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ORIGINAL RESEARCH

Microbial diversity and dynamics of Pecorino di Filiano PDO, a traditional cheese of Basilicata region (Southern Italy)

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The evolution of microbial populations of 'Pecorino di Filiano' (PF) cheese was investigated during ripening in natural cave and storeroom. 62.5% of isolates grow at 45 and 15 °C and 77.7% showed high salt concentrations tolerance. Brevibacterium linens *was dominant in surface samples.* Lactobacillus delbrueckii *subsp.* bulgaricus *and* Lactobacillus paracasei *subsp.* paracasei *were more frequently isolated both surface and core samples, while* Leuconostoc lactis *and* Leuconostoc mesenteroides *subsp.* mesenteroides *prevailed among* Leuconostoc *isolates. Our results suggest the importance of the ripening environment of cheeses and how a biological ecosystem affects and produces the typical features of artisanal products.*

Keywords 'Pecorino di Filiano' PDO cheese, Microbial dynamics, Ripening environment, Molecular identification.

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INTRODUCTION

'Pecorino di Filiano' (PF) is an artisanal cheese, produced in north-western areas of Basilicata region (Southern Italy) and bearing a Protected Designation of Origin (PDO) in compliance with the national legislation (EU 1992; EU 2007). Since 1600, the ripening process has been performed in natural tuff caves that give the freshness and unique features to the final product. In PDO cheeses, the autochthonous microflora plays a major role in the determination of the cheese specificity, so its diversity within the area of origin has been the subject of studies (Duthoit *et al.* 2003); however, no research has ever been performed on PF, neither the technique for producing this cheese nor the microbial composition of the cheese has been previously described.

Two microbial groups are important in cheese manufacture and ripening: starter lactic acid bacteria (LAB) responsible for acid development during cheese manufacture and secondary flora, specific to particular cheese varieties and with proteolytic and lipolytic enzymes significant for texture, flavour, chemical composition and quality of final product (Caridi *et al.* 2003). Therefore, it is important to know the microflora evolution in the PF cheese, which production is allowed only within a well-defined geographical area, with traditional manufacturing and ripening processes, to protect the microbial biodiversity and features of quality and authenticity (Rantsiou *et al.* 2008). The aim of this study was to investigate the presence and the evolution of microbial populations during PF cheese ripening by comparison with cheese samples manufactured with the same technology but ripened in two different environments.

MATERIALS AND METHODS

Cheese manufacturing and sampling procedure

PF cheese was produced in a small plant in the PDO area of Basilicata region using traditional techniques without the use of starter cultures. Manufacturing procedures specified under the PDO were applied (EU 2007). Briefly, the raw ewe's milk was heated at 68 °C for 20 s, then cooled at 36–40 °C and coagulated by adding paste lamb rennet (100 g/q). After milk coagulation, the curd was cut into grains of about 0.3–0.5 cm^3 and pressed manually into cylindrical form with subsequent removal of whey. Afterwards, the curd was heated at 60–65 °C for 10 min and held at room temperature until 12 h, when it was salted. After the salting stage, the resulting cheeses were divided

into two batches: one placed in a natural cave and another in a storeroom with controlled temperature (12–14 °C) and relative humidity (80–85%) for 120 days of ripening. Samplings were taken, from the surface and the core of products, after salting ($t = 0$) at the mid-ripening ($t = 30$ days) and then at the end of ripening ($t = 120$ days). Samplings were collected in duplicate and used for the analyses.

Microbiological analyses

Ten grams of each sample was transferred into a sterile stomacher bag, 10-fold diluted with sterile saline/peptone water (8.5 g/L NaCl and 0.1 g/L bacteriological peptone) and homogenised for 2 min in a stomacher (Lab-Blender, Seward, London). Further decimal dilutions were made and inoculated on agar plates of appropriate and selective media. Total bacterial counts were evaluated on Plate Count Agar (PCA) incubated at 10 and 30 °C for 24 h; presumptive LAB were enumerated on MRS agar in anaerobic conditions incubated at 30 °C for 48 h; total coliforms on Violet Red Bile Agar (VRBA) incubated at 37 °C for 24 h; faecal coliforms in Violet Red Bile Agar supplemented with 4-methylumbelliferyl-β-D-glucuronide (VRBA-MUG) incubated at 44 °C for 24 h; enterococci on Kanamycin Aesculin Azide Agar (KA) added with 0.020 g/L kanamycin, incubated at 37 °C for 24 h; presumptive mesophilic cocci on M17 agar supplemented with 5 g/L lactose, incubated at 30 °C for 48 h under anaerobic conditions; and presumptive thermophilic cocci on Baird Parker Medium Agar supplemented with egg yolk tellurite emulsion, incubated at 37 °C for 24–48 h. Tests were carried out in duplicate, and then, after counting, means and standard deviations were calculated, and the results were expressed as log (cfu/g).

