Food Microbiology 59 (2016) 66–75

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

A large factory-scale application of selected autochthonous lactic acid bacteria for PDO Pecorino Siciliano cheese production

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ARTICLE INFO

Article history:
Received 13 March 2016
Received in revised form
11 May 2016
Accepted 17 May 2016
Available online 18 May 2016

Keywords:
Non starter lactic acid bacteria
RAPD-PCR
Starter lactic acid bacteria
Traditional cheese
Typicality

ABSTRACT

The main hypothesis of this study was that the autochthonous lactic acid bacteria (LAB) selected for their dairy traits are able to stabilize the production of PDO (Protected Denomination of Origin) Pecorino Siciliano cheese, preserving its typicality. The experimental plan included the application of a multi-strain lactic acid bacteria (LAB) culture, composed of starter (*Lactococcus lactis* subsp. *lactis* CAG4 and CAG37) and non starter (*Enterococcus faecalis* PSL71, *Lactococcus garviae* PSL67 and *Streptococcus macedonicus* PSL72) strains, during the traditional production of cheese at large scale level in six factories located in different areas of Sicily. The cheese making processes were followed from milk to ripened cheeses and the effects of the added LAB were evaluated on the microbiological, chemico-physical and sensorial characteristics of the final products. Results highlighted a high variability for all investigated parameters and the dominance of LAB cocci in bulk milk samples. The experimental curds showed a faster pH drop than control curds and the levels of LAB estimated in 5-month ripened experimental cheeses (7.59 and 7.27 Log CFU/g for rods and cocci, respectively) were higher than those of control cheeses (7.02 and 6.61 Log CFU/g for rods and cocci, respectively). The comparison of the bacterial isolates by randomly amplified polymorphic DNA (RAPD)-PCR evidenced the dominance of the added starter lactococci over native milk and vat LAB, while the added non starter LAB were found at almost the same levels of the indigenous strains. The sensory evaluation showed that the mixed LAB culture did not influence the majority of the sensory attributes of the cheeses and that each factory produced cheeses with unique characteristics. Finally, the multivariate statistical analysis based on all parameters evaluated on the ripened cheeses showed the dissimilarities and the relationships among cheeses. Thus, the main hypothesis of the work was accepted since the quality parameters of the final cheeses were stabilized, but all cheeses maintained their local typicality.

1. Introduction

Pecorino Siciliano is an Italian cheese enjoying the Protected Designation of Origin (PDO) status produced in throughout the Sicily region (25,711 km^2) in South Italy. Pecorino Siciliano is a semi-hard cheese manufactured following traditional techniques from raw ewe's milk without any addition of bacterial starters, according to the protocol of production (GURI n. 295/1955). In these conditions, cheese production relies on lactic acid bacteria (LAB) present in milk, on those transferred by the equipments and from the dairy environments (Settanni and Moschetti, 2014) that together with pH, salt content, ripening conditions and chemical changes occurring during ripening contribute to the microbiological stability of the final product (Johnson et al., 1990). Raw milk is generally employed to produce extra-hard cheeses that are ripened for a long period, until 24 months or even longer (Gobbetti, 2004). Aged, ripened cheeses retain their sensorial traits for long time thanks their low pH, low water activity, and low redox potential (Ledenbach and Marshall, 2010). Despite the stressing chemico-physical parameters that characterize ripened cheese, Todaro

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http://dx.doi.org/10.1016/j.fm.2016.05.011
0740-0020/

et al. (2011) found undesired potentially pathogenic microorganisms in ripened PDO Pecorino Siciliano cheese samples.

Pecorino cheese production is widespread in South and Central Italy, but several regional differences are found. The raw materials are directly responsible for the characteristics of this cheese typology (Suzzi et al., 2015; Tofalo et al., 2015), while the indigenous milk microbiota might be defining for the final safety of the ripened products (Schirone et al., 2011, 2013; Guarcello et al., 2015). The differences in the fermenting LAB of the several Pecorino cheeses depend strongly on the technology of transformation and on the use of a natural whey starter culture (Di Cagno et al., 2003).

In cheese making production, starter cultures are generally added in order to rapidly dominate over the native microbiota, but the indiscriminate use of starter strains might determine a flattening of the taste in the final products, with the risk that the obtained cheeses may no longer be distinguishable by production technology and/or geographical origin. The application of autochthonous microorganisms, that are adapted to the production area (environment), the local raw materials (substrates) and the traditional protocol (technology), provide a cheese with the typical characteristics that cannot be reproduced elsewhere (Settanni and Moschetti, 2014).

Recently, same studies have been published on selection of LAB strains to be used as starter for Pecorino Siciliano cheese production (Randazzo et al., 2006, 2007; Franciosi et al., 2009), valuating their effects on volatile compounds (Randazzo et al., 2008) and on microbiological characteristics (Settanni et al., 2013). In particular, in the latter work Settanni et al. (2013) evaluated the effect of different starter lactic acid bacteria (SLAB), alone or in combination with non SLAB (NSLAB), on the microbiological quality and sensory traits of 5-month ripened cheeses.

The aim of the present work was to evaluate the suitability of a multiple strain SLAB/NSLAB culture on the final characteristics of PDO Pecorino Siciliano cheese produced in several areas of Sicily different for pedoclimatic conditions, sheep breed, pasture and dairy farmer.

2. Materials and methods

2.1. Microorganisms and characteristics of milk

The mixed LAB culture used in this study was composed of two SLAB strains (*Lactococcus lactis* subsp. *lactis* CAG4 and CAG37) and three NSLAB strains (*Lactococcus garvieae* PSL67, *Enterococcus faecalis* PSL71 and *Streptococcus macedonicus* PSL72), isolated from PDO Pecorino Siciliano cheeses (Todaro et al., 2011), previously selected for their technological performances and already applied at factory-scale level in a restricted area of western Sicily (Agrigento province) (Settanni et al., 2013).

LAB cultures were grown in M17 broth (Oxoid, Basingstoke, UK) for 18 h and centrifuged at 5000 $\times g$ for 5 min. The cells were washed twice in Ringer's solution (Sigma-Aldrich, Milan, Italy) and re-suspended in the same solution till reaching an optical density (OD) of ca. 1.00, determined by means of a 6400 Spectrophotometer (Jenway Ltd., Felsted Dunmow, UK) at 600 nm wavelength, which approximately corresponds to a concentration of 10^9 CFU/mL, as verified by plate counting.

Bulk raw ewes' milk samples were analysed for somatic cell count (SCC), fat, protein, casein, and lactose using the infrared method (Combi-foss 6000, Foss Electric, Hillerod, Denmark). Urea content was determined by enzymatic method using the difference in pH (CL-10 Plus, Eurochem, Roma, Italy), pH values using a HI 9025 pH-meter (Hanna Instruments, Ann Arbor, MI, USA).

