic acid bacteria

SLAB identified in this study and NSLAB previously isolated from PDO Pecorino Siciliano cheese (Todaro et al., 2011) were evaluated for their ability to acidify milk and to undergo autolysis. LAB cultures were grown overnight in M17 or MRS medium and centrifuged at 5000 × *g* for 5 min. The cells were suspended in and washed with Ringer's solution. The acidifying capacity was assayed in 10 mL full fat ultra-high temperature treated (UHT) milk inoculated with 1% (v/v) of cell suspension, to reach a final concentration of about 10^7 cfu mL^{-1} and incubated at 30 °C. Measurements of pH were carried out at 2 h intervals for the first 8 h and then 24, 48 and 72 h after inoculation.

Autolysis of whole cells was determined in buffer solution (potassium phosphate, 50 mmol L^{-1}, pH 6.5) following the method of Mora, Musacchio, Fortina, Senini, and Manachini (2003) using a 6400 Spectrophotometer (Jenway Ltd., Felsted Dunmow, UK) at 600 nm. Optical density (OD) was measured at 2-h intervals for the first 8 h and then 24, 48 and 72 h after inoculation.

2.3. Genotypic differentiation and identification of starter lactic acid bacteria

Before genetic identification was carried out, the presumptive SLAB isolates showing the best acidifying and autolytic performance were differentiated at strain level by random amplification of polymorphic DNA-PCR (RAPD-PCR) analysis as reported by Settanni et al. (2012). Cell lysis for DNA extraction was performed on overnight cultures by the Instagene Matrix kit (Bio-Rad, Hercules, CA, USA) as described by the manufacturer.

Genotypic identification was carried out by 16S rRNA gene sequencing following the scheme applied by Settanni et al. (2012).

2.4. Experimental cheese production

The strains within SLAB and NSLAB groups showing the best acidifying and autolytic performance, alone or in combination, were selected to be used in cheese production. Cells were centrifuged and washed as described above and re-suspended in Ringer's solution till reaching an OD of approximately 1.00, which corresponds to a concentration of 10^9 cfu mL^{-1} as evaluated by plate count.

Cheese trials were carried out at a dairy factory located in Menfi (Italy), which was one of the providers of both curd and ripened PDO Pecorino Siciliano cheeses used to isolate SLAB and NSLAB, respectively. This dairy factory produces PDO Pecorino Siciliano cheeses daily employing the same wooden vat for the last seven years. The bulk milk (250 L) used for the experimental cheese making was first put in contact with the traditional wooden vat, under manual agitation, for 15 min, which represents the time that commonly occurs before rennet addition. After that, the milk was transferred into seven plastic vats (37 L each); the milk in six of the vats was inoculated with LAB (described under Section 3.4.) to obtain the experimental cheeses (EC1–EC6), while the milk in one vat was supplemented with the same volume of Ringer's solution without bacteria and represented the control vat to obtain the control cheese (CC). SLAB and NSLAB were inoculated at a final concentration of approximately 10^7 and 10^3 cfu mL^{-1}, respectively. The cheese productions followed then the traditional protocol (Fig. 1) and the cheeses were ripened for five months. The cheese trials were carried out in duplicate in two consecutive weeks.

2.5. Analysis of the experimental cheeses

Temperature and pH of milk and curd samples were measured by a portable pH meter (waterproof pHTestr 30, Eutech Instruments, Nijkerk, The Netherlands). The different samples (milks and curds) to be microbiologically investigated were collected during cheese production in sterile containers, immediately lowered in temperature and transported for 90 min under refrigeration with a portable fridge to the laboratory of Agricultural Microbiology (University of Palermo). Other curd samples were collected to be followed for pH decrease and LAB counts during the first hours (2, 4, 6, 8, 24 and 48) after production and were kept at ambient temperature during transport and for 48 h. After a 5-month ripening period, the 14 cheeses were sampled and subjected to the same analyses as performed on the refrigerated curd samples mentioned above.

The decimal dilutions of milk (10 mL) samples were prepared in Ringer's solution. The first dilution of curd (10 g) and cheese (25 g) samples was performed in sodium citrate solution as described above, while further serial dilutions were carried out in Ringer's solution. The total mesophilic count (TMC), total psychrotrophic counts (TPC), number of Enterobacteriaceae, enterococci, pseudomonads, positive coagulase staphylococci (PCS), rod and coccus LAB, yeasts and clostridia were estimated as reported by Settanni et al. (2012). The microbiological counts were carried out in duplicate.

Detection of *Listeria monocytogenes* was carried out on 25 g of cheese sample, after pre-enrichment, as described by Mucchetti et al. (2008).

The concentration of salt (NaCl) in the final cheeses was determined by the Volhard method (AOAC, 1975).

Microbial data were statistically analysed by the STATISTICA software (StatSoft Inc., Tulsa, OK, USA) using a generalised linear model (GLM) including the effects of sample; the Student "*t*" test was used for mean comparison. The post-hoc Tukey method was applied for pairwise comparison. Significance level was $P < 0.05$.