Twenty-five/thirty colonies were randomly selected using a Harrison disk (Harrigan and McCance 1976) from MRS, M17 and PCA agar count plates of each batch at different stages of ripening. For a preliminary screening, colonies were checked for Gram staining, colony pigmentation, cell morphology evaluated by phase contrast microscopy and the presence of catalase. The isolates were maintained as frozen stock in reconstituted skim milk (11% w/v) at −80 °C. A final number of 296 isolates were available for following analyses.

DNA extraction and RAPD analysis

The selected isolates were subjected to the total DNA extraction from a single colony using the IstaGene™ Matrix (Bio-Rad Laboratories, Hercules, CA, USA) following the supplier's instruction. One hundred nanograms of the DNA was submitted to RAPD analysis as described by Bonomo *et al.* (2008).

Physiological characterisation

184 isolates, randomly picked from clusters of RAPD analysis, were submitted to phenotypic characterisation using a set of 28 physiological and biochemical tests including the preliminary screening described earlier. The strains were examined for different physiological features as already described in details by Parente *et al.* (2001). All tests were performed in duplicate.

Reference strains

Twenty-one reference strains were used for molecular identification: *Streptococcus (St.) thermophilus* DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) 20479; *Enterococcus (E.) faecium* DSM 20477; *E. faecalis* ATCC (American Type Culture Collection, Manassas, Virginia, USA) 14433; *E. durans* ATCC 11576; *E. gallinarum* LMG (Laboratorium voor Microbiologie, Universiteit Gent, UGent, Belgium) 13129; *Brevibacterium (B.) linens* DSM 20158; *Lactobacillus (L.) delbrueckii* subsp. *bulgaricus* DSM 20081; *L. paracasei* subsp. *paracasei* DSM 4905; *L. curvatus* DSM 20019; *L. plantarum* DSM 20174; *L. acidophilus* DSM 20079; *Lactococcus (Lc.) lactis* subsp. *lactis*, DSM 20481; *Lc. garviae* DSM 20684; *Leuconostoc (Leu.) mesenteroides* subsp. *mesenteroides* DSM 20240; *Leu. mesenteroides* subsp. *cremoris* DSM 20346; *Leu. paramesenteroides* DSM 20288; *Leu. fallax* DSM 20189; *Leu. citreum* DSM 5577; *Leu. lactis* DSM 20202; *Leu. dextranicum* DSM 20484; *Pediococcus (P.) acidilactici* DSM 20333.

Molecular identification

Cocci-shaped isolates were identified by the amplification of 16S-23S rDNA intergenic spacer region (ISR) as described by Bonomo *et al.* (2009). Rod-shaped isolates were identified by the restriction analysis of amplified 16S rDNA gene (ARDRA) as described by Bonomo *et al.* (2008), using restriction enzymes *Sma*I and *Taq*I (New England BioLabs, County Road Ipswich, MA, USA), for strains with cream, yellow and red/orange colonies, or *Hae*III and *Cfo*I for the others. Species-specific PCR assays were performed for rapid and reliable identification and differentiation of *Leu. lactis* as described by Lee *et al.* (2000) and *P. acidilactici* as described by Mora *et al.* (1997).

Statistical analysis

The banding patterns were captured with GelDoc 2000 Apparatus (Bio-Rad), digitised in Diversity Database™ software (Bio-Rad Laboratories Ltd., Watford, Herts, UK) and processed for detection of the bands, and calculation of similarity in the band profiles was based on Pearson product-moment correlation coefficient. Dendrograms were obtained by means of the Unweighted Pair Group Method using Arithmetic Average (UPGMA) clustering algorithm (Vauterin and Vauterin 1992). A coefficient of correlation of 85% was arbitrarily selected to distinguish the clusters.

Statistical analysis was performed using Systat 10.0 for Windows (SPSS, Chicago, IL, USA). Significant differences and correlations between counts of microbial groups were evaluated by analysis of variance (ANOVA) ($P < 0.05$) to observe the microbial loads of the batches at each sampling points. All

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other data were statistically analysed by the variance analysis followed by a Student's t-test with significant differences at $P < 0.05$.

Reagents and media

Unless otherwise specified, all reagents were obtained from Sigma-Aldrich (Milan, Italy), while bacteriological media and ingredients were obtained from Oxoid (Basingstoke, Hampshire, UK).

RESULTS

Microbial population dynamics

Total bacterial counts, initially high and similar in surface and core samples, decreased reaching lower values at the end of ripening in both environments (Table 1). Presumptive mesophilic cocci counts showed a similar trend, reaching the highest value ($\sim 10^9$ cfu/g) after 30 days in both environments. Total coliforms showed an initial number rather high, then it progressively decreased with a very low count in natural cave, while it was nondetectable in storeroom product. Faecal coliforms behaved similarly with high count at the beginning of ripening and a very low presence at the end of process. Enterococci counts showed initially a low presence and kept the same values until the end of process in storeroom; in natural cave, they reached a high value at the end of ripening with a major presence in surface.

