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ORIGINAL PAPER

Effect of different salting technologies on the chemical and microbiological characteristics of PDO Pecorino Siciliano cheese

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Abstract The present work was carried out to evaluate the effect of two salting technologies [dry salting (DS) and the combined dry-brine salting (DBS)] on the chemico-physical and microbiological characteristics of PDO Pecorino Siciliano cheeses of different final weight (6 and 12 kg). Dry matter was significantly influenced by both salting process and final size. Twelve kilogram cheeses treated by DBS showed higher protein content with higher soluble nitrogen per cent than 6 kg cheeses. Salt content was in the range 3.1–4.0% on dry matter. The colour did not show significant differences for any of the factors, but 12 kg cheeses subjected to DS showed higher yellow index than the other cheeses. The resistance at 30% of strain was influenced by cheese size, with 6 kg cheeses showing higher resistances than 12 kg cheeses. All cheeses were dominated by coccus LAB, but pseudomonads and Enterobacteriaceae showed comparable levels of about 10^5 cfu/g. Significant microbiological differences were evidenced only for enterococci and yeasts concerning the final cheese size. Thirteen species of LAB, belonging to five genera (*Enterococcus*, *Lactobacillus*, *Lactococcus*, *Pediococcus* and *Streptococcus*), were identified, but several spoilage/pathogenic species were also identified, especially *Pseudomonas putida*, *Citrobacter freundii* and *Stenotrophomonas maltophilia*. LAB isolates were preliminary evaluated for their physiological characteristics in view of developing autochthonous starters to improve the microbiological quality of PDO Pecorino Siciliano cheese.

Keywords Chemico-physical parameters · Lactic acid bacteria · Raw ewes' milk cheese · Salting process · Spoilage microorganisms

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Introduction

Any Italian hard cheese made from ewes' milk is known as 'Pecorino'. Pecorino cheese is produced throughout Italy and the name often indicates the geographical origin. Outside the national borders, the most known Italian Pecorino cheese is 'Pecorino Romano', but there are other Pecorino cheeses, which also enjoy a protected designation of origin (PDO) status, that are being appreciated by foreigner consumers, in particular, 'Pecorino Sardo', 'Pecorino Siciliano' and 'Pecorino Toscano'. In general, Pecorino cheeses are characterized by a certain salty taste.

Pecorino Siciliano cheese is, probably, the oldest European cheese [3], the PDO disciplinary goes back to 1955 (GURI n. 295 of 12-22-1955) and provides the use of entire ewe's raw milk produced in Sicily, the use of traditional wooden equipment, the application of dry salting and a ripening period of at least 4 months. The dry salting technology is performed by manual aspersion of salt onto the cheese surface. However, this dry salting method produces high variability on cheese salt content.

PDO Pecorino Siciliano cheese is obtained without the addition of bacterial starters. Thus, the microflora acting during cheese making and ripening is indigenous, deriving from milk or the transformation environment and, for this reason, it may be considered autochthonous. The presence of indigenous microorganisms provides characteristic

features that link their presence to the typicality of a given cheese [18]. This is particularly true for the artisanal cheeses, which are produced in restricted areas, whose territory and habits, as well as the pedoclimatic conditions and anthropogenic activities (not reproducible elsewhere), are the expression of history and tradition.

After curdling of raw milk, several microbial groups may be found in the fat-protein matrix obtained [21]. During ripening, the complexity of the microbial structure associated with curd diminishes, since lactic acid bacteria (LAB) produce organic acids, mainly lactic acid, determining the decrease in pH till 4.9–5.3 that, besides the presence of salt, low moisture, low temperatures and a deficiency of nutrients makes the environment stressing for many microorganisms [46]. In these conditions, the microbiology of aged raw milk cheeses is typically represented by non-starter LAB (NSLAB) [43]. Several studies have been forwarded to the description of NSLAB communities in Italian raw milk ewes' cheeses [12, 14, 41, 49]. However, some other microbial groups, such as Enterobacteriaceae, have been reported in different Mediterranean cheeses made from ewes' raw milk and subjected to a short time of ripening, [23, 36]. The presence of Enterobacteriaceae is supposed to influence the final characteristics of cheese [7].