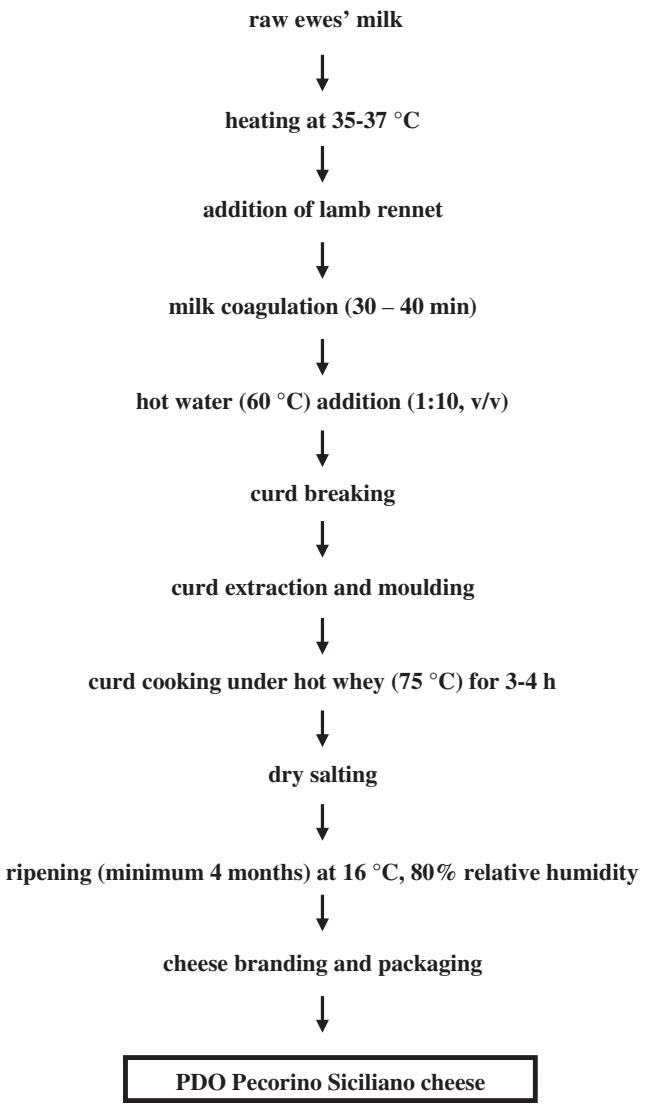

Fig. 1. Flow diagram of PDO Pecorino Siciliano cheese production.

2.6. Sensory analysis

To evaluate the influence of the several bacterial inocula in the definition of the final characteristics of cheese, the different cheese productions, at 5-month ripening, were subjected to the sensory evaluation.

To define the sensory profile of the experimental cheeses, a descriptive panel of nine graders (five females and four males, 30–50 years old) performed the organoleptic evaluation of the cheeses. All panellists were trained at CoRFiLac (Ragusa, Italy), which is a consortium whose research activities are focused on the local dairy products (www.corfilac.it), and participate at their sensory profiling and other types of sensory analysis during the whole year. All the graders were familiar with the descriptive sensory analysis of the Pecorino cheese variety and were specifically trained for the PDO evaluation of cheese products during the previous years of PDO Pecorino Siciliano certification. The score chart used for the expert panellists of CoRFiLaC in this study was the same as used for the certification of the PDO Pecorino Siciliano. The scores of the nine graders were reported in the score chart sequentially for appearance (colour, oil, eyes after cutting of the cheese, uniformity attributes), smell (odour, pasture, unpleasant attributes), taste (taste, salt, spicy, bitter) and consistency (soft/hard, saliva-evoking, dispersion attributes). The evaluations were acquired with the software Compusense five v4.6 (Compusense, Guelph, Canada).

The sensory tests were carried out following the ISO (2003) indications. The graders were not informed about the experimental design and had no specific information about the individual cheese samples tested and operated in individual chambers (ISO 2007). The seven cheeses made in each of the two consecutive weeks were tested in a randomised order of presentation. The samples (pieces of about $3 \times 3 \times 2$ cm in size) were left at ambient temperature (ca. 20 °C) for 60 min before administration and they were presented in coded white plastic plates. Two evaluation sessions were performed.

Sensory evaluations were statistically analysed using the GLM procedure in SAS 2004, version 9.1.2 (Statistical Analysis System Institute Inc., Cary, NC, USA). The discrimination efficiency of the attributes for each assessor was tested by a 2-factor analysis of variance (ANOVA), with graders ($i = 1…9$) and experimental cheeses ($j = 1…7$) as fixed factors. Least square means (LSM) were compared using T test ($P < 0.05$).

3. Results and discussion

3.1. Isolation and phenotypic grouping of starter lactic acid bacteria from curd samples

The samples of PDO Pecorino Siciliano curd, acidified for approximately 24 h at ambient temperature, as performed at the dairy factory in the routine cheese production, contained between 7.5 and 8.9 log cfu g^{-1} of presumptive coccus LAB, while presumptive rod LAB were in the range 6.2–8.2 log cfu g^{-1}. On the basis of appearance (colour, morphology, edge, surface and elevation) at least 3–5 identical colonies per curd sample were randomly picked up from M17 plates, forming a total of 129 cultures. They were considered presumptive LAB, as being Gram-positive and catalase-negative.