Presumptive LAB had high levels at the beginning of ripening, and then they presented a slightly reduced number at the end. A slight difference between surface and core samples in

Table 1 Log counts of the microbial groups during ripening process of the artisanal 'Pecorino di Filiano' cheese in a natural cave and in a store-room; t = 0: after salting stage; t = 1: after 30 days (mid-ripening); t = 2: after 120 days (end of ripening). Samplings were collected in duplicate and used for the analyses

		t = 30 days		*t = 120 days*	
Micro-organisms	*t = 0*	*Store-room*	*Natural cave*	*Store-room*	*Natural cave*
Total bacterial counts (10°C)					
Surface	9.41 ± 0.04	9.95 ± 0.07	9.94 ± 0.14	7.15 ± 0.03	8.56 ± 0.08
Core	8.54 ± 0.05	8.68 ± 0.08	7.59 ± 0.07	6.34 ± 0.15	6.00 ± 0.02
Total bacterial counts (30°C)					
Surface	9.95 ± 0.07	9.12 ± 0.11	9.95 ± 0.23	7.74 ± 0.07	9.74 ± 0.22
Core	8.99 ± 0.15	7.92 ± 0.09	7.67 ± 0.07	7.11 ± 0.11	7.01 ± 0.14
Faecal coliforms					
Surface	5.08 ± 0.24	4.49 ± 0.03	5.18 ± 0.12	ND	2.57 ± 0.07
Core	6.40 ± 0.15	1.69 ± 0.06	1.00 ± 0.02	ND	2.43 ± 0.11
Total coliforms					
Surface	6.04 ± 0.17	5.04 ± 0.02	5.79 ± 0.21	ND	2.62 ± 0.11
Core	6.62 ± 0.08	1.78 ± 0.11	1.30 ± 0.08	ND	2.68 ± 0.08
Counts on Baird Parker agar at 37°C					
Surface	4.95 ± 0.21	6.39 ± 0.06	6.59 ± 0.08	5.02 ± 0.13	6.68 ± 0.09
Core	6.52 ± 0.14	6.41 ± 0.12	6.42 ± 0.22	5.18 ± 0.11	5.32 ± 0.06
Enterococci					
Surface	3.20 ± 0.07	3.48 ± 0.03	4.73 ± 0.06	3.08 ± 0.04	7.04 ± 0.12
Core	3.04 ± 0.04	3.36 ± 0.08	3.84 ± 0.11	3.20 ± 0.06	5.67 ± 0.07
Counts on MRS agar at 30°C					
Surface	6.83 ± 0.09	7.08 ± 0.12	6.98 ± 0.07	5.45 ± 0.14	5.18 ± 0.05
Core	9.00 ± 0.02	7.83 ± 0.05	7.52 ± 0.03	6.38 ± 0.09	6.38 ± 0.06
Counts on M17 agar at 30°C					
Surface	9.20 ± 0.11	9.81 ± 0.07	9.92 ± 0.05	7.89 ± 0.11	9.45 ± 0.21
Core	8.90 ± 0.02	7.87 ± 0.04	7.16 ± 0.08	6.11 ± 0.03	6.78 ± 0.12

Values are expressed in log cfu/g. Each number is the mean ± standard deviation of two samples taken from the same batch.
ND, not detected.

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both environments was observed. Presumptive thermophilic cocci increased during ripening and kept equivalent values until the end. The preliminary phenotypic evaluation allowed to hypothesise that some surface isolates of PF belonged to *B. linens* as they were catalase positive, showed coryneform morphology and produced yellow to deep orange–red colonies.

RAPD analysis

All isolates were grouped into 15 different clusters or single strains. The sample population after salting stage was distributed in almost all clusters and was dominated by a heterogeneous coccal population and different groups of lactobacilli. Cocci were also the dominant population after 30 days, where a more complex bacterial community was found, because different RAPD profiles were detected (belongings cluster shown in Table 2). Lactic acid bacteria were significantly present during all ripening process, especially in the core samples ripened both in natural cave and storeroom, and their distribution was dominant in five clusters (I, VII, VIII, XII, XIII) and single strains. Finally, after 120 days, the bacterial population was distributed in ten clusters with a fair balance between cocci and bacilli strains. RAPD fingerprints allowed to characterise the dominant populations during ripening process and to select randomly strains of different populations with high biodiversity from the different clusters obtained.

Physiological characterisation

A total of 184 isolates (100 from surface and 84 from core) were randomly picked from clusters obtained by RAPD analysis for physiological characterisation. Table 2 shows some of physiological features tested to carry out the preliminary characterisation and identification. Isolates showed different abilities to face various growth condition; 62.5% were able to grow at 45 °C and even at 15 °C, 77.72% tolerated high salt concentrations (4% and 6%), and the most of strains not able to grow in presence of salt were cocci-shaped. 63.58% of isolates hydrolysed esculin, while 72.82% was not able to hydrolyse arginine. Moreover, sixty-six isolates showed a hetero-fermentative metabolism, producing gas from glucose, and all isolates produced L-lactate either alone or together with D-lactate and presented different fermentation patterns.