With the aim to standardize the salt content and to evaluate the microbiological quality of PDO Pecorino Siciliano cheese, the influence of different salting methodologies was evaluated on the chemico-physical characteristics, microbiology, LAB and spoilage microbial composition of cheeses of two different sizes.

Materials and methods

Cheese production and sample collection

Milk employed for cheese productions was obtained from sheep reared in three farms located in western Sicily (Agrigento and Trapani provinces). Cheese manufacturing was performed in three dairy factories (A, B and C) annexed to the farms. Milk bulk comprised the milk from evening (kept refrigerated under slow stirring) and morning milking. Cheeses were produced between 10 May and 25 May (2010), following the disciplinary of PDO Pecorino Siciliano cheese (Reg. CE n. 1107/96) at two different final weights (6 and 12 kg). Two salting processes were applied to the cheeses: dry salting (DS) and the combined dry-brine salting (DBS). DS technique involved the shedding of 3% of dry salt per kg of cheese, after 48 h from cheese making, and a further 1% of dry salt per kg of cheese after 15 days. DBS technique involved the immersion in brine after 48 h from cheese making for 24 h for the 12 kg cheeses and for 12 h for the 6 kg cheeses. For both cheese sizes, the addition of a further 1% of dry salt per kg cheese was performed.

During the first 2 months of ripening, all cheeses were brushed weekly to remove moulds; the temperature and relative humidity (RH) of the storage chamber were 16 °C e 85%, respectively. Afterwards, cheeses were kept for 3 months into a cave characterized by a temperature of 16 °C and 90% RH. Sixty cheeses, 30 of 12 kg and 30 of 6 kg, were produced in the three dairy factories.

Three cheeses per size (6, and 12 kg), salting process (DS, DBS) and dairy factory (A, B, C), forming a total of 36 cheeses, were sampled after 5 months of ripening. Samples were collected as follows: 300 g were randomly taken from different portions of cheese and subjected to grating for chemical and microbiological analysis.

Chemical and physical measurements

Samples of cheeses were analysed for moisture, fat, protein (total nitrogen × 6.38) and ash content according to IDF Standards [30–33]. Salt content was determined by the Volhard method [2]. Cheeses colour was measured with a Minolta chroma meter (CR-300, Minolta, NJ 07446 USA), and results were expressed as lightness L*, redness a* and yellowness b* in the CIEL*a*b* system. Texture profile was determined at 21 °C. Each cylinder of cheese was subjected to a load cell force of 90.9 kg and compression at 30 and 40% using an Instron Universal Testing Machine (model 5000; Instron Corporation, Canton, MA). Analysis was performed in triplicate.

Microbiological analysis

Cheese samples were homogenized in sodium citrate (2% w/v) solution (cheese/diluent 1:9) by means of a stomacher (Laboratory Blender Stomacher 400, Seward, UK) for 2 min at the highest speed. Further decimal dilutions were prepared in Ringer's solution (Oxoid, Milan, Italy). Cell suspensions were plated and incubated as follows: total mesophilic count (TMC) on plate count agar (PCA) added with 1 g/L skimmed milk (SkM), incubated aerobically at 30 °C for 72 h; total psychrotrophic counts (TPC) on PCA-SkM, incubated aerobically at 7 °C for 7 days; Enterobacteriaceae on violet red bile glucose agar (VRBGA), incubated anaerobically at 37 °C for 24 h; enterococci on kanamycin aesculin azide (KAA) agar, incubated aerobically at 37 °C for 24 h; pseudomonads on *Pseudomonas* agar base (PAB) supplemented with 10 mg/mL cetrimide fucidin, incubated aerobically at 20 °C for 48 h; positive coagulase staphylococci (PCS) on Baird Parker (BP) added with R.P.F. supplement, incubated aerobically at 37 °C for 48 h; rod LAB on de Man-Rogosa-Sharpe (MRS) agar,

acidified at pH 5.4 with lactic acid (5 M), incubated anaerobically at 30 °C for 48 h; coccus LAB on M17 agar, incubated anaerobically at 30 °C for 48; yeasts on yeast glucose chloramphenicol (YGC) agar, incubated aerobically at 25 °C for 48 h. Clostridial content was estimated by the most probable number (MPN) technique using a 3 × 3 scheme [21]. All media were purchased from Oxoid. Microbiological counts were carried out in triplicate.