2.2. Cheese production and sampling points

Cheese productions were performed in six dairy factories (Table 1) producing PDO Pecorino Siciliano cheese daily, located in different production areas throughout the Sicily region and gathered into a consortium for the protection of this traditional PDO cheese production. The sampling points, the number of samples analysed and the analysis performed are reported in Table 2.

Bulk milk (100 L) used for the production of experimental cheeses (EC) in each factory was collected from a higher volume of milk left, under manual agitation for approximately 15 min, in a wooden vat and transferred into two plastic vats (50 L each) representing two different trials. One vat was inoculated with the mixed LAB culture (500 mL) to reach, in the final volume of milk, the concentration of 10^7 and 10^3 CFU/mL for SLAB and NSLAB, respectively, and used to produce the experimental cheese (EC). The second vat was added with 500 mL of Ringer's solution without bacteria to obtain the control cheese (CC). Both bulks were then subjected to the traditional cheese making provided by the production protocol of PDO Pecorino Siciliano cheese (GURI, 1955) and ripened for 5 months as follows: 2 months in a storage chamber at 16 °C and 85% of relative humidity (RH) and then 3 months into a natural cave at approximately 16 °C and 90% of RH. The cheese trials were carried out in duplicate in two consecutive weeks in April 2014.

All production processes were entirely monitored; samples were collected from bulk milk at delivery (BM1), bulk milk after resting in wooden vat (BM2), bulk milk with inoculum (BM3), curds just after curdling, acidified curds and ripened cheeses. The wooden vat surfaces (400 cm^2) were sampled, just before cheese production took place, as reported by Didienne et al. (2012) using UV-treated paper squares positioned halfway up the side and on the bottom. Curds were collected after whey discharge, just before reversal in the rattan baskets where they assumed the final shape. Furthermore, in order to follow the curd acidification, two samples of curd were collected for each production and kept at ambient temperature for 7 days. One curd sample was subjected to the monitoring of pH, performed by the portable pH meter (waterproof pHTestr 30, Eutech Instruments, Nijkerk, The Nederlands) at 2-h intervals for the first 8 h and then after 1, 2, 3 and 7 days from milk curdling. The second sample of curd was used for microbiological analysis. Cheeses were sampled after five months of ripening.

2.3. Microbiological analyses

The bulk milk samples, (10 mL), curds (10 g) and wooden vat surfaces (1 mL of the cell suspension obtained after homogenisation of the gauze) collected during cheese making, and the cheese samples (25 g), after 5 months of ripening, were subjected to serial decimal dilution in Ringer's (Sigma-Aldrich, Milan, Italy) solution. Curds and cheeses were homogenised in sodium citrate solution (2% w/v) by a stomacher (BagMixer® 400, Interscience, Saint Nom, France) for 2 min at the highest speed. This step is necessary because sodium citrate is a calcium-sequestering agent that determines the disruption of casein micelles (Corredig et al., 2003) allowing the accessibility to the microorganisms associated to fat globules (Griffiths, 2000). Microbial suspensions were plated and incubated as follows: total psychrotrophic counts (TPC) on Plate Count Agar plus Skimmed Milk, incubated aerobically at 7 °C for 7 days; *Enterobacteriaceae* counts on Violet Red Bile Glucose Agar, aerobically incubated at 37 °C for 24–48 h; enterococci on Kanamycin Aesculin Azide aerobically incubated at 37 °C for 24–48 h; coagulase-positive staphylococci (CPS), on Baird Parker supplemented with Rabbit Plasma Fibrinogen (RPF) supplement and

Table 1
Characteristics of dairy factories involved in the study.

Factories	Cities (province)[a]	Vats		Sheep breed
		Age (years)	Type of wood	
I	Aidone (EN)	8	oak	Valle del Belìce
II	Ramacca (CT)	1	oak	Comisana
III	Salemi (TP)	9	douglas	Valle del Belìce
IV	Santa Margherita del Belìce (AG)	5	douglas	Valle del Belìce
V	Castronovo di Sicilia (PA)	6	douglas	Valle del Belìce
VI	Santo Stefano di Quisquina (AG)	1	oak	Valle del Belìce

[a] AG, Agrigento; CT, Catania; EN, Enna; PA, Palermo; TP, Trapani.

Table 2
Sampling points[a] and analyses performed during cheese production.

Analyses	Sampling points									
	WV	BM1	BM2	BM3	Curd T0		Curd T7		Cheese	
					Ctr	Exp	Ctr	Exp	Ctr	Exp
pH		■			■	■	■	■	■	■
SSC		■								
Plate counts	■	■	■	■	■	■	■	■	■	■
Molecular typing							■	■	■	■
Chemical composition		■							■	■
a_w									■	■
Sensory tests									■	■

Abbreviations: WV, wooden vat; BM1, bulk milk at delivery; BM2, bulk milk after resting in wooden vat; BM3, bulk milk after inoculum; Ctr, control; Exp, experimental; SSC, somatic cell counts; a_w, water activity.
[a] Two samples were analysed for each production. The number of replicates for each analysis is reported in the text.

incubated under aerobic conditions at 37 °C for 48 h; total mesophilic counts (TMC) on Plate Count Agar plus 1 g/L of skimmed milk, incubated aerobically at 30 °C for 72 h; LAB cocci on M17 agar, anaerobically incubated at 30 °C for 48 h; LAB rods on de Man-Rogosa-Sharpe (MRS) agar, acidified at pH 5.4 with lactic acid (5 M), anaerobically incubated at 30 °C for 48 h; yeasts on dichloran rose Bengal chloramphenicol agar, aerobically incubated at 25 °C for 48 h; *Pseudomonas* spp. on Pseudomonas Agar Base supplemented with 10 mg/mL of cetrimide fucidin and aerobically incubated at 25 °C for 48 h. Plate counting was carried out in duplicate. The spores of clostridia were estimated by the most probable number (MPN) technique using a 3 × 3 scheme: undiluted samples and decimal dilutions were pasteurized at 85 °C for 15 min, inoculated into reinforced clostridial medium supplemented with 1.4% (v/v) Na-lactate (Merck, Darmstadt, Germany), sealed with paraffin:Vaseline (1:6) and incubated at 37 °C for 7 days. All media were purchased from Oxoid.