Molecular identification of strains

The selected 184 isolates were also submitted to molecular identification. The identification by ISR-PCR obtained nine different profiles for cocci-shaped strains. 34, 12 and 6 isolates belonged, respectively, to the species *Leu. mesenteroides* subsp. *mesenteroides*, *P. acidilactici* and *Leu. paramesenteroides*. The remaining isolates were identified as *Leu. lactis* (5), *Leu. fallax* (3), *Leu. citreum* (3), *Lc. lactis* (2), *Leu. dextranicum* (1) and *Leu. mesenteroides* subsp. *cremoris* (1). Among lactic acid rods, the ARDRA-PCR evidenced that *L. paracasei* subsp. *paracasei* was the predominant species (29 isolates).

Other species isolated in small amounts were *L. delbrueckii* subsp. *bulgaricus* (9), *L. acidophilus* (1) and *L. curvatus* (1). Moreover, species-specific PCR assays were performed for an exact identification of *Leu. lactis* and *P. acidilactici* species, and then a differentiation of *P. acidilactici* from the phylogenetically related *P. pentosaceus* species was obtained, and sixteen strains were identified as *P. acidilactici,* while fourteen isolates belonged to *Leu. lactis* species. *B. linens* was dominant in surface samples showing a constant presence in both ripening environments with a frequency of 46% among isolates (Figure 1). *L. delbrueckii* subsp. *bulgaricus* and *L. paracasei* subsp. *paracasei* were the populations of *Lactobacillus* more frequently isolated both surface and core samples, even if the percentages were not higher than 3% and 7% on surface and 6% and 16% in core, respectively. *L. paracasei* subsp. *paracasei* proved a good presence after 30 days of ripening that comes down to a very low percentage in surface samples, while it showed the highest frequency in core samples at the end of ripening.

L. curvatus, *L. acidophilus*, *Lc. lactis* and *Leu. dextranicum* were occasionally isolated with low percentage (Figure 1). *Leu. lactis* and *Leu. mesenteroides* subsp. *mesenteroides* prevailed among *Leuconostoc* isolates; *Leu. lactis* was present after salting, and its frequency decreased during the other ripening stages with the same percentages in both samples and in both ripening environments, while *Leu. mesenteroides* subsp. *mesenteroides* showed an important presence at the end of ripening (Figure 1). One *Leu. mesenteroides* subsp. *cremoris* isolate was found in a 120 days ripened cheese sample, while *Leu. citreum* and *Leu. fallax* were present only after 30 days of ripening in core samples ripened in natural cave and in storeroom, respectively. Moreover, *P. acidilactici* was found in surface samples only at the end of ripening with a frequency of 10%, while in core samples, this species was present during all ripening stages with an important presence in 120 days ripened cheese samples.

In Figure 2, the frequency of isolation of each species in correlation with the different selective-isolation-strain media is reported. M17 and MRS agar showed a low selectivity towards lactococcal and leuconostoc isolates obtained from both media with comparable percentages. On the contrary, pediococci grew preferably on M17 agar plates, while MRS agar was quite discriminating for lactobacilli. The incubation temperature used in the study allowed to obtain a growth differentiation among species isolated belonging to mesophilic microflora.

DISCUSSION

This study was undertaken to follow the evolution of microflora during PF cheese ripening. The cheese microflora plays a critical and essential role in the development of the unique characteristics of each cheese variety, with a strong impact on the appearance, flavour and texture. Microbiological counts obtained from PF samples displayed a significant presence of

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Table 2 Physiological characterization of isolates taken after salting (t = 0), at the mid-ripening (t = 30 days) and at the end of ripening (t = 120 days). Isolates were taken from the surface and the core of the 'Pecorino di Filiano' cheese ripened in a natural cave and in a storeroom. Tests were carried out in duplicate

Isolate(s)	Origin of isolates[a]	Morphology	CO_2 from glucose	Growth at		Growth at		Hidrolysis		Dextran production	Identified as[b]	Cluster number[c]
				15 °C	45 °C	4%NaCl	6%NaCl	Esculin	Arginine			
Surface cheese after salting (t = 0)												
G3, G4, G8, G10, G12, G14	PCA	Cocci	0	1	1	1	1	0	0	0	*Brevibacterium (B.) linens*	V
G11	PCA	Cocci	0	1	1	1	1	0	0	0	*B. linens*	XI
G6, G15	MRS	Bacilli	0	0	1	0	0	0	0	0	*Lactobacillus (L.) delbrueckii* subsp. *bulgaricus*	V
G1	MRS	Bacilli	0	1	1	1	1	1	1	0	*L. curvatus*	V
G5	M17	Cocci	0	1	0	1	1	1	1	0	*Lactococcus (Lc.) lactis*	V
T2	MRS	Cocci	1	1	1	1	1	1	0	1	*Leuconostoc (Leu.) dextranicum*	VIII
T1, T3, T4, T5, T6, T8, T9, T10, T11	MRS	Cocci	1	1	1	1	1	1	1	1	*Leu. lactis*	VIII
Surface cheese at the mid-ripening (t = 30 days)												
Natural cave												
G31, G32, G33, G38, G41, R4	PCA	Cocci	0	1	1	1	1	0	0	0	*B. linens*	IV
G34	PCA	Cocci	0	1	1	1	1	0	0	0	*B. linens*	III
G40, G42, G43	PCA	Cocci	0	1	1	1	1	0	0	0	*B. linens*	I
T30, T32, T34, T35, T42	MRS	Bacilli	0	1	1	1	1	1	0	0	*L. paracasei* subsp. *paracasei*	II
T13	MRS	Bacilli	0	1	1	1	1	1	0	0	*L. paracasei* subsp. *paracasei*	XII
T41	M17	Cocci	1	0	0	1	1	1	1	1	*Leu. lactis*	Single-strain
G35,G36, G39, G44, G45	PCA	Cocci	0	1	1	1	1	0	0	0	*B. linens*	IV
Storeroom												
G18, G19, G20, G21, G22, G24, G25	PCA	Cocci	0	1	0	1	1	0	0	0	*B. linens*	IV
G16	PCA	Cocci	0	1	0	1	1	0	0	0	*B. linens*	II
G17	PCA	Cocci	0	1	0	1	1	0	0	0	*B. linens*	I
G26, G27, G28, G30	PCA	Cocci	0	1	0	1	1	0	0	0	*B. linens*	IV
T12	MRS	Cocci	1	1	1	1	1	1	1	1	*Leu. lactis*	VIII
T15, T19, T20, T27	MRS	Bacilli	0	1	1	1	1	1	0	0	*L. paracasei* subsp. *paracasei*	XIII
T14	MRS	Bacilli	0	1	1	1	1	1	0	0	*L. paracasei* subsp. *paracasei*	X