Phenotypic characterisation allowed the separation of all coccus LAB isolates collected from the acidified curds into three groups: group I (102 isolates) included LAB forming short chains that were able to grow at 15 °C and at pH 9.2; group II (21 isolates) included LAB forming short chains that were able to grow at 15 and 45 °C, at pH 9.2 and in presence of 6.5% NaCl; group III (6 isolates) included LAB forming long chains that were able to grow at 45 °C, but not under the other conditions tested. All isolates were characterised by a homofermentative metabolism of lactose, which is a basic characteristic for application in cheeses for which the presence of eyes is undesired.

3.2. Evaluation of acidification and autolysis of starter and non-starter lactic acid bacteria

About 30% of the SLAB isolates of each phenotypic group, or at least one isolate per curd for the less numerous groups, forming a total 40 isolates, were randomly chosen and, together with 22 NSLAB previously isolated and identified from ripened PDO Pecorino Siciliano cheese (Todaro et al., 2011), subjected to the evaluation of their aptitudes in cheese making. The acidification capacity and the autolysis were tested, so that the optimal SLAB were characterised by a fast and appropriate acidification and a rapid autolysis. Optimal NSLAB, however, showed opposite performances (Franciosi, Settanni, Cavazza, & Poznanski, 2009).

The results of the acidification and autolysis of the 62 LAB (results not shown) indicated that eight SLAB cultures (CAG4, CAG5, CAG12, CAG23, CAG25, CAG37, CAG60 and CAG70) showed a rapid decrease of milk pH (Fig. 2A), but only two of them (CAG4 and CAG37) were characterised also by a rapid autolysis (Fig. 2B).