2.4. Monitoring of the added strains

The monitoring of the SLAB and NSLAB strains added before milk coagulation was performed applying the random amplified polymorphic DNA (RAPD)-PCR technique. After plate counting, the colonies developed from the highest dilutions of curds on M17 agar and those of cheeses on M17 and MRS agar were randomly collected based on their appearance (at least three identical colonies), purified by successive sub-culturing on the same media and tested for Gram reaction, performed by the KOH (3%, w/v) method, and catalase activity, determined with H_2O_2 (5%, v/v). The broth cultures, overnight growth, were subjected to DNA extraction using the InstaGene Matrix kit (Bio-Rad, Hercules, CA, USA) following the manufacturer's instruction. PCRs were carried out in a 25-μL reaction mix using single the primers M13, as described by Settanni et al. (2012). The amplicons were separated by electrophoresis on 1.5% (w/v) agarose gels (Gibco BRL, Cergy Pontoise, France). GeneRuler 100 bp Plus DNA ladder (M Medical Srl, Milan, Italy) was used as molecular size marker. The gels were stained with the SYBR® safe DNA (Molecular probes, Eugene, OR, USA) and visualised by UV *trans*-illumination. The RAPD patterns were analysed using GelCompar II software version 6.5 (Applied-Maths, Saint-Marten-Latem, Belgium). The recognition of the added strains was performed by comparison between polymorphic profiles obtained from strains isolated from experimental curd samples after 7 days of acidification and those of *Lactococcus lactis* subsp. *lactis* CAG4 and CAG37, and by comparison between polymorphic profiles obtained from isolated strains from experimental cheeses, after 5 months of ripening, and profiles of *Lactococcus garvieae* PSL67, *Enterococcus faecalis* PSL71 and *Streptococcus macedonicus* PSL72.

2.5. Chemico-physical composition of cheese samples

Cheese samples were analysed for: cheese yield, dry matter (DM), protein (N x 6.38), fat and ash content according to International Dairy Federation (IDF) standards [4A:1982 (IDF, 1982), 25:1964 (IDF, 1964a), 5B:1986 (IDF, 1986) and 27:1964 (IDF, 1964b), respectively]. Soluble nitrogen (N) was determined on an aqueous filtrate using the Kjeldahl method (MAF, 1986), NaCl according to the IDF procedure (17A:1972; IDF, 1972). Determination of pH values was performed by the HI 9025 pH-meter. Water activity (a_w) was determined at 23 °C at the surface of each sample slice by using an activity-meter instrument (Rotronic Int., USA).

2.6. Sensory analyses

Twelve cheese samples were analysed in order to define and detect differences in sensorial profiles between EC and CC products. A set of 21 descriptive attributes was developed by a panel of 10 assessors members at CoRFiLaC. The panellists were selected and trained using procedures consistent with international standards for the training of descriptive panels (ISO 8589, 2007) in order to

recognize specific attribute for Pecorino cheese. Each attribute was presented as a separate unstructured line scale that recorded panellist responses in increments of 0.1 between 1 (leftmost position) and 15 (rightmost position). The cheese samples were cubed (approximately 1 cm each side) and presented on white paperboard plates. The panellists also had available an entire transverse slice of each cheese for evaluating appearance attributes. The attributes were organized into: aroma (odour intensity, pasture, unpleasant); taste (intensity of taste, salt, spicy, bitter); structure of surface (colour, oil, hole, uniformity of structure); and texture (soft/tough, pasty, dispersion). The samples were identified using random three-digit codes. The panellists were not provided with information regarding the treatment of each sample. The sensory data were collected and elaborated by the Compusense five v.4.6 software (Compusense, 2003).

Triangle tests were also performed on cheese samples, in order to evaluate differences between CC and EC products. The sensory evaluation was assessed by testing the five senses of the products and was conducted according to the ISO 4120 (2004). The test room, having 6 boxes, was regulated with a comfortable temperature and humidity and it was free from any extraneous odour and noise. The samples were tasted in a randomized complete block design and identified by a specific code. No information was given to the assessors about the origin of the samples. Results of sensory evaluation were analysed according to UNI ISO 4120. Test of preference was carried out according to the ISO 5495: 2005 ($\alpha = 0.05$).

2.7. Statistical univariate and explorative multivariate analyses

Milk chemical and physical data and microbial loads of wooden vats were statistically analysed with the following ANOVA linear model: $Y_{ik} = \mu + Factories_i + \varepsilon_{ik}$ where $Factories_i$ is the fixed factor "dairy factor" (i: 1–6); milk microbial loads during processing were analysed with the following ANOVA linear model: $Y_{ijk} = \mu + Factories_i + Milk_j + \varepsilon_{ijk}$ were $Milk_j$ is the fixed factor "milk treatment" (j: Bulk milk, Bulk milk after resting, Milk + LAB culture); curd (T0 and T7) and cheese microbial loads were analysed with the following hierarchic ANOVA linear model: $Y_{ijlk} = \mu + Factories_i + Matrix_j + Treatment_{l(j)} + \varepsilon_{ijlk}$ were $Matrix_l$ is the fixed factor "food matrix" (j: Curd at 0 day, Curd at 7 days, cheese at 5 months of ripening), $Treatment_j$ is the fixed factor "cheese treatment" (l: experimental cheese (EC) and control cheese (CC)), cheese chemical and physical data were analysed with the following ANOVA linear model: $Y_{ijk} = \mu + Factories_i + Treatment_j + \varepsilon_{ijk}$; Sensorial parameters were analysed with the following ANOVA linear model: $Y_{ijlk} = \mu + Treatment_i + Factories_j + Panellist_l + \varepsilon_{ijlk}$ were $Panellist_l$ is the fixed factor "expert judges" (1–10).

The Student "t" test was used for means comparisons at $P < 0.05$ and $P < 0.01$ significance level, while the statistical software used was SAS 9.1.2 (Proc GLM).

To better understand the relationship among data obtained from cheeses produced in the different factories, an explorative multivariate analysis was carried out. The different productions were grouped by a hierarchical cluster analysis (HCA) performed as described by Todeschini (1998), selecting parameters as reported by Martorana et al. (2015). Furthermore, data were subjected to the principal component analysis (PCA). The input matrix used for PCA consisted of the microbial loads of the several groups investigated, the chemico-physical data as well as the scores from sensory analysis. XLStat software version 7.5.2 (Addinsoft, New York, USA) for excel was used for data processing and graphic construction.

3. Results

3.1. Chemico-physical parameters of milk and curd acidification

The composition of BM1 of each factory is showed in Table 3. Fat, protein and casein percentages were within 5.77–6.71, 5.20–6.14 and 4.06–4.82, respectively. Among these parameters, protein and casein contents showed the highest variability among factories. Urea concentrations, between 26.85 and 46.35 mg/dL, showed consistent differences among milk samples such as SCC values that showed relevant differences among milk samples from different factories.

In Fig. 1 the average values of the acidification, both in experimental and control curds, during the first 7 days, are reported. All curd samples, independently on the addition of the mixed SLAB-NSLAB culture, were characterised by an initial pH value of 6.4–6.5. The experimental curd samples showed a faster pH drop than the corresponding curd control samples, from the 4th hour of data recording. The fastest pH decrease was registered for the experimental curd samples, from factories V and VI, that showed values 0.3–0.4 points lower than the other experimental curds after 4 and 6 h (data not shown). However, the control curd samples V and VI were also characterised by a lower pH than the other control curds from 4 to 8 h. The differences among the pH values of experimental and control curds were still consistent after 24 h, but decreased with time; only 0.1 difference was found from 48 h onward.