Isolation of LAB and phenotypic grouping

After growth, colonies of various shapes (at least 5 with identical morphology) were randomly picked from count plates used for LAB enumeration (MRS, M17 and KAA) and transferred into the corresponding broth media, with the exception of KAA which was replaced by M17. The isolates were purified by successive sub-culturing. The purity of the cultures and cell morphology was checked microscopically. Gram-positive (Gregersen KOH method) and catalase-negative (determined by transferring fresh colonies from a Petri dish to a glass slide and adding 5% H_2O_2) isolates were stored in glycerol at −80 °C until further experimentations.

Rod- and coccus-shaped LAB cultures were first grouped on the basis of cell disposition, growth at 15 and 45 °C and CO_2 production from glucose. The last test was carried out in the optimal growth media (MRS for rod LAB and M17 for coccus LAB) containing all components except citrate. M17 contained glucose in place of lactose. The assay consisted of LAB inoculation into test tubes sealed with H_2O agar (2%, w/v). The strains negative to the assay were inoculated into test tubes containing the optimal growth media prepared with a mixture of pentose carbohydrates (xylose, arabinose and ribose, 8 g/L each) in place of glucose. Coccus isolates were further sub-grouped on the basis of their growth at pH 9.6 and in the presence of 6.5% (w/v) NaCl.

Isolation of presumptive spoilage microorganisms

In order to better evaluate the presence of unwanted bacteria, colonies (about three with different morphology) originated from the highest dilutions of cell suspensions showing the highest numbers of TPC, Enterobacteriaceae, pseudomonads and yeasts, for each dairy factory, were picked up from the corresponding agar media, purified, microscopically investigated and conserved as reported above.

Genotypic identification of cheese isolates

Cell lysis for DNA extraction was performed by the Instagene Matrix kit (Bio-Rad, Hercules, CA) as described by the manufacturer. Crude cell extracts were used as template for PCR.

The isolates representative of each phenotypic group of LAB were identified by the amplification of the gene 16S/23S rRNA ITS (in the range 200–400 bp) performed as described by White et al. [51], while the presumptive spoilage microorganisms were identified by the 16S rRNA gene amplification (approximately 1,600 bp) following the protocol reported by Weisburg et al. [50]. Yeasts were analysed by the restriction fragment length polymorphism (RFLP) of the 5.8S ITS rRNA gene (in the range 400–1,050 bp): the DNA fragments were amplified following the methodology of Esteve-Zarzoso et al. [15] and the amplicons digested with the endonucleases *Cfo*I, *Hae*III and *Hinf*I (MBI Fermentas, St. Leon-Rot, Germany) at 37 °C for 8 h.

The amplicons to be sequenced were purified by the QIAquick purification kit (Quiagen S.p.a., Milan, Italy) and sequenced using the same primers employed for PCR amplification. DNA sequences were determined by the dideoxy chain termination method with the DNA sequencing kit (Perkin-Elmer Cetus, Emeryville, CA, USA) according to the manufacturer's instructions. The sequences were compared by a BLAST search in GenBank/EMBL/DDBJ database.

Statistical analysis

Chemical, physical and microbiological data were analysed with the ANOVA linear model according to a repeated measure design (GLM procedure of SAS 9.1.2 software, 2004) which included the effects of dairy factories (A, B and C), the interaction between salting processes (DS and DBS) and cheese size (6 and 12 kg) and the cheeses for each thesis (1.3) as repeated measure. Comparison among LS means was performed by t test; differences were considered significant at $P < 0.05$.

Results

Chemical and physical parameters

Chemical composition and physical parameters of Pecorino cheeses are reported in Table 1. Dry matter was significantly influenced by the salting process and the cheese size: DS determined a higher loss of water from cheeses and the size inversely affected dry matter content. The 12 kg cheeses subjected to DBS showed higher protein content with higher soluble nitrogen per cent than 6 kg cheeses.