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Table 2 Continued

Isolate(s)	Origin of isolates[a]	Morphology	CO_2 from glucose	Growth at 15 °C	Growth at 45 °C	Growth at 4%NaCl	Growth at 6%NaCl	Hidrolysis Esculin	Hidrolysis Arginine	Dextran production	Identified as[b]	Cluster number[c]
T16, T17, T18, T25, T26	PCA	Bacilli	0	1	0	1	1	0	0	0	*B. linens*	XIII
Surface cheese at the end of ripening (t = 120 days)												
Natural cave												
G62, G66, G73, G75	PCA	Cocci	0	1	1	1	1	0	0	0	*B. linens*	II
G72	PCA	Cocci	0	1	1	1	1	0	0	0	*B. linens*	XI
G65	PCA	Cocci	0	1	1	1	1	0	0	0	*B. linens*	XII
G61	MRS	Bacilli	0	0	1	0	0	0	0	0	*L. delbrueckii* subsp. *bulgaricus*	V
G69, G71	M17	Cocci	1	1	0	0	0	1	0	1	*Leu. mesenteroides* subsp. *mesenteroides*	V
G68	MRS	Bacilli	0	1	1	1	1	1	0	0	*L. paracasei* subsp. *paracasei*	V
G63, G64, G67, G70, G74	M17	Cocci	0	1	1	1	1	1	1	0	*Pediococcus (P.) acidilactici*	V
Storeroom												
T45	MRS	Bacilli	0	1	1	1	1	1	0	0	*L. paracasei* subsp. *paracasei*	VII
T43	M17	Cocci	0	1	1	1	1	1	1	0	*P. acidilactici*	V
G49, G48, G50, G51, G55, G56, G57, G58, G59	MRS	Cocci	1	1	0	0	0	1	0	1	*Leu. mesenteroides* subsp. *mesenteroides*	V
G60	MRS	Cocci	1	1	0	1	1	1	0	1	*Leu. mesenteroides* subsp. *mesenteroides*	V
G53, G55, G46, G47	M17	Cocci	0	1	1	1	1	1	1	0	*P. acidilactici*	V
T44	M17	Cocci	1	1	1	1	1	1	1	1	*Leu. lactis*	VII
G54	MRS	Bacilli	0	0	1	0	0	1	0	0	*L. acidophilus*	XI
Core cheese after salting (t = 0)												
H2	MRS	Bacilli	0	0	1	0	0	0	0	0	*L. delbrueckii* subsp. *bulgaricus*	X
H29	MRS	Bacilli	0	0	1	0	0	0	0	0	*L. delbrueckii* subsp. *bulgaricus*	IX
H12, H13, H14, H15	MRS	Cocci	1	1	0	0	0	1	0	1	*Leu. mesenteroides* subsp. *mesenteroides*	III
H5, H6, H8, H9	M17	Cocci	1	1	1	1	1	1	1	1	*Leu. lactis*	X