3.2. Microbiological analyses

Microbiological analyses were carried out throughout cheese production from milk to ripened cheese. The surfaces of wooden vats used for collection of milk in the dairy factories were also analysed (Table 4). The surface of vat VI was characterised by a TPC value below the detection limit, whereas the other vat surfaces showed TPC ranging between 3.25 and 5.38 Log CFU/cm^2. TMC were detected in all vat surfaces reaching the maximum level (5.47 Log CFU/cm^2) in the vat I and the lowest (4.20 Log CFU/cm^2) in the vat IV. All vat surfaces displayed the presence of both cocci and rods LAB. Except in vat IV, the LAB cocci detected were always higher than LAB rods. Yeast counts were below the detection limit in the surfaces of both vat IV and VI and ranged between 2.40 and 3.88 Log CFU/cm^2 in the other vats. The spores of clostridia were not detected in any vat. *Enterobacteriaceae* counts were below the detection limit for the vat I and reached the highest value (3.20 Log CFU/cm^2) in vat I. Enterococci ranged values between 2.7 and 3.8 Log CFU/cm^2 and *Pseudomonas* spp. showed values below the

Table 3
Somatic cell counts and chemical composition of bulk milk samples.

Parameters	Factories						SEM	P
	I	II	III	IV	V	VI		
	(n. 2)	(n. 2)	(n. 2)	(n. 2)	(n. 2)	(n. 2)		
SCC (Log)	6.08	3.95	6.32	5.86	5.99	6.10	0.01	**
Fat (%)	6.71	6.53	6.14	5.77	6.29	6.40	0.25	*
Protein (%)	5.82	6.14	5.60	5.20	5.64	5.60	0.02	***
Casein (%)	4.58	4.82	4.38	4.06	4.44	4.40	0.01	***
Fat + Protein (%)	12.53	12.66	11.74	10.97	11.93	12.01	0.25	*
Lactose (%)	4.50	4.66	4.43	4.46	4.57	4.59	0.06	ns
Urea (mg/dL)	36.50	33.30	29.20	26.85	36.90	46.35	2.54	*
pH	6.61	6.47	6.51	6.64	6.57	6.65	0.06	ns

Abbreviations: SEM, standard error of means; n., number of samples; SCC, somatic cell count.
Statistical significance: *P ≤ 0.05; **P < 0.01; ***P < 0.001; ns = not significant.

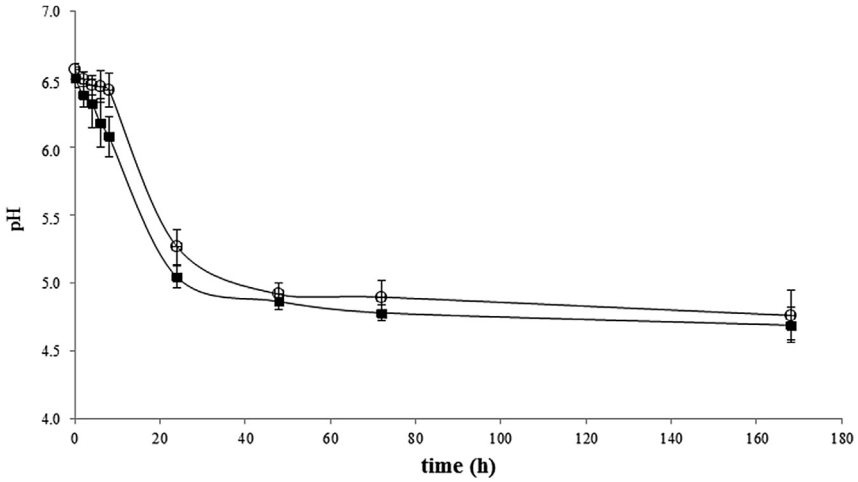

Fig. 1. pH decrease during 7-days of curd acidification. Symbols: ○, average values of control curds; ■, average values of experimental curds.

Table 4
Microbiological characteristics of wooden vat surfaces (as Log CFU/cm^2).

Parameters	Factories						SEM	P
	I	II	III	IV	V	VI		
	(n. 2)	(n. 2)	(n. 2)	(n. 2)	(n. 2)	(n. 2)		
TPC	4.55	4.52	5.38	3.25	3.74	<2	0.25	***
Enterobacteriaceae	3.20	2.18	1.49	1.39	1.44	<1	0.48	ns
Enterococci	3.76	3.60	3.19	3.03	2.68	3.22	0.89	ns
CPS	<1	3.28	2.73	2.98	2.75	3.19	0.82	ns
TMC	5.47	4.71	5.37	4.20	4.77	4.45	0.40	ns
LAB cocci	6.03	4.87	5.81	4.43	2.82	4.80	0.83	ns
LAB rods	5.02	4.50	3.95	4.70	2.38	4.50	0.60	ns
Yeasts	2.78	3.88	2.40	<2	3.41	<2	0.44	***

Abbreviations: SEM, standard error of means; n., number of samples; TPC, total psychrotrophic count; CPS, coagulase positive staphylococci; TMC, total mesophilic count; LAB, lactic acid bacteria.
[a] ***P < 0.001; ns = not significant; data within a column followed by the same letter are not significantly different according to Tukey's test.

detection limit in all vat surfaces. CPS exhibited the highest count in surface of vat II and the lowest (below detection limit) in vat I.

The BM1 samples (Table 5) were basically characterised by levels of LAB cocci similar to TMC, indicating a dominance of this group of LAB in the raw materials. Moreover LAB cocci were one Log unit higher than LAB rods in all analysed samples. Yeast counts were always higher than 3 Log CFU/mL and lower densities were displayed by enterococci, CPS and *Enterobacteriaceae* counts. The BM2 samples showed higher densities for both LAB groups, TPC and TMC, while cell densities of the other microbial groups detected remain quite constant.

Table 5
Least Square Means of microbial counts (as Log CFU/g) in milk samples during processing.

Microbial groups	BM1	BM2	BM3	SEM	P	
	(n. 12)	(n. 12)	(n. 12)		Factories	Milk
TPC	5.09 Bc	5.72 ABb	6.34 Aa	0.31	ns	**
Enterobacteriaceae	2.41	2.50	2.90	0.34	**	ns
Enterococci	2.73	2.67	3.48	0.29	**	ns
CPS	2.56	2.55	2.90	0.31	**	ns
TMC	5.94 a	6.23 ab	6.70 b	0.24	**	*
LAB cocci	5.61 A	6.11 A	7.29 B	0.29	*	***
LAB rods	4.78 A	5.33 A	6.62 B	0.28	**	***
Yeasts	3.14	3.10	3.04	0.26	***	ns

Abbreviations: BM1, bulk milk at delivery; BM2, bulk milk after resting in wooden vat; BM3, bulk milk after inoculum; SEM, standard error of means; n., number of samples; TPC, total psychrotrophic count; CPS, coagulase-positive staphylococci; TMC, total mesophilic count; LAB, lactic acid bacteria.
Statistical significance: *P ≤ 0.05; **P < 0.01; ***P < 0.001; ns = not significant; a, b, c means with different letter differ for P ≤ 0.05; A, B, C, means with different capital letter differ for P ≤ 0.01.