Even though the size and the salting process did not show significant differences, the following trends were registered: 6 kg size and DS salting process determined a higher salt uptake than 12 kg and DBS.

Table 1 Chemical and physical characteristics of PDO Pecorino Siciliano cheeses of different size subjected to different salting technologies

Determination	6 kg cheese		12 kg cheese		S.E.	*P* value	
	DBS	DS	DBS	DS		DF	SP*CS
Dry matter (%)	65.6 Aa	67.0 Ab	63.6 Bc	65.0 Ba	0.45	***	***
Protein (% DM)	44.3 A	44.3 A	46.4 B	45.0 A	0.54	***	*
Soluble N (% DM)	1.7 a	1.6 a	2.0 b	1.8 ab	0.10	***	*
N sol./N tot (%)	23.9	23.1	26.9	25.9	1.41	**	NS
Ether extract (% DM)	43.4	43.3	42.6	43.4	0.62	***	NS
NaCl (% DM)	3.7	4.0	3.1	3.5	0.29	NS	NS
Ash (% DM)	9.9	10.2	9.1	9.7	0.39	*	NS
L*, Lightness	73.88	72.16	73.32	75.06	0.91	NS	NS
a*, red index	4.64	4.80	4.66	4.77	0.11	**	NS
b*, yellow index	11.86 a	11.21 a	12.00 a	13.16 b	0.38	NS	**
CS 30% (N/mm^2)	0.12 a	0.13 a	0.09 b	0.08 b	0.01	NS	**
CS 40% (N/mm^2)	0.22 A	0.26 B	0.18 C	0.17 C	0.01	***	***

CS compressive stress at 30 or 40% of strain

DS dry salting, *DBS* combined dry-brine salting, *DF* dairy factory, *SP* salting process, *CS* cheese size

P value: *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; *NS* not significant. On the row: different letter A, B, C = $P \leq 0.01$; a, b, c = $P \leq 0.05$

The colour did not show significant differences for both factors considered; only 12 kg cheeses subjected to DS showed a higher yellow index than the other cheeses. Instead, the resistance at 30% of strain, in compression test, was influenced by the cheese size, since the 6 kg cheeses showed higher resistances than 12 kg cheeses; moreover, when the resistance at 40% was considered, a significant effect related to the salting process was showed by the 6 kg cheeses which displayed a higher resistance for DS rather than DBS.

Microbiological analysis

Several microbial (pro-technological and spoilage) populations (Table 2) were investigated in the PDO Pecorino Siciliano cheese samples.

TMC were almost superimposable with the counts of coccus LAB. This data showed that the dominant microbial group of each cheese analysed was represented by coccus LAB. In general, TPC and rod LAB were less concentrated than those coccal-shaped. Enterococci were the LAB less concentrated in all samples. Yeasts represented the microbial group present at the lowest concentrations for each cheese typology, while pseudomonads and Enterobacteriaceae showed levels comparable with those of LAB.

Statistical differences were evidenced for enterococci and yeasts which were more concentrated and less concentrated, respectively, in 12 kg cheeses independently on the salting technology applied. No statistical differences were found for the other microbial groups with regard to the size and the salting technologies. The dairy factories determined significant differences among cheese productions.

Isolation and grouping of LAB

A total of 1,323 pure cultures were isolated and propagated and 1,171 cultures were further characterized since being Gram-positive and catalase-negative. Only 117 presumptive LAB were rods, while almost 90% of them ($n = 1{,}054$), including some cultures developed in MRS, were coccus.

The phenotypic characterization allowed the separation of the cultures into seven groups (Table 3), five for cocci and two for rods. The group most numerous was group III which included almost the 50% of the presumptive LAB. The distinction between the groups VI and VII was possible, thanks to the CO_2 formation from glucose. However, the unequivocal determination of the fermentative metabolism of LAB included in the group VII needed the evaluation of their growth in the presence of pentose sugars, which showed their facultative heterofermentative metabolism.