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Table 2 Continued

Isolate(s)	Origin of isolates[a]	Morphology	CO_2 from glucose	Growth at		Growth at		Hidrolysis		Dextran production	Identified as[b]	Cluster number[c]
				15 °C	45 °C	4%NaCl	6%NaCl	Esculin	Arginine			
R9	M17	Cocci	0	1	1	1	1	1	1	0	*P. acidilactici*	XII
H3	M17	Cocci	1	0	1	0	0	0	0	1	*Leu. lactis*	XIII
Core cheese at the mid-ripening (t = 30 days)												
Natural cave												
H31, H36, H38	MRS	Cocci	1	1	1	1	1	1	0	0	*Leu. citreum*	XI
R24, R25, R26, R27, R28	M17	Cocci	0	1	1	1	1	1	1	0	*P. acidilactici*	XI
Storeroom												
R20	M17	Cocci	0	0	0	1	1	1	1	0	*P. acidilactici*	VI
H17	MRS	Cocci	0	1	0	1	1	1	1	0	*Lc. lactis*	IX
H16, H18, H19	MRS	Cocci	1	1	1	1	1	0	0	0	*Leu. fallax*	IX
R13, R22	MRS	Bacilli	0	1	1	1	1	1	0	0	*L. paracasei* subsp. *paracasei*	X
R17	MRS	Bacilli	0	1	1	1	1	1	0	0	*L. paracasei* subsp. *paracasei*	XII
R10, R11, R12	MRS	Cocci	1	1	1	0	0	0	0	0	*Leu. paramesenteroides*	XII
R21	MRS	Cocci	1	1	1	0	0	0	0	0	*Leu. paramesenteroides*	X
Core cheese at the end of ripening (t = 120 days)												
Natural cave												
R44, R47, R46, R49, R51	MRS	Bacilli	0	1	1	1	1	1	0	0	*L. paracasei* subsp. *paracasei*	V
R48, R52	MRS	Bacilli	0	1	1	1	1	1	0	0	*L. paracasei* subsp. *paracasei*	I
R53	M17	Cocci	1	1	1	1	1	1	1	1	*Leu. lactis*	I
H72, H74	M17	Cocci	1	0	0	1	1	1	0	1	*Leu. mesenteroides* subsp. *mesenteroides*	V
H68, H76	MRS	Cocci	1	1	0	0	0	1	0	1	*Leu. mesenteroides* subsp. *mesenteroides*	V
H77, H78, H82	MRS	Cocci	1	1	0	1	1	1	0	1	*Leu. mesenteroides* subsp. *mesenteroides*	V
R57	M17	Cocci	1	0	0	1	1	1	0	1	*Leu. mesenteroides* subsp. *mesenteroides*	V
R45, H80	M17	Cocci	0	1	1	1	1	1	1	0	*P. acidilactici*	VI

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Table 2 Continued

Isolate(s)	Origin of isolates[a]	Morphology	CO_2 from glucose	Growth at 15 °C	Growth at 45 °C	Growth at 4%NaCl	Growth at 6%NaCl	Hidrolysis Esculin	Hidrolysis Arginine	Dextran production	Identified as[b]	Cluster number[c]
R56	M17	Cocci	1	1	0	0	0	1	0	1	*Leu. mesenteroides* subsp. *mesenteroides*	VII
R50, R54, R55, R58	MRS	Bacilli	0	1	1	1	1	1	0	0	*L. paracasei* subsp. *paracasei*	VII
Storeroom												
R30, R39	M17	Cocci	1	1	1	0	0	0	0	0	*Leu. paramesenteroides*	I
R31, R32, R33, R34, R37, R38	M17	Cocci	0	1	1	1	1	1	1	0	*P. acidilactici*	I
R40, R43	M17	Cocci	0	1	1	1	1	1	1	0	*P. acidilactici*	V
H56	MRS	Cocci	1	1	1	1	1	0	0	0	*Leu. mesenteroides* subsp. *cremoris*	V
H60, H66	M17	Cocci	1	1	0	0	0	1	0	1	*Leu. mesenteroides* subsp. *mesenteroides*	V
H57, H59, H65	M17	Cocci	1	1	0	1	1	1	0	1	*Leu. mesenteroides* subsp. *mesenteroides*	V
H58	M17	Cocci	1	1	0	0	0	1	0	1	*Leu. mesenteroides* subsp.. *mesenteroides*	V
H53, H54, H61, H63	MRS	Bacilli	0	0	1	0	0	0	0	0	*L. delbrueckii sub. bulgaricus*	V
H55	M17	Cocci	0	1	1	1	1	1	1	0	*P. acidilactici*	V
R35, R41	M17	Bacilli	0	1	1	1	1	1	0	0	*L. paracasei sub. paracasei*	VII
R36, R29, R42	M17	Cocci	1	1	0	0	0	1	0	1	*Leu. mesenteroides sub. mesenteroides*	VII
H62	M17	Cocci	1	0	0	1	1	0	0	1	*Leu. lactis*	IX

All tests were carried out in duplicate.

[a]Isolates originated from different media (MRS, M17, PCA).

[b]Identification of selected strains by different amplification reactions.

[c]Cluster number indicates the RAPD-PCR cluster to which strains belong.