A consistent increase in concentration of LAB and, consequently, TPC and TMC, was detected after the addition of the mixed LAB culture. In particular, the BM3 samples showed a LAB cocci count of 7.29 Log CFU/mL (Table 5).

The curds were analysed after milk curdling and after 7 days of acidification, and the cheese samples were investigated after a ripening period of 5 months (Table 6). After coagulation, all microbial groups, in all curd samples, showed levels of about 1 Log unit higher than the corresponding milk samples. The TMC and both the LAB groups in experimental curd samples were higher than those of the control curds. In particular, LAB cocci reached 8.20 Log CFU/g in the experimental curds at T0. After 7 days of acidification, the LAB counts increased in all trials, reaching the highest values in experimental curd samples, even though the highest increasing were detected for both LAB groups in the control curd samples. During curd acidification, *Enterobacteriaceae*, CPS and yeast counts did not change. Regarding enterococci, an increase was registered only in control curd samples. After ripening, the EC samples were still characterised by the highest levels of LAB and TMC, while TPC were under the detection limit. The addition of the LAB culture significantly influenced the levels of LAB, TMC, TPC and enterococci, while did not affect CPS, *Enterobacteriaceae*, and yeasts. Interestingly, the levels of LAB cocci, enterococci and CPS were statistically different among the factories. Pseudomonad counts were below the detection limit in all analysed samples and the spores of clostridia, absent in all vats, were detected at very low levels (0.2–1.0 CFU/mL) in the BM1 samples from factories I, II and V, but not in curd and cheese samples (results not shown).

3.3. Persistence of the added SLAB and NSLAB strains

The RAPD profiles of the isolates collected after plate counts were compared to those of the pure cultures of the added LAB, in order to evaluate their persistence. As an example, Fig. 2 reports the RAPD-PCR profiles of the LAB isolated from the curds and the cheeses produced at Factory I. The direct comparison of the polymorphic profiles of the curd isolates allowed the recognition of both *L. lactis* subsp. *lactis* CAG4 and CAG37 (Fig. 2B), at similar

Table 6
Least Square Means of microbial counts (Log CFU/g) in curd samples and in 5-month ripened cheeses.

Media	Curd T0		Curd T7		Cheese		SEM	P		
	Ctr	Exp	Ctr	Exp	Ctr	Exp		Factories	Matrix	Treatment (matrix)
	(n.12)	(n. 12)	(n. 12)	(n. 12)	(n. 12)	(n. 12)				
TPC	6.39 a	7.04 b	7.50	7.92	0.84 A	0.00 B	0.22	ns	***	**
Enterobacteriaceae	3.78	3.57	3.93	3.55	3.02	2.68	0.36	ns	*	ns
Enterococci	3.88	3.93	4.50 a	3.84 b	5.09 a	4.35 b	0.22	***	**	*
CPS	3.53	3.28	3.66	3.38	4.19	4.04	0.27	***	*	ns
TMC	7.13 A	7.79 B	7.90 a	8.30 b	7.00 A	7.63 B	0.17	ns	***	***
LAB cocci	6.99 A	8.20 B	8.47	8.66	6.61 A	7.27 B	0.17	**	***	***
LAB rods	6.05 A	7.53 B	8.22	8.55	7.02 a	7.59 b	0.18	ns	***	***
Yeasts	3.90	4.18	4.05	3.68	2.44	2.53	0.19	ns	***	ns

Abbreviations: Ctr, control; Exp, experimental; TPC, total psychrotrophic count; CPS, coagulase positive staphylococci; TMC, total mesophilic count; LAB, lactic acid bacteria; SEM, standard error of means; n., number of samples.
Statistical significance: *P ≤ 0.05; **P < 0.01; ***P < 0.001; ns = not significant; a, b, c means with different letter differ for P ≤ 0.05; A, B, C, means with different capital letter differ for P ≤ 0.01.