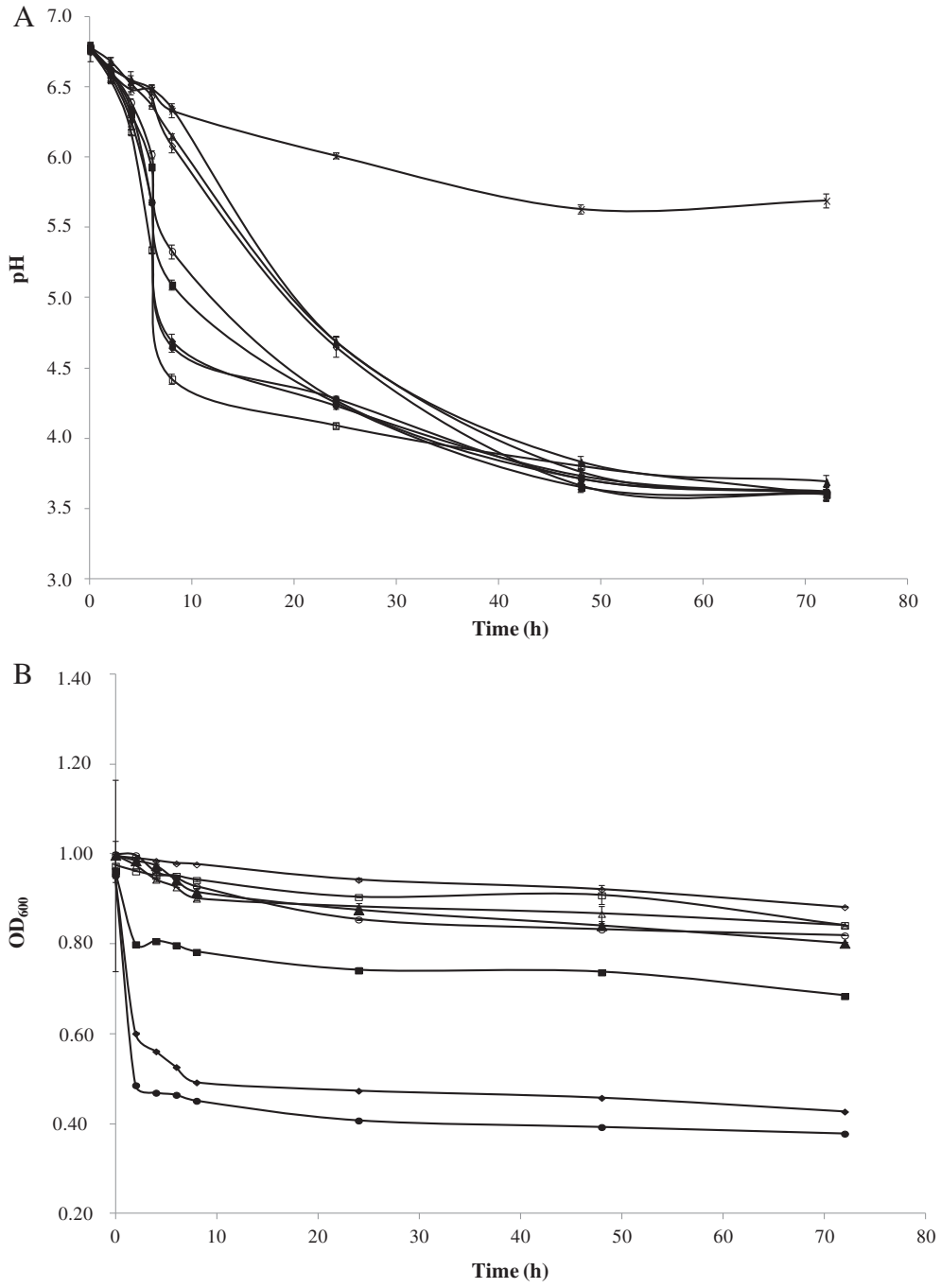

Fig. 2. Acidification (A) and autolysis (B) of SLAB isolated from acidified PDO Pecorino Siciliano curds: ◆, isolate CAG4; ■, isolate CAG5; ▲, isolate CGA12; ◇, isolate CAG23; △, isolate CAG25; ●, isolate CAG37; □, isolate CAG60; ○, isolate CAG70; ×, isolate CAG76 (slow acidifier, added as control in A). Bars represent standard deviation of the mean. Vertical bars not visible are smaller than symbol size.

Regarding NSLAB, identified at species level by Todaro et al. (2011), the combination of both parameters indicated the strains PSL67, PSL71 and PSL72 as the weakest acidifiers with pH 6.14, 6.16 and 6.21, respectively, at 24 h after inoculation with the slowest autolytic activity with 0.088, 0.085, 0.096 OD decrease, respectively, after 72 h. These three strains were identified as: *Lactococcus garvieae*, *Enterococcus faecalis* and *Streptococcus macedonicus*, respectively. The three strains were chosen not only for their technological potential: *E. faecalis* has been reported to be linked to the typicality of final products (Foulquié Moreno, Sarantinopoulos, Tsakalidou, & De Vuyst, 2006); *L. garvieae* and *S. macedonicus* are found in raw milk (Franciosi et al., 2009) and they are commonly isolated from several Italian cheeses, including PDO Pecorino Siciliano cheeses (Todaro et al., 2011), and possess properties useful to provide cheese typicality during ripening (Fortina et al., 2007; Settanni, Franciosi, Cavazza, Cocconcelli, & Poznanski, 2011).

3.3. Recognition of starter lactic acid bacteria at strain and species level

The eight isolates of the SLAB group characterised by a strong and fast decrease of milk pH were analysed by RAPD-PCR and

recognised as different strains (Fig. 3). All eight strains listed above belonged to the phenotypic group I. Strains CAG4 and CAG37, which showed the fastest autolysis, isolated from two distinct curds, were subjected to the analysis of 16S rRNA gene sequencing. Both strains were identified as *Lactococcus lactis* subsp. *lactis* (Acc. No. KC351901, KC351902), which is commonly found during the acidification of several cheeses and used as mesophilic starter (Settanni & Moschetti, 2010).

3.4. Evolution of chemical parameters and microbial populations during experimental cheese making and ripening

L. lactis subsp. *lactis* CAG4 and CAG37 were selected as starter cultures, while *L. garvieae* PSL67, *E. faecalis* PSL71 and *S. macedonicus* PSL72 were chosen as secondary adjunct cultures in cheese making. The experimental PDO Pecorino Siciliano cheese productions were carried out in a dairy factory whose ripened cheese were characterised by high numbers of undesired (pathogenic/spoilage) bacteria (Todaro et al., 2011). The raw ewes' milk used was characterised by a concentration of TMC of 6.2 log cfu mL^{-1}, which is higher than the limit for the "good microbiological quality" in Europe for raw ewes' milk (<500,000 cfu mL^{-1}) to be processed into cheese with a manufacturing process that does not involve any heat treatment (CE, 2004).

Cheese productions were performed with different inocula of SLAB (alone or in combination) or SLAB and the three species of NSLAB as listed below: CC, control cheese not inoculated; EC1, with *L. lactis* CAG4; EC2, with *L. lactis* CAG37; EC3, with *L. lactis* CAG4/*L. lactis* CAG37; EC4, with *L. lactis* CAG4 and *L. garvieae* PSL67/*E. faecalis* PSL71/*S. macedonicus* PSL72; EC5, with *L. lactis* CAG37 and *L. garvieae* PSL67/*E. faecalis* PSL71/*S. macedonicus* PSL72; EC6, with *L. lactis* CAG4/*L. lactis* CAG37 and *L. garvieae* PSL67/*E. faecalis* PSL71/*S. macedonicus* PSL72.

The pH drop followed during 48 h after curd production is shown in Fig. 4. All inoculated curds (EC) showed a faster decrease of pH than the control curd (CCu). No statistical significant differences in acidification were found between the curds inoculated with *L. lactis* subsp. *lactis* CAG4 and CAG37 alone (EC1 and EC2) or in combination with NSLAB (EC4 and EC5). However, when *L. lactis* subsp. *lactis* CAG4 and CAG37 where inoculated together (EC3 and EC6) the fastest pH drop was observed.