Identification of LAB

The isolates representative of each phenotypic group, at least one per factory, were subjected to the genotypic identification. The BLAST search for the 16S/23S rRNA ITS sequences analysed evidenced a percentage of identity with sequences available in the NCBI database of at least 97% for 18 isolates, while seven isolates showed a lower similarity level (Table 4). All isolates were confirmed to belong to the group of LAB, since although not recognized at species level, the seven isolates remained un-speciated shared a certain similarity within the genera *Enterococcus*,

Table 2 Microbial concentrations (log cfu/g) of PDO Pecorino Siciliano cheeses of different size subjected to different salting technologies

Microbial group	6 kg cheese		12 kg cheese		S.E.	*P* value	
	DBS	DS	DBS	DS		DF	SP*CS
TMC	5.37	5.45	5.83	5.35	0.20	***	NS
TPC	4.78	4.92	4.87	4.42	0.28	*	NS
Enterobacteriaceae	5.02	5.08	5.18	4.18	0.38	**	NS
Enterococci	2.65 a	2.78 a	3.52 b	3.42 b	0.23	***	*
Pseudomonads	5.26	5.17	4.68	4.55	0.25	NS	NS
Rod LAB	4.73	5.02	5.23	5.04	0.21	***	NS
Coccus LAB	5.35	5.62	5.80	5.27	0.20	***	NS
Yeasts	2.33 A	2.75 A	1.62 B	1.47 B	0.22	NS	***

DS dry salting, *DBS* combined dry-brine salting, *DF* dairy factory, *SP* salting process, *CS* cheese size

P value: *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; *NS* not significant. On the row: different letter A, B, C = $P \leq 0.01$; a, b, c = $P \leq 0.05$

Table 3 Phenotypic grouping of LAB isolates collected from PDO Pecorino Siciliano cheese of different size subjected to different salting technologies

Characters	Clusters						
	I ($n = 37$)	II ($n = 189$)	III ($n = 565$)	IV ($n = 151$)	V ($n = 112$)	VI ($n = 73$)	VII ($n = 44$)
Morphology	Coccus short chain	Coccus short chain	Coccus short chain	Coccus tetrads	Coccus long chain	Rod	Rod
Growth							
15 °C	−	+	+	+	−	+	+
45 °C	+	+	+	+	+	−	−
pH 9.6	+	+	+	+	−	n.d.	n.d.
6.5% NaCl	−	−	+	+	−	n.d.	n.d.
CO_2 from glucose	−	−	−	−	−	+	−
Growth in the presence of pentose carbohydrates	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	+

n.d. not determined

Lactobacillus, *Lactococcus* and *Streptococcus*. The species clearly identified were nine, included into five genera. The isolates recognized as *Lactococcus garvieae* showed different phenotypic characteristics.

In Table 4, it is also reported the speciographic distribution of LAB among PDO Pecorino Siciliano cheeses. In general, the species more frequently identified were lactococci followed by enterococci and, at a lesser extent, pediococci. In terms of species, the differences found between the cheeses with a diverse final weight were more evident than those registered with regard to the salting technology (Table 4).

Identification of the presumptive spoilage microorganisms

Due to the high concentrations estimated on the media used for the counting of putative spoilage microorganisms, they were deeply investigated at species level. A total of 25 isolates were identified by means of the 16S rRNA gene sequencing which succeeded to find a homology ≥97% in GenBank for 19 sequences. *Pseudomonas putida* was the species most frequently identified; it was found in the cheese of all three factories in both final size. Also *Stenotrophomonas maltophilia* and, among the Enterobacteriaceae family, *Citrobacter* spp. and *Serratia* spp were among the psychrotrophic species. The isolates from VRBGA were confirmed to belong to the Enterobacteriaceae family.

Within the group of spoilage, yeasts were also considered. Those registered at the highest numbers were *Pichia membranifaciens* with a 5.8S-ITS amplicon of approximately 500 bp and the restriction profile 250 + 80 + 50 bp with *Cfo*I, 330 + 90 + 50 bp with *Hae*III and 290 + 145 + 90 bp with *Hinf*I and *Yarrowia lipolytica* with a 5.8S-ITS amplicon of 380 bp and the restriction profile 200 + 170 bp with *Cfo*I, 380 with *Hae*III and 200 + 180 bp with *Hinf*I.