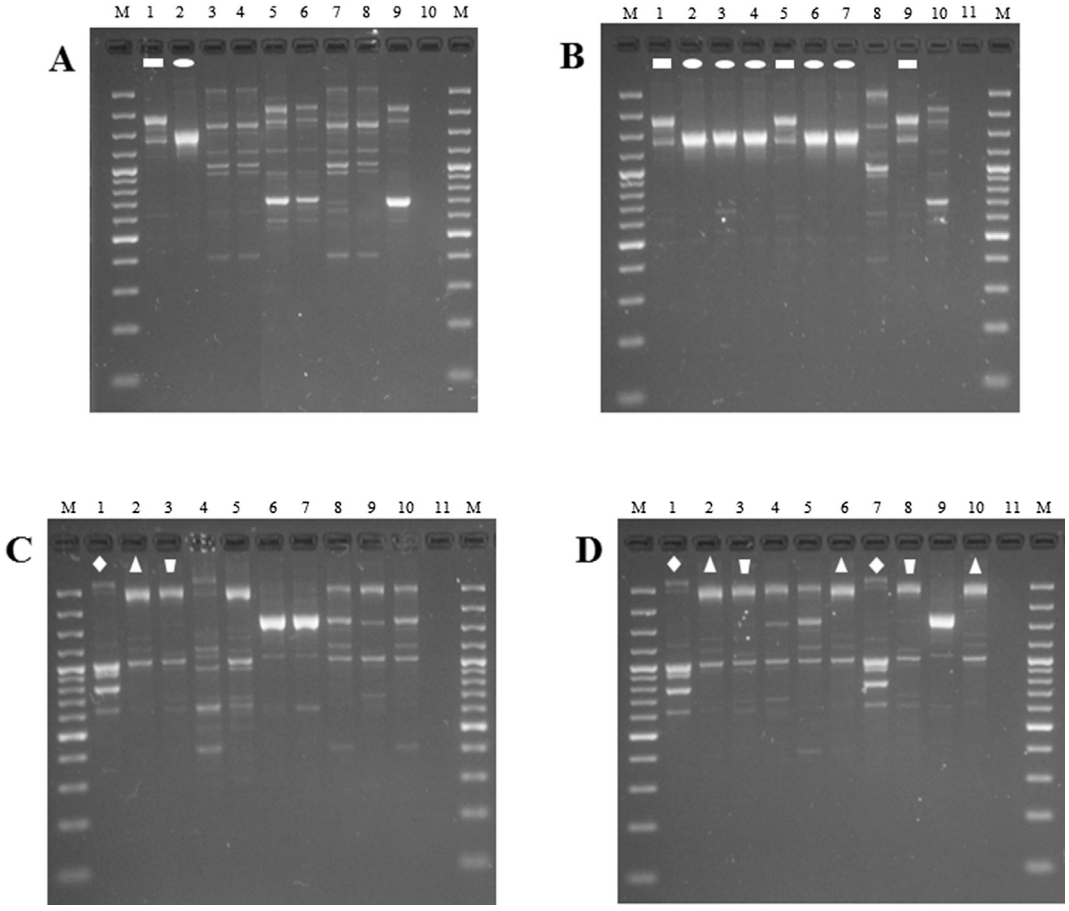

Fig. 2. RAPD-PCR profiles of LAB isolated from the highest dilutions of curds (at 7 d) and cheeses (at 5 months) produced at Factory I obtained with primer M13. A, control curd; lanes: 1, *Lactococcus lactis* subsp. *lactis* CAG4; 2, *L. lactis* subsp. *lactis* CAG37; 3–9, curd isolates; 10, negative control. B, experimental curd; lanes: 1, *Lactococcus lactis* subsp. *lactis* CAG4; 2, *L. lactis* subsp. *lactis* CAG37; 3–10, curd isolates; 11, negative control. C, control cheese; lanes: 1, *Lactococcus garvieae* PSL67; 2, *Enterococcus faecalis* PSL71; 3, *Streptococcus macedonicus* PSL72; 4–10, cheese isolates; 11, negative control. D, experimental cheese; lanes: 1, *L. garvieae* PSL67; 2, *E. faecalis* PSL71; 3, *S. macedonicus* PSL72; 4–10, cheese isolates; 11, negative control. Lanes M, GeneRuler 100 bp plus DNA ladder. Lanes with the same symbols (rectangle, oval, rhombus, triangle and trapezoid) indicate the same strains.

levels, clearly evidencing their dominance in the acidified experimental curds over native milk and vat LAB. The two SLAB were not found in the control curds (Fig. 2A). The isolates from the EC samples showed a codominance of *L. garvieae* PSL67, *E. faecalis* PSL71 and *S. macedonicus* PSL72 with other strains (Fig. 2D), demonstrating the ability of the added NSLAB to persist during ripening. The three RAPD profiles of the added NSLAB were not recognised in any isolate from the CC samples (Fig. 2C), confirming that they were not present in the raw milk and/or in the dairy environment.

3.4. Chemico-physical and sensory characteristics of the cheeses

Chemico-physical cheese composition is reported in Table 7. The

Table 7
Chemico-physical composition of cheese samples.

Parameters	CC (n. 12)	EC (n. 12)	SEM	P	
				Treatment	Factories
Cheese yield (%)	17.41	17.61	0.43	ns	**
Dry matter (%)	67.57	67.11	0.52	ns	ns
Protein (% of DM)	46.12	46.03	0.75	ns	ns
Soluble N/total N	21.92	22.75	0.75	ns	ns
Ether extract (% of DM)	41.88	41.38	1.09	ns	ns
pH	5.76 a	5.66 b	0.03	*	**
a_w	0.92	0.91	0.004	ns	*
Ash (% of DM)	9.04	9.10	0.21	ns	***
Salt (%)	5.39	6.11	0.36	ns	**

Abbreviations: CC, control cheese; EC, experimental cheese; SEM, standard error of means; n., number of samples; a_w, water activity.
Statistical significance: *P ≤ 0.05; **P < 0.01; ***P < 0.001; ns = not significant; a, b, means within group (C, E) with different letter differ for P ≤ 0.05.

effect of the addition of the LAB culture significantly influenced only pH values in the final cheeses which was lower (5.66) for the EC samples. Among factories, significant differences were also found for cheese yield, a_w, ash and salt percentage values. In particular, a higher salt percentage was registered in the EC samples.

Results of sensory evaluation for all cheese samples are reported in the Table 8. The addition of the selected LAB influenced significantly the colour, the formation of eyes, the uniformity of the structure and the unpleasant taste. In particular, the judges found less holes in the EC samples and recognised a difference for the uniformity of structure between Ec and CC samples. The cheese samples obtained from different factories did not show statistically difference in odour and taste. Except soft/hard texture, the differences among panellists were significant for almost all other cheese attributes. Hence, this analysis evidenced that each factory produced cheeses with unique characteristics.

The cheese samples were also subjected to the triangle test (Table 9). A good discrimination was found for 50% of the samples analysed. In detail, significant differences ($\pi = 0.50$; $P < 0.01$) in colour, odour and taste attributes, were found between EC and CC samples produced at the factories II, III and IV. Moreover, the cheeses that reached the highest preference were those produced with the addition of the starter culture at the factory III.

Table 8
Sensorial parameters in CC and EC samples.

Descriptors	CC (n. 12)	EC (n. 12)	SEM	P		
				Treatment	Factories	Panellist
Colour	6.30 A	6.62 B	0.09	**	**	***
Oil	3.42	3.28	0.10	ns	*	***
Hole	3.68 A	2.88 B	0.14	***	***	*
Uniformity	11.43 a	11.95 b	0.16	*	***	*
Odour intensity	8.36	8.49	0.10	ns	ns	***
Pasture	5.42	5.30	0.09	ns	*	***
Unpleasant	1.93 a	1.65 b	0.10	*	***	***
Taste intensity	8.29	8.47	0.10	ns	ns	*
Salt	5.06	4.97	0.10	ns	**	***
Spicy	6.34	6.12	0.12	ns	**	***
Bitter	2.07	2.12	0.10	ns	***	**
Soft/hard	7.01	6.84	0.11	ns	***	ns
Pasty	11.06	11.05	0.11	ns	**	***
Dispersion	5.05	5.15	0.11	ns	***	***

Abbreviations: CC, control cheese; EC, experimental cheese; SEM, standard error of means; n., number of samples.
Statistical significance * P ≤ 0.05; **P < 0.01; ***P < 0.001; ns = not significant; a, b, means with different letter differ for P ≤ 0.05.

3.5. Multivariate statistical analyses

HCA classified the productions in accordance to their mutual dissimilarity and relationship based on the values of the 31 parameters evaluated through microbiological, chemico-physical and sensory characterization of the cheeses (Fig. 3A). CC and EC samples form the factories II and IV clustered closely, whereas the CC and EC of the other factories were quite distant. Furthermore, the majority of the cheeses were characterised by high scores of dissimilarity with the most distant being control and experimental cheeses of the factories III, VI and I.