The bulk milk, after resting in the wooden vat, hosted 6.5 log cfu mL^{-1} of TMC and it was dominated by coccus LAB (Table 1). After inoculation, all experimental vats showed a concentration of coccus LAB 1 log cycle higher than the control vat (results not shown). Except rod LAB, whose concentration was affected by the addition of the selected SLAB; all other microbial populations did not show differences in the levels detected for the different vats. After coagulation, a 10-fold increase in concentration was registered for the majority of the microbial groups (Table 1). In contrast, the almost complete disappearance of clostridia was evidenced by the most probable number (MPN) technique. At seven days from inoculation, curds evolved almost similarly, but CCu was characterised by higher levels of Enterobacteriaceae and pseudomonads than ECs. Furthermore, all curds were dominated by LAB and, regarding this population, the differences between CCu and ECs were less evident, showing that the indigenous LAB were able to develop during the seven days after curd production. After five months of ripening, several microbiological data were almost superimposable among the different vats (Table 1), including CC, but the concentration of Enterobacteriaceae and pseudomonads, undetectable for all ECs, were 3.7 log cfu g^{-1} in CC.

A significant reduction of Enterobacteriaceae concentration caused by *L. lactis* subsp. *lactis* has been reported for Serra de Estrela cheese made from raw ewes' milk (Macedo, Tavares, & Malcata, 2004), whereas no previous study has evaluated the inhibitory effect of the addition of lactococci on the growth of pseudomonads in raw ewes' milk cheeses. Regarding pseudomonads, known agents of food spoilage, the higher the pH the higher their concentrations (Hayes, 1995); thus, the rapid decrease of pH due to the activity of

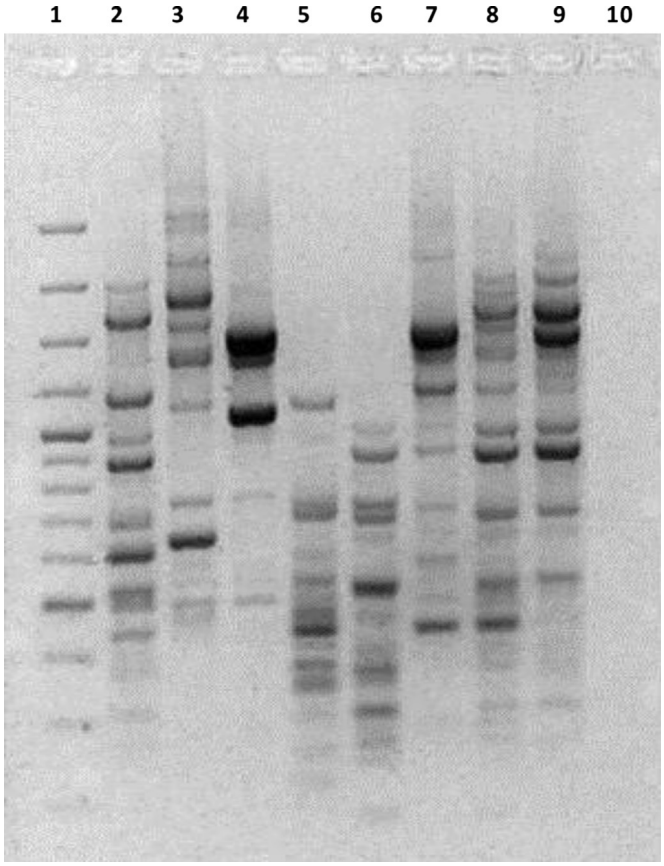

Fig. 3. RAPD-PCR profiles of rapid acidifying SLAB isolated from PDO Pecorino Siciliano cheese obtained with primer M13. Lanes: 1, GeneRuler 100 bp plus DNA ladder; 2, strain CAG4; 3, strain CAG5; 4, strain CAG12; 5, strain CAG23; 6, strain CAG25; 7, strain CAG37; 8, strain CAG60; 9, strain CAG70; 10, negative control.

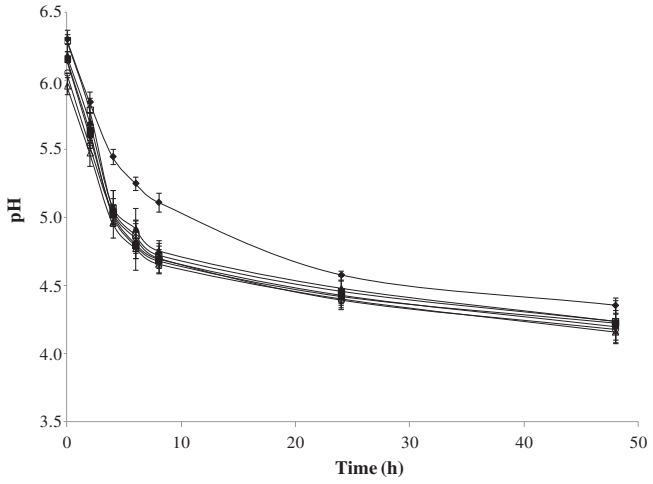

Fig. 4. pH during the acidification of experimental PDO Pecorino Siciliano cheese curds. Abbreviations: CCu, control curd; EC, experimental curd: ◆, CCu; □, EC1; ▲, EC2; ■, EC3; ●, EC4; ○, EC5; △, EC6. Bars represent standard deviation of the mean. Vertical bars not visible are smaller than symbol size.