Table 4 Species of LAB detected in PDO Pecorino Siciliano cheeses of different size subjected to different salting technologies

Species	Phenotypic group	16S/23S rRNA ITS			Cheese factory		
		bp	% homology	Acc. No.	A	B	C
Enterococcus spp.	II	357	<97	JN696685		6 kg-DS	
E. durans	III	340	99	JN696686			12 kg-DS
E. faecalis	II	330	99	JN696687		6 kg-DBS	
E. faecalis	III	329	99	JN696688	6 kg-DS		
E. faecium	III	330	99	JN696689			6 kg-DBS
E. faecium	III	325	100	JN696690			12 kg-DBS
Lactobacillus spp.	VII	260	<97	JN696691	12 kg-DBS		
L. brevis	VI	423	99	JN696692	12 kg-DBS		
L. brevis	VI	429	99	JN696693	12 kg-DBS		
Lactococcus spp.	III	290	<97	JN696694	6 kg-DS		
Lactococcus spp.	III	330	<97	JN696695	6 kg-DS		
Lactococcus spp.	III	230	<97	JN696696			6 kg-DBS
Lactococcus spp.	III	320	<97	JN696697		6 kg-DBS	
Lc. garvieae	III	227	99	JN696698		6 kg-DBS	
Lc. garvieae	III	235	97	JN696699	6 kg-DS		
Lc. garvieae	I	232	99	JN696700	6 kg-DBS		
Lc. garvieae	II	202	99	JN696701		6 kg-DBS	
Lc. garvieae	II	320	100	JN696702		6 kg-DS	
P. acidilactici	IV	349	98	JN696703			12 kg-DS
P. acidilactici	IV	236	98	JN696704	6 kg-DBS		
P. pentosaceus	IV	328	98	JN696705		6 kg-DS	
P. pentosaceus	IV	224	98	JN696706		12 kg-DBS	
Streptococcus spp.	V	275	<97	JN696707		6 kg-DBS	
S. infantarius	V	323	98	JN696708			12 kg-DS
S. macedonicus	V	222	98	JN696709			12 kg-DBS

E., *Enterococcus*; *L.*, *Lactobacillus*; *Lc.*, *Lactococcus*; *P.*, *Pediococcus*; *S.*, *Streptococcus*; *DS* dry salting, *DBS* combined dry-brine salting

Discussion

The effect of salting process and cheese size determined significant differences on cheese dry matter content corresponding to different moisture levels of the cheeses. It is well known that, at the same time of ripening, bigger cheeses show a higher water content; as expected, in this work, 12 kg cheeses were characterized by a higher moisture than 6 kg cheeses. Chemical composition reported in Table 1 resulted in agreement with previous studies carried out on PDO Pecorino Siciliano [34] and other Italian raw ewes' milk cheeses [8]. The significant higher soluble nitrogen of 12 kg cheeses subjected to DBS could be linked to the higher total nitrogen, as confirmed by N soluble/N total ratio, which did not result to be different among cheeses.

Even if the salt percentage did not show significant differences between the factors considered, 6 kg cheeses presented higher salt content, probably due to the higher superficial area exposed to the salt. In fact, salt absorption increases with increasing surface area/volume (SA/V) ratio of the cheese [27].

Cheese colour, expressed as L*, a* and b* index, resulted similar with those reported by Cozzi et al. [9], although determined on a different cheese (Asiago d'allevo, northern Italy). The yellowness of cheeses coming from pasture depends on the yellow colour originate from β-carotene and related carotenoid compounds, which are transferred from fresh plants to milk and cheese. In our study, the significant higher yellow index found in the 12 kg-DS cheeses could be due to different microbial activities, as suggested by other authors [6].