The results of PCA showed seven eigen-values higher than 1, with the first four accounting for 70.75% of total variability. However, Factor 1 and 2 together explained 42.96% of total variability. For this reason, the 31 variables were expressed as linear combination of the first two factors. The score plot (Fig. 3B) clearly showed the far distance among the cheeses produced in the different factories and confirmed the strict correlation between the CC and the corresponding EC samples from the factories II and IV. For other factories, the CC samples were quite different from the EC samples. In particular, the highest differences were found for the factory I, followed by the factory VI for which the EC sample was distant from their CC sample along Factor 1 which has the highest incidence on the total variability. Another high difference was showed by the cheeses of the factory III, although they differed mainly along the Factor 2 which has a lower incidence than Factor 1 on the total variability. The lowest effects along Factor 1 were registered for the factories II, IV and V. The highest differences for the CC and EC samples form the factory III were mainly explained by some sensory attributes (salt, spicy, bitter, unpleasant aroma, holes and uniformity of structure) as shown by the loading plot (Fig. 3C). The differences registered for the factories I and VI are mainly imputable to soft/hard texture, oil, dispersion and pasty. As a matter of fact, the addition of the mixed LAB culture influenced the final characteristics of the cheeses.

4. Discussion

The complex interactions among LAB are crucial for the final characteristics of ripened cheese (Settanni and Moschetti, 2010). The different species found during cheese making process are characterised by different ability to grow in a changing substrate (Gatti et al., 2014). For this reason, SLAB, that mainly participate to the fermentation process, are superseded by NSLAB which are implicated almost exclusively in the ripening process.

Although cheeses produced from raw milk with traditional wooden equipment are generally contaminated by undesirable spoilage bacteria (Dogan and Boor, 2003; Munsch-Alatossava and Alatossava, 2006), the traditional wooden vats, commonly used for milk transformation, are a primary source of pro-technological LAB (Lortal et al., 2009; Scatassa et al., 2015). A possible innovation to improve the quality of traditional PDO cheeses is represented by the addition of autochthonous LAB as starter cultures, which are able to safeguard the typicality of the cheese as well as to preserve the traditional manufacture technology (Settanni et al., 2013).

LAB are indicated as “autochthonous” when they were isolated from a typical cheese and, consequently, they are strongly adapted to the production area and the traditional technology (Settanni and Moschetti, 2014). The application of these bacteria at high numbers, at a certain extent, might ensure their dominance over the indigenous milk LAB inhibiting several undesired (spoilage and/or pathogenic) microorganisms.

In the present work, a mixture of LAB, including SLAB and NSLAB, was applied to a large-scale level, for PDO Pecorino Siciliano cheese production. *L. lactis* subsp. *lactis* CAG4 and CAG37

Table 9
Difference between CC and EC samples evaluated by triangle test and test of preference.

Factories	Triangle test CC *vs* EC			Test of Preference[a]		
	Correct answer	Assessor (n)	Difference between samples[b]	CC	EC	Significance
I	9	26	no	3	6	no
II	17	30	yes	9	8	no
III	28	30	yes	4	24	yes
IV	14	25	yes	4	10	no
V	7	25	no	3	4	no
VI	11	27	no	7	4	no

Abbreviations: n, number; CC, control cheese; EC, experimental cheese.
[a] Calculated on correct answers.
[b] Significance $\alpha = 0.05$ (5% risk ISO 4120: 2004).

represented the SLAB inocula, since this species is a common mesophilic LAB applied as starter for cheese production (Fox et al., 2004). The NSLAB strains, chosen for their dairy traits, represented three different species: *E. faecalis*, *S. macedonicus* and *L. garvieae*. In particular, *E. faecalis* has been reported to be positively linked to the typicality of traditional cheeses (Foulquié Moreno et al., 2006). *L. garvieae* and *S. macedonicus*, that constitute a common part of raw milk (Franciosi et al., 2009), are frequently isolated from several Italian cheeses (Fortina et al., 2003; Pacini et al., 2006), including PDO Pecorino Siciliano cheeses (Todaro et al., 2011).

Cheese manufacturing was performed in six factories located in different areas of the Sicily using the same strains previously proven effective at stabilizing Pecorino Siciliano cheese in a restricted area (Settanni et al., 2013).