Table 1
Microbial loads of samples collected through experimental PDO Pecorino Siciliano cheese production.[a]

Samples	pH	Media									
		PCA-SkM 7 °C	PCA-SkM 30 °C	VRBGA	KAA	PAB	BP	MRS	M17	YGC	RCM[b]
Bulk milk	6.34 ± 0.02	4.9 ± 0.1	6.2 ± 0.2	3.3 ± 0.5	3.5 ± 0.6	3.6 ± 0.2	2.9 ± 0.3	4.9 ± 0.1	6.0 ± 0.2	<1	1.6
Wooden vat surface	n.d.	4.1 ± 0.2	6.2 ± 0.9	1.5 ± 0.3	3.3 ± 0.8	0.5 ± 0.3	<1	3.7 ± 0.1	5.7 ± 0.7	1.8 ± 0.4	1.6
Bulk milk in wooden vat	6.34 ± 0.05	5.0 ± 0.5	6.5 ± 0.5	3.9 ± 0.4	3.9 ± 0.2	3.3 ± 0.2	2.8 ± 0.1	5.0 ± 0.1	6.1 ± 0.3	<1	1.6
Curds at T_0											
CCu	6.30 ± 0.03	6.0 ± 0.6	6.8 ± 0.3	4.6 ± 0.2	4.6 ± 0.2	4.3 ± 0.3	3.6 ± 0.1	5.8 ± 0.5	6.9 ± 0.2	<2	1.6
EC1	6.30 ± 0.08	5.8 ± 0.1	8.4 ± 0.6	4.8 ± 0.2	4.7 ± 0.5	4.0 ± 0.4	3.5 ± 0.2	7.4 ± 0.6	8.3 ± 0.6	<2	1.6
EC2	6.18 ± 0.03	5.5 ± 0.2	8.5 ± 0.4	4.8 ± 0.0	4.4 ± 0.3	3.9 ± 0.4	3.7 ± 0.3	7.2 ± 0.3	8.5 ± 0.4	<2	1.6
EC3	6.16 ± 0.06	6.2 ± 0.2	8.4 ± 0.4	4.6 ± 0.2	4.4 ± 0.2	4.1 ± 0.6	3.6 ± 0.3	7.6 ± 0.2	8.4 ± 0.5	<2	1.6
EC4	6.15 ± 0.07	6.0 ± 0.7	8.1 ± 0.5	4.6 ± 0.2	4.7 ± 0.1	3.8 ± 0.2	3.8 ± 0.6	7.3 ± 0.2	8.5 ± 0.4	<2	1.6
EC5	6.05 ± 0.02	5.5 ± 0.4	8.5 ± 0.2	4.6 ± 0.4	4.6 ± 0.2	4.2 ± 0.6	3.9 ± 0.4	7.5 ± 0.2	8.0 ± 0.2	<2	1.6
EC6	5.96 ± 0.06	5.6 ± 0.5	8.5 ± 0.6	4.7 ± 0.2	4.7 ± 0.3	4.2 ± 0.2	4.0 ± 0.3	7.1 ± 0.4	8.0 ± 0.7	<2	1.6
Curds after 7 d											
CCu	4.23 ± 0.09	5.3 ± 0.5	6.9 ± 0.3	5.8 ± 0.7	4.8 ± 0.4	4.9 ± 0.2	2.7 ± 0.3	7.8 ± 0.4	7.8 ± 0.7	<2	0
EC1	4.18 ± 0.07	6.5 ± 0.3	7.6 ± 0.3	3.4 ± 0.2	4.5 ± 0.2	4.0 ± 0.5	2.6 ± 0.3	8.0 ± 0.3	8.1 ± 0.3	<2	0
EC2	4.20 ± 0.06	6.8 ± 0.8	7.7 ± 0.4	3.8 ± 0.3	4.4 ± 0.3	3.9 ± 0.2	2.3 ± 0.3	8.2 ± 0.3	8.2 ± 0.4	<2	0
EC3	4.16 ± 0.05	6.9 ± 0.4	7.9 ± 0.3	3.7 ± 0.2	4.3 ± 0.4	3.9 ± 0.4	2.3 ± 0.3	8.3 ± 0.4	8.4 ± 0.4	<2	1.6
EC4	4.18 ± 0.09	6.7 ± 0.6	7.6 ± 0.5	3.8 ± 0.4	4.9 ± 0.4	4.2 ± 0.4	2.6 ± 0.5	8.3 ± 0.4	8.5 ± 0.5	<2	0
EC5	4.18 ± 0.06	6.5 ± 0.6	7.5 ± 0.3	3.4 ± 0.5	4.7 ± 0.5	4.1 ± 0.2	2.4 ± 0.2	8.4 ± 0.2	8.4 ± 0.3	<2	0
EC6	4.18 ± 0.06	7.2 ± 0.6	7.8 ± 0.6	3.5 ± 0.5	4.5 ± 0.5	4.1 ± 0.4	2.3 ± 0.1	8.7 ± 0.6	8.8 ± 0.3	<2	0
Ripened cheeses											
CC	5.57 ± 0.06	3.2 ± 0.1	7.7 ± 0.1	3.7 ± 0.1	5.1 ± 0.3	3.7 ± 0.4	<2	7.6 ± 0.4	7.3 ± 0.2	<2	0
EC1	5.47 ± 0.03	<2	7.4 ± 0.3	<1	5.4 ± 0.5	<2	<2	7.6 ± 0.2	7.3 ± 0.1	<2	0
EC2	5.57 ± 0.02	<2	7.4 ± 0.5	<1	5.5 ± 0.3	<2	<2	7.4 ± 0.1	7.2 ± 0.5	<2	0
EC3	5.62 ± 0.04	<2	7.2 ± 0.6	<1	5.7 ± 0.6	<2	<2	7.5 ± 0.4	7.4 ± 0.4	<2	0
EC4	5.51 ± 0.02	<2	7.4 ± 0.3	<1	5.6 ± 0.2	<2	<2	7.6 ± 0.3	7.4 ± 0.4	<2	0
EC5	5.51 ± 0.06	<2	7.5 ± 0.3	<1	5.5 ± 0.6	<2	<2	7.4 ± 0.3	7.2 ± 0.3	<2	0
EC6	5.55 ± 0.06	<2	7.8 ± 0.1	<1	5.5 ± 0.6	<2	<2	7.2 ± 0.3	7.4 ± 0.1	<2	0
Statistical significance[c]	$P < 0.001$	$P < 0.001$	$P < 0.05$	$P < 0.001$	$P < 0.001$	$P < 0.001$	–	$P < 0.001$	$P < 0.001$	–	–