Salting process and cheese size determined significant differences on the rheological properties of PDO Pecorino Siciliano, in particular, on compressive stress. The 12 kg cheeses showed a lower compressive stress values than 6 kg cheeses due to the higher moisture, while salting methods determined higher compressive stress values for DS cheeses that resulted saltier than DBS cheeses. Numerous investigators have studied the effects of salt

Table 5 Spoilage bacteria detected in PDO Pecorino Siciliano cheeses of different size subjected to different salting technologies

Species	16S rRNA			Cheese factory			Isolation medium
	bp	% homology	Acc. No.	A	B	C	
Citrobacter spp.	396	<97	JN696710			12 kg-DS	PCA-SkM 7 °C
C. freundii	415	100	JN696711	12 kg-DBS			VRBGA
C. freundii	445	100	JN696712			12 kg-DS	VRBGA
C. freundii	445	100	JN696713			12 kg-DS	VRBGA
Enterobacter spp.	390	97	JN696714		12 kg-DBS		VRBGA
Enterobacter spp.	400	<97	JN696715		12 kg-DBS		VRBGA
Es. coli	445	99	JN696716	12 kg-DBS			VRBGA
K. oxytoca	390	99	nd		6 kg-DS		VRBGA
Pseudomonas spp.	427	<97	JN696717			12 kg-DS	PCA-SkM 7 °C
Ps. putida	399	100	JN696718	12 kg-DBS			PAB
Ps. putida	435	99	JN696719	12 kg-DBS			PAB
Ps. putida	398	100	JN696720	6 kg-DBS			PCA-SkM 7 °C
Ps. putida	398	99	JN696721			12 kg-DS	PAB
Ps. putida	399	100	JN696722	6 kg-DBS			PCA-SkM 7 °C
Ps. putida	403	100	JN696723		6 kg-DS		PAB
Ps. putida	385	100	JN696724		6 kg-DS		PCA-SkM 7 °C
Ps. putida	412	100	JN696725		6 kg-DS		PAB
Ps. putida	395	98	JN696726		6 kg-DS		PAB
Ps. vranovensis	297	100	JN696727			12 kg-DS	PAB
Serratia spp.	388	98	JN696728			12 kg-DS	VRBGA
Serratia spp.	400	98	JN696729			12 kg-DS	PCA-SkM 7 °C
Ser. grimesii	392	99	JN696730			12 kg-DS	VRBGA
Sten. maltophilia	450	100	JN696731	6 kg-DBS			PCA-SkM 7 °C
Sten. maltophilia	439	98	JN696732	12 kg-DBS			VRBGA
Sten. maltophilia	450	100	JN696733		6 kg-DS		PCA-SkM 7 °C

C., *Citrobacter*; *Es.*, *Escherichia*; *K.*, *Klebsiella*; *Ps.*, *Pseudomonas*; *Ser.*, *Serratia*; *Sten.*, *Stenotrophomonas*; *nd* not deposited, *DS* dry salting, *DBS* combined dry-brine salting

concentration on the rheological properties such as firmness, fracture stress, fracture strain and/or sensory hardness. These studies have shown that increases in salt, within the range 0.4–12% (w/w), determine an increase of firmness and sensory hardness of various cheeses [26].

The microbiological results obtained in this work showed that coccus LAB dominated any cheese samples analysed, while the counts registered for rod LAB were slightly lower. This trend was observed in other similar Italian cheeses [4] and the concentration levels of rod LAB was, on average, in the same order of magnitude of those reported by other authors [25, 39]. In a previous work carried out on the microbial ecology of Pecorino Siciliano cheese produced in winter and spring [48], data, collected at latest at 90 days of ripening, showed that mesophilic rod and coccus LAB were approximately at the same level (about 10^7 cfu/g).

Enterococci, which are strictly linked to the typicality of ripened cheeses [18] and, for this reason, often selected for their positive roles in cheese making [19, 20], were considered among the pro-technological bacteria. The counts determined on the medium specifically employed for enterococci showed that they were less concentrated, of about two log cycles, than other LAB. Our data almost agreed with those observed for different ripened raw ewes' milk cheeses analysed approximately at the same period of ageing [39], even though higher levels were reported for Pecorino Siciliano cheese investigated at 3-month ripening [48]. Enterococci have also been detected at higher levels than ours at 12-month ripening for some Pecorino-type cheeses [14].