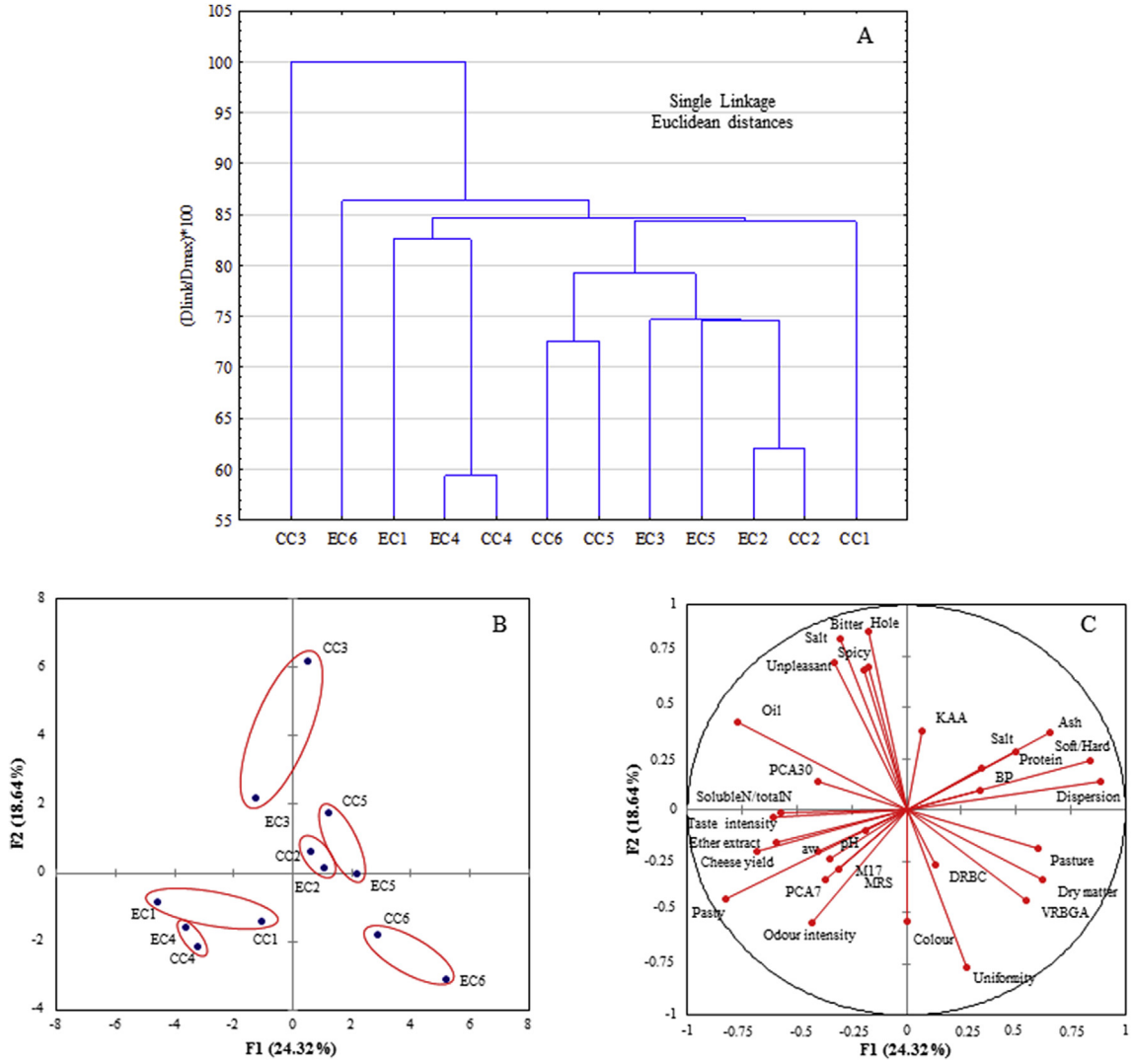

Fig. 3. Dendrogram resulting from HCA analysis (A) and score plot (B) and loading plot (C) of PCA analysis based on microbiological, chemico-physical and sensory data from 5-month ripened cheeses. Abbreviations: CC, control cheese; EC, experimental cheese.

The microbiological investigation started with the characterization of the wooden vat surfaces and results, related to microbial groups detected, are closed to results reported for wooden vat used in other traditional raw ewes' milk cheese process (Didienne et al., 2012; Scatassa et al., 2015). In particular, LAB cocci, including several species responsible for the rapid acidification of milk, were found dominant in all wooden vat surfaces, in agreement with previous studies (Settanni and Moschetti, 2010). Furthermore, the low levels of members of *Enterobacteriaceae*, CPS and the absence of pseudomonads and spores of clostridia, confirmed previous observations (Licitra et al., 2007; Lortal et al., 2009).

High levels of microorganisms in raw ewes' milk are quite common (Barron et al., 2001). In the present work although the TMC values of raw milks were slightly higher than the limit for the raw ewes' milk (CE, 2004) the final microbiological quality of cheeses was in accordance with results previously reported (Randazzo et al., 2008), confirming that quality of final product is not depending exclusively from milk but is also a consequence of the interaction between milk, wood equipment and microbiota (Settanni et al., 2013).

All samples displayed the presence of LAB cocci before and after contact with the wooden vat surfaces. Similar results were previously reported by Settanni et al. (2013) and by Gaglio et al. (2016a) in PDO Pecorino Siciliano and in PDO Vastedda della valle del Belìce processing, respectively. High levels of enterococci in milk has been related to milking equipment (Gelsomino et al., 2002), and their fast growth to the high adaptation abilities of these bacteria (Medina et al., 2001). Fat, protein and casein percentages resulted within the parameters of sheep milk produced in Sicily (Todaro et al., 2014). The variability observed among milk parameters of the dairy factories is linked to different environmental factors (such as breeds, altitude, management, etc.) (Boyazoglu and Morand-Fehr, 2001).

The kinetics of acidification of both CC and EC samples were different for the six monitored processes, showing a trend similar to that previously reported for the same LAB culture (Settanni et al., 2013), with a faster decrease of pH in experimental curds. The application of RAPD-PCR technique on strains isolated from experimental curd samples, after 7 days of acidification, showed the dominance of the *L. lactis* added strains. The same technique indicated also the presence of the added NSLAB strains in the EC samples after 5 months of ripening.

The differences between EC and CC samples were not statistically different for the counts of *Enterobacteriaceae* and CPS, but a decreasing trend for both undesired groups was observed in the ripened EC.

The chemico-physical composition of the EC samples was not influenced by the addition of microbial culture, with the exception of pH, which was lower in EC samples. Although lower pH values are reported to modify the bitterness (McSweeney and Sousa, 2000), in the present study no evidence was reported in this regard, in agreement with previous observations (Settanni et al., 2013). Furthermore, the chemical composition of the cheeses produced in the present work was respectful of the PDO Pecorino Siciliano cheese production disciplinary.

The sensory analysis showed that the addition of the selected LAB significantly influenced only the colour, the formation of eyes, the uniformity of the structure and the unpleasant taste of the final products. Furthermore, the judges found some differences regarding the structure between EC and CC samples. *L. lactis* subsp. *lactis* CAG4 and CAG37 at 10^7 CFU/mL did not modify the aroma of the experimental cheese, as previously observed (Settanni et al., 2013). Other authors (Centeno et al., 2002) reported that single strains of *L. lactis* showed different attitude to enhance the flavour intensity of raw ewes' milk cheeses, even at high inoculation levels. The triangle test showed the highest preference by the judges towards the cheeses produced with the mixed LAB culture at the factory III.

In order to better understand the effect of the selected mixed culture on the characteristics of the cheeses at 5-month ripening, a multivariate analysis, including HCA and PCA, was performed with the microbiological, chemico-physical and sensory parameters evaluated on the final cheeses. HCA is a graphical representation of a matrix of distances such as the dendrogram where the objects (cheeses) are joined together in a hierarchical ascendant analysis from the closest one, i.e. the most similar, to the furthest apart, which is the most different (Gaglio et al., 2016b). PCA is a potent tool to condense the information retrieved by the parameters monitored during cheese production into a reduced number of factors. In the majority of factories, EC and CC samples were quite distant from one another. The most distant cheeses were those from factory III obtained with the LAB mixed culture. Their position on the score plot was mainly explained by a few sensory attributes (salt, spicy, bitter, unpleasant aroma, holes and uniformity of structure).

Some sensory attributes are strongly related to the regional typicality of Pecorino cheeses. E.g. bitter and acidic notes are revealed in Canestrato Pugliese produced in Apulia, sweet and buttery tastes are associated to Fiore Sardo made in Sardinia, Pecorino Romano manufactured in the area around Rome and Sardinia is characterised by a salty perception (Di Cagno et al., 2003), while Pecorino di Farindola produced in a restricted area of Abruzzo region is recognizable for the bitterness, saltiness, but also for fruity and grassy notes (Suzzi et al., 2015) derived from the activity of *Kluyveromyces marxianus* associated to this cheese (Fasoli et al., 2015).

In conclusion, the results of this study indicated that the addition of the mixed LAB culture influenced positively the final features of PDO Pecorino Siciliano cheeses, but the cheeses produced in the different factories were characterised by different profiles. Thus, the inoculation of *Lactococcus lactis* subsp. *lactis* CAG4 and CAG37, *Lactococcus garvieae* PSL67, *Enterococcus faecalis* PSL71 and *Streptococcus macedonicus* PSL72, in mixture, stabilized the microbiological characteristics of PDO Pecorino Siciliano cheese, preserving the typicality of the productions of each factory.

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