[a] Units are log cfu mL^{-1} for milk samples, log cfu g^{-1} for curds and cheeses, log cfu cm^{-2} for wooden vat surface. Results indicate mean values ± S.D. of four plate counts (carried out in duplicate for two independent productions). Abbreviations: PCA-SkM 7 °C, plate count agar added with skimmed milk incubated at 7 °C for total psychrotrophic counts; PCA-SkM 30 °C, plate count agar added with skimmed milk incubated at 30 °C for total mesophilic counts; VRBGA, violet red bile glucose agar for Enterobacteriaceae; KAA, kanamycin aesculin azide agar for enterococci; PAB, *Pseudomonas* agar base for pseudomonads; BP, Baird Parker for positive coagulase staphylococci; MRS, de Man–Rogosa–Sharpe agar for mesophilic rod LAB; M17 agar for mesophilic coccus LAB; YGC, yeast glucose dichloran rose bengal chloramphenicol agar for yeasts; RCM, reinforced clostridial medium for clostridia; n.d., not determined. Sample designation: CC, control cheese; EC1, with *L. lactis* CAG4; EC2, with *L. lactis* CAG37; EC3, with *L. lactis* CAG4/*L. lactis* CAG37; EC4, with *L. lactis* CAG4 and *L. garvieae* PSL67/*E. faecalis* PSL71/*S. macedonicus* PSL72; EC5, with *L. lactis* CAG37 and *L. garvieae* PSL67/*E. faecalis* PSL71/*S. macedonicus* PSL72; EC6, with *L. lactis* CAG4/*L. lactis* CAG37 and *L. garvieae* PSL67/*E. faecalis* PSL71/*S. macedonicus* PSL72.
[b] As estimated by MPN.
[c] Statistical significance is referred to ripened cheeses.

SLAB reduced the *Pseudomonas* spp. numbers. Although the stressing conditions of cheese during ripening should determine the reduction of Enterobacteriaceae population, the presence of these bacteria in raw ewes' milk cheeses at consistent levels is not a rare finding (Prodromou, Thasitou, Haritonidou, Tzanetakis, & Litopoulou-Tzanetaki 2001; Tavaria & Malcata, 2000). *L. monocytogenes* was not detected in any cheese.

The concentration of salt was registered at 6.59, 6.45 and 6.64% (w/w) when the strain *L. lactis* subsp. *lactis* CAG37 was employed as starter culture in EC2, EC3 and EC5, respectively. Lower levels of salt were determined in CC (5.01%, w/w) and in cheese with *L. lactis* subsp. *lactis* CAG4 as starter, 5.13 and 5.87% (w/w) for EC1 and EC4, respectively. The faster and stronger acidification caused by *L. lactis* subsp. *lactis* CAG37 determined a higher syneresis of the curd and the subsequent higher salt concentration (Salvadori del Prato, 1998) in EC2, EC3 and EC5 than CC.