The different cheeses produced in this experimentation were also evaluated for their hygienic characteristics. PCS and butyric clostridia were not found in any sample. With the exception of yeasts, whose levels were in the range of those known for similar aged products [16, 39], but lower than those previously reported for Pecorino Siciliano ripened for 3 months [48], the other microbial populations (TPC, Enterobacteriaceae and pseudomonads) were detected at relatively high levels. In fact, in comparison

with other raw ewes' milk cheeses, our data showed higher levels of the unwanted bacteria, especially Enterobacteriaceae. A level in the range 10^5–10^6 cfu/g is common for this kind of cheeses at around 2–3 months of ripening [36, 40, 45], but the concentration of Enterbacteriaceae greatly decreases after that period [11, 29, 38], since their growth is affected by the stressing cheese conditions.

Even though pseudomonads, the most frequently psychrotrophic bacteria of raw milk [28], due to their intense proteolytic and lypolytic activities [10] are implicated with spoilage of milk and milk-derivates [44], they are not generally searched in ripened cheeses. In case of milk, the spoilage occurs when their concentrations are around 10^7 cfu/mL and, for this reason, *Pseudomonas* spp. are sometimes investigated at the beginning of dairy fermentations [35, 47], but no more during ripening, since their number decreases. Our data showed a different trend: pseudomonads were found at consistent levels after 5-month ripening in PDO Pecorino Siciliano cheeses. These findings underlined the importance of better investigate on the different microbial populations detected during the characterization of the several productions of PDO Pecorino Siciliano cheese.

LAB were phenotypically divided into seven groups, and 13 species belonging to five LAB genera were identified. Except streptococci, all LAB found in this study are generally reported to be associated with Italian raw ewes' milk cheeses [8, 14, 39, 49].

Among the *Streptococcus* species detected, *S. macedonicus* is of dairy origin and has been used as secondary adjunct culture in cheese making [42], while *S. infantarius*, in particular, the subspecies S. *infantarius* subsp. *infantarius*, has been found at high numbers in Gariss, a camel's fermented milk [1]. Regarding lactococci, the only species clearly identified was *Lc. garvieae*, which is associated with raw milk [19] and cheeses, where some selected strains are also employed as secondary adjunct cultures [17].

Lactobacillus brevis is among the obligately heterofermentative species of the NSLAB group responsible for the ripening of several cheeses [43]. A very low percentage of lactobacilli was revealed by genetic identification, despite the high counts detected on the medium (MRS) used for mesophilic rod LAB. These results confirmed our practical observations that LAB cocci are able to develop colonies on the above media, even though at lower levels than those estimated on the medium (M17) generally used for coccus LAB counting. This reduction in number could be due to the lower pH of MRS used in this study (final pH 5.4).

The presence of pediococci, basically *P. acidilactici* and *P. pentosaceus*, in mature cheeses is almost common [5], but it is not frequently reported in raw ewes' milk cheeses. Three species of *Enterococcus* genus (*E. durans*, *E. faecalis* and *E. faecium*) were identified in the present study. Although the presence of enterococci may be attributed to faecal contaminations [24], it is desirable since they, especially in traditional long-term ripened cheeses, strongly contribute to the aromatic characteristics of the final products. Furthermore, the three *Enterococcus* species found in this work are commonly associated with food fermentation [22].

Spoilage and/or potentially pathogenic microorganisms were also investigated at species levels. The species *C. freundii*, *Enterobacter* spp., *Es. coli* and *K. oxytoca* have been already reported for this type of cheese, but not after the first month of ripening [39]. Also *Serratia* genus was isolated during the ripening of Italian Pecorino cheeses [7]. Although *Stenotrophomonas* spp. was revealed in raw milk [13], this is the first work reporting the presence of *Stenotrophomonas maltophilia* in a cheese product. An unwanted species frequently detected was *Pseudomonas putida*. *Pseudomonas* spp. have been found associated to raw ewes' milk cheeses of 2 months [37], but the identification of *Ps. putida* at high numbers from 5-month ripened Pecorino cheese has never been reported before.

Due to the high numbers of undesired microorganisms, especially those potentially pathogenic for consumers, found in the different productions of PDO Pecorino Siciliano cheese, the revision of the production protocol is suggested. The addition of autochthonous LAB as starter bacteria deserves a particular attention in order to improve the hygienic conditions of this cheese. Other Italian PDO raw ewes' milk cheese producers (e.g. Pecorino Romano and Pecorino Toscano) adopted this strategy to obtain final products characterized by a high microbiological quality.

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