J. Agr. Sci. Tech. (2011) Vol. 13: 585-600

Microbiological and Physicochemical Properties of Pecorino Romano Cheese Produced Using a Selected Starter Culture

N. P. Mangia[1*], M. A. Murgia[1], G. Garau[1], and P. Deiana[1]

ABSTRACT

The effect of a selected autochthonous starter culture made up by *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *lactis* and *Lactobacillus helveticus* on the microbiological and physicochemical properties of Pecorino Romano cheese during ripening was investigated. The suitability of the experimental starter culture was tested at industrial scales in cheese-making trials of Pecorino Romano. Pecorino Romano cheese manufactured by use of *scotta-fermento* served as control. The lactic microflora increased significantly in experimental cheeses as compared to the control and this was also accompanied by a substantial decrease of spoilage microorganisms in experimental cheeses. Free amino acids (FAAs) were more abundant in experimental cheeses, arginine+γ-aminobutyric acid and leucine in particular. These differences could be likely due to a different enzymatic activity of the selected starter culture as compared to the *scotta-fermento* used in the control trials. Oleic (C18:1), palmitic (C16:0), butyric (C4), stearic (C18:0) and myristic (C14:0) acids were the most abundant Free Fatty Acids (FFAs) detected in both brand of cheeses at the end of ripening. Overall, the level of FFAs in experimental and control cheeses did not show significant differences, even if the average values in experimental cheeses were always slightly higher than those recorded for the control. Moreover, the average content of FFAs of Pecorino Romano was found the lowest when compared with the other Sardinian PDO cheeses; most likely the high content of sodium chloride and the low a_W of Pecorino Romano influenced all the lipase activities, even those present in the rennet paste. Despite this, the employment of the selected starter culture revealed useful to improve the physico-chemical features of Pecorino Romano while preserving its tipicity.

Keywords: Autochthonous starter culture, Free amino acids, Free fatty acids, Pecorino Romano cheese, Thermophilic microflora.

INTRODUCTION

Pecorino Romano is the most important Italian ewe's milk cheese deserving the Protected Denomination of Origin (PDO) status (Regolamento CE n°1107/96). The ancient Romans introduced the manufacturing procedure to different regions in Europe where the production of the cheese took place during the Middle Ages. In 1897 the production of Pecorino Romano cheese was introduced in Sardinia (Italy). Since 1981, the production and trade of this cheese are under the supervision of the *Consorzio per la tutela del formaggio Pecorino Romano*, made up of the producers in order to guarantee the compliance of the cheeses with the PDO rules. Although the PDO Pecorino Romano can also be manufactured in the Lazio region and in the province of Grosseto (Tuscany, Italy), more than 90% of the current cheese production (33,425 t) (Assolatte, 2007) occurs in Sardinia. The majority of the Sardinian Pecorino Romano production is destined for the USA markets (Assolatte, 2007).

[1] Department of Environmental Agricultural Sciences and Agro-Food Biotechnologies, University of Sassari, 07100 Sassari, Italy.
[*] Corresponding author, e-mail: nmangia@uniss.it

[DOR: 20.1001.1.16807073.2011.13.4.13.2]

PDO Pecorino Romano is a cooked hard-paste cheese manufactured of whole ewe milk that can be thermised at approximately 68°C for 15-17 seconds and inoculated with natural Lactic Acid Bacteria (LAB) cultures. The coagulation is achieved in approximately 20 minutes through adding lamb rennet paste (usually 40 g/100L of milk). The curd is then cut into small grains (approximately 0.3 cm-side), cooked for 15-20 minutes at 48°C, and pressed (1-2 kg for each kg of curd) to have the whey drained out. The pressed curd is then cut into blocks that are poured into moulds and kept at a constant temperature of 35-38°C until the pH achieves a value of approximately 5.40. The cheese is salted for at least 70 days (the first 8 days in brine) at 12°C and 80% relative humidity and then ripened for 5 (table-cheese) or 8 (grating-cheese) months.

Nowadays, the addition of LAB in the form of *scotta-fermento* is a common procedure in