3.5. Sensory evaluation

The sensory evaluation carried out by the expert graders recognised both control cheeses produced in this study as typical PDO Pecorino Siciliano cheeses. The sensory profiles of the experimental cheeses compared with the control cheese (Table 2) showed that only five attributes (colour, eyes, taste, salt and saliva-evoking) were significantly different among cheeses. The most notable differences were evidenced by saliva-evoking and eyes. The highest number and diameter of eyes were displayed by the control cheese. The presence of fewer eyes in the experimental cheeses may be the effect of a rapid inhibition of coliforms in these cheeses. The inoculation with *L. lactis* subsp. *lactis* CAG4 (EC1) showed fewer differences from the control cheese, especially for colour, oil, odour intensity, unpleasant, salt and dispersion. Thus, the addition of *L. lactis* subsp. *lactis* CAG4 did not alter the typicality of the final cheese.

The finding that inocula of *L. lactis* subsp. *lactis* at 10^7 cfu mL^{-1} did not modify the aroma of cheese is not surprising; Centeno, Tomillo, Fernández-García, Gaya, and Nuñez (2002) reported that not all strains of *L. lactis* were able to enhance the flavour intensity of raw ewes' milk cheeses, even though their levels of inoculation were high. In our study, the bulk milk hosted a concentration level of TMC even higher (more than 1 log cycle) than that of the milk processed by Centeno et al. (2002).

The addition of NSLAB was not effective in the modification of the sensory characteristics of the final cheeses. This could be due to the low level of inocula (approximately 10^3 cfu mL^{-1} of milk) chosen to avoid the interference in the acidification process or negative influences in the mature cheeses by NSLAB (Franciosi et al., 2008).

Table 2
Sensory characteristics of experimental PDO Pecorino Siciliano cheeses (LSM) ripened for five months.[a]

Attributes	Cheese samples							SEM	Significance	
	CC	EC1	EC2	EC3	EC4	EC5	EC6		Graders	Cheese
Colour	6.48^{A}	6.38^{A}	6.40^{A}	5.95^{AB}	6.56^{A}	5.66^{B}	5.96^{AB}	0.19	*	*
Oil	3.10	3.00	2.56	2.90	2.94	2.81	2.92	0.13	***	ns
Eyes	2.93^{A}	2.53^{A}	2.09^{B}	2.63^{A}	2.25^{AB}	2.29^{AB}	2.60^{A}	0.17	***	**
Uniformity	11.89	12.19	12.38	11.86	12.36	11.82	11.80	0.20	***	ns
Odour intensity	7.87	7.98	8.07	8.44	8.02	8.43	8.43	0.17	***	ns
Pasture	5.02	5.25	5.08	5.13	5.05	5.30	5.12	0.14	***	ns
Unpleasant	1.63	1.67	1.82	1.87	1.73	1.64	1.86	0.16	***	ns
Taste intensity	7.97^{ab}	8.12^{ab}	7.79^{a}	8.14^{b}	8.18^{b}	8.42^{b}	8.15^{b}	0.13	***	*
Salt	4.62^{a}	4.77^{a}	5.35^{b}	5.01^{ab}	4.57^{a}	5.06^{ab}	5.02^{ab}	0.22	ns	*
Bitter	6.05	6.35	6.31	6.03	5.68	6.40	6.02	0.24	**	ns
Spicy	1.64	1.86	1.70	1.77	1.65	1.91	1.75	0.13	***	ns
Soft/hard	6.29	6.05	6.80	6.39	6.28	6.48	6.38	0.18	**	ns
Saliva-evoking	11.17^{ac}	11.74^{b}	10.79^{c}	11.30^{ab}	11.45^{ab}	11.38^{ab}	11.31^{ab}	0.18	***	**
Dispersion	5.02	4.96	5.39	4.51	4.62	5.26	4.50	0.22	***	ns

[a] Abbreviations are: LSM, least square means; SEM, standard error of means. Sample designation: CC, control cheese; EC1, with *L. lactis* CAG4; EC2, with *L. lactis* CAG37; EC3, with *L. lactis* CAG4/*L. lactis* CAG37; EC4, with *L. lactis* CAG4 and *L. garvieae* PSL67/*E. faecalis* PSL71/*S. macedonicus* PSL72; EC5, with *L. lactis* CAG37 and *L. garvieae* PSL67/*E. faecalis* PSL71/*S. macedonicus* PSL72; EC6, with *L. lactis* CAG4/*L. lactis* CAG37 and *L. garvieae* PSL67/*E. faecalis* PSL71/*S. macedonicus* PSL72. Graders and cheese significance are given as: $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$; ns, not significant. Lowercase and uppercase superscript letters indicate different statistical significances at, respectively, P values of ≤ 0.05 and $P \leq 0.01$.

4. Conclusions

The addition of selected autochthonous LAB in the raw ewes' milk showing a low hygienic quality determined the production of PDO Pecorino Siciliano cheese characterised by acceptable hygienic conditions. In particular, the individual addition of *L. lactis* subsp. *lactis* CAG4 determined also the preservation of the typical sensory profile of this traditional cheese. Studies are being prepared to test the resistance of this strain to the most common dairy viruses and to evaluate its performances in the several dairy factories producing PDO Pecorino Siciliano cheese, which are gathered into a consortium for the protection of this traditional cheese production.

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