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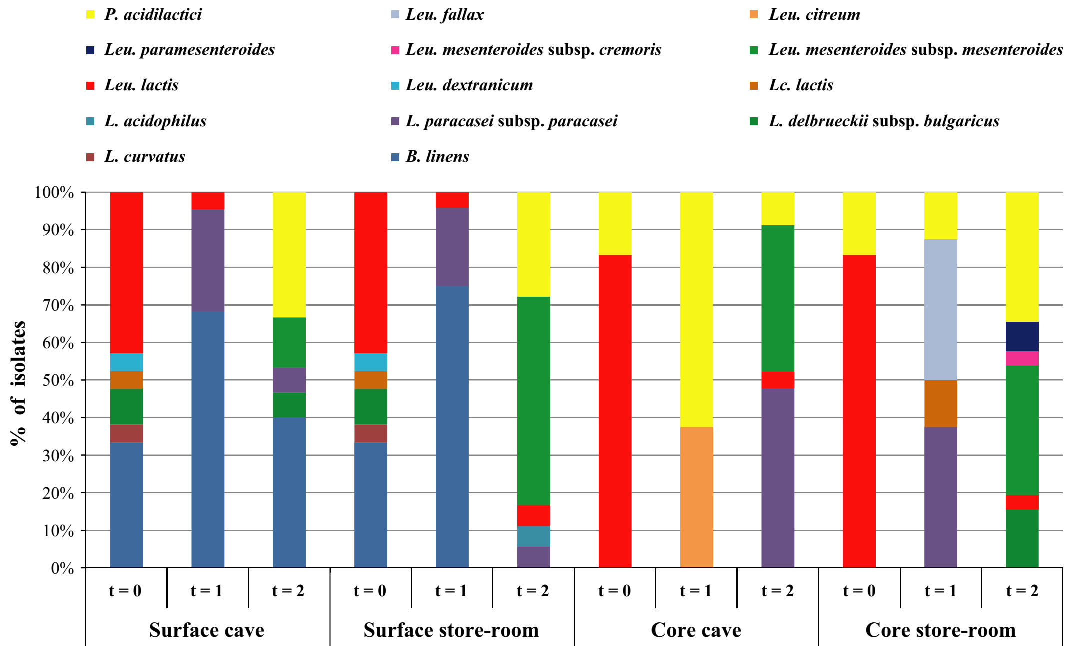

Figure 1 Frequency of isolation of different species in samples from the surface and the core of PF cheese at three different sampling times (t = 0, t = 1, t = 2) ripened in a natural cave and in store-room.

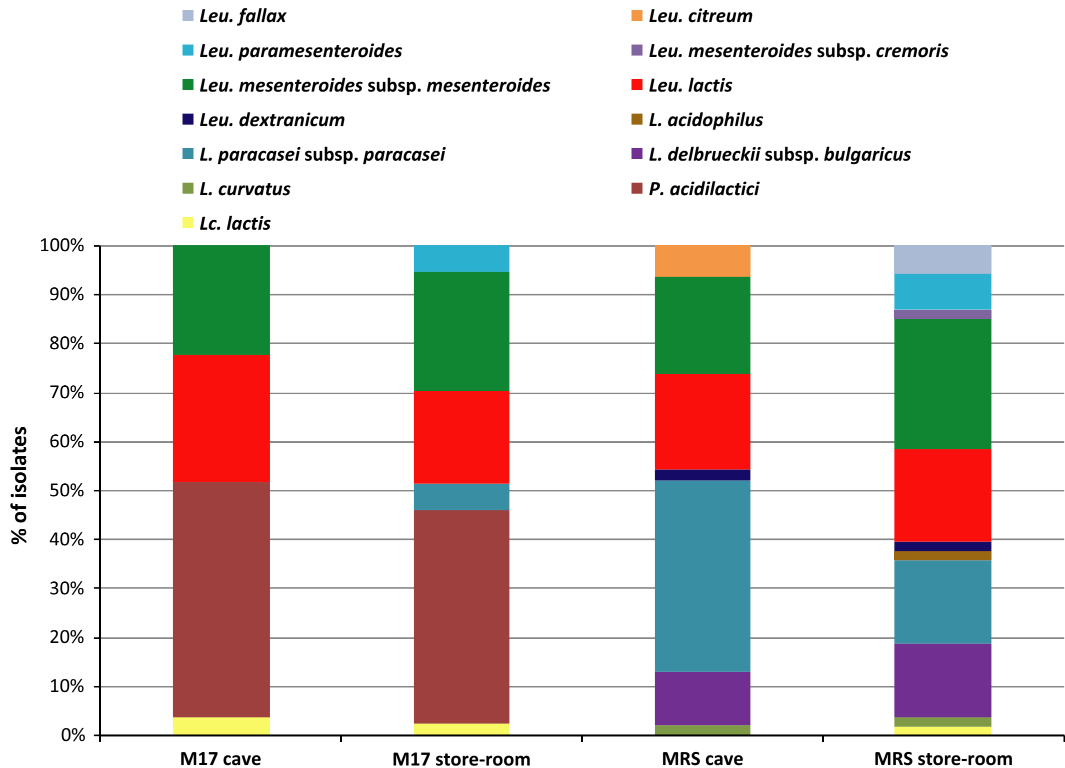

Figure 2 Prevalence of different species among isolates randomly selected on M17 agar and MRS agar plates in samples ripened in a natural cave and in store-room.

all microbial groups targeted with values comparable to those reported for other Italian ripened cheeses (Randazzo *et al.* 2006; Dolci *et al.* 2008). The high values of lactobacilli and staphylococci were attributable to their ability to develop at low pH and to resist high salt concentrations. There were no relevant differences between the two ripening environments,

underlining the contribution of these groups to final typical product. The elevated presence of specific microbiological groups evidences the important selective effect of ripening conditions and that the environment of production area greatly affects characteristics of traditional cheeses. The trend observed in the evolution of streptococcal community was comparable with that reported by other authors for raw milk cheeses (Caridi *et al.* 2003; Marino *et al.* 2003; Dolci *et al.* 2008).

The presence and the values detected for enterococci were not surprising because their presence in cheeses made from raw milk has been previously described (Caridi *et al.* 2003; Dolci *et al.* 2008). The presence of enterococci during the late stage of PF ripening suggests that they may play an important role in the ripening of this type of cheese; their role as a relevant component of natural cultures involved in the fermentation of artisanal cheeses has been described in detail, and *E. faecium* has been included in the list of the LAB starters by the International Dairy Federation (Giraffa 2002). Our results showed that enterococci had behaviour similar to that observed in other studies (Casaburi *et al.* 2007; Cenci-Goga *et al.* 2008) and confirmed the observation of Giraffa (2002) that established enterococci can survive and grow during fermentation of meat and dairy products, especially in foods without the use of competitive starter cultures.

As expected, the presence of coliforms was elevated at the beginning of ripening because of the raw milk was used, their number decreased progressively as a consequence of pH drop during ripening, and they were not detected or were present in a very low count at the end of this process.

Despite being indicators of faecal contamination or of insufficient thermal treatment in pasteurised milk cheeses, some authors suggested that high counts of coliforms might contribute to the development of the characteristic aromas of raw milk cheeses, which are desirable for this type of product (Marino *et al.* 2003).

Among lactic acid rods, *L. paracasei* subsp. *paracasei* was the species most frequently isolated during PF manufacture as already reported by others authors who frequently isolated this species from artisanal cheeses (Baruzzi *et al.* 2000; Marino *et al.* 2003). Amount of other LAB is commonly found during cheese ripening, which promotes in particular the development of heterofermentative lactobacilli belonging to the so-called secondary culture strongly contributing to flavour development (Baruzzi *et al.* 2000).

Our results revealed the strong selective effect of the stringent typical conditions of the natural caves on the indigenous microflora. Samples collected from the surface and the core of products proved the presence of a lower number of species but with a higher frequency of isolation, when ripened in natural cave. This suggests the importance of the ripening environment of cheeses and how a biological ecosystem, such as a natural cave, affects and produces the typical features of artisanal products.

The elevated frequency of isolation of *B. linens* evidenced and confirmed the importance already observed in dairy products because of its ubiquitous presence on the surface of a variety of smear surface-ripened cheeses. The growth of *B. linens* on the surface was thought to be an essential prerequisite for the development of the characteristic colour, flavour and aroma of different cheeses. The distinctive characteristics of cheeses and the number, type and growth rate of the surface microflora are influenced by physical and chemical characteristics intrinsic to the cheese (pH, water activity, redox potential, composition and size), the ripening environment and its parameters (ripening temperature, relative humidity) and the technological conditions during manufacture. This underlines that the environment of ripening cheese has a significant role for the growth and development of specific species such as *B. linens* whose the physiological and metabolic activities are significantly strain dependent as demonstrated in the past years; in fact the reported differences in the biochemical properties and the number of its extracellular proteinases, differences for its amino peptidase, esterase and lipase activities are a clear example (Rattray and Fox 1999).

The good frequency of *L. paracasei* subsp. *paracasei* observed during ripening was in accordance with other studies in which this species together *L. plantarum* showed a high frequency and occurred in dairy ecosystems and dominated the bacterial microflora (Dolci *et al.* 2008).

Our results confirmed previous data indicating the presence of *Leuconostoc* strains in the majority of raw milk French cheeses and other European cheeses (Hemme and Foucaud-Scheunemann 2004). In dairy technology, the importance of *Leuconostoc* strains is widely recognised and their role in the formation of aroma and texture of certain dairy products is essential. The presence of *Leuconostoc* in numerous cheese varieties made without addition of *Leuconostoc* starter is regular, in particular in raw milk cheeses and various authors isolated from raw milk cheeses strains belonging to the different *Leu. mesenteroides* subspecies known for their aroma potential and thus their important role to determine the unique features of the final product (Hemme and Foucaud-Scheunemann 2004).

This study represent an important approach to the understanding of microbial dynamics in PF cheese characterised by typical cheesemaking without the addition of any starter culture. The combined approach, based on phenotypic and genotypic techniques, increases the resolution of each independent device, allowing the detectable levels of strain heterogeneities to be widened. Autochthonous microflora of cheeses represents a heritage that has to be protected and conserved, as typical products possess quality characteristics mainly owing to the presence of specific micro-organisms. Therefore, it is very important to study natural microflora more closely and to understand their contribution to the flavour formation. These studies yield strains with promising and useful properties, which will make them applicable as starters for product innovation, with improvement in flavour properties and enhancement of PF cheese manufacture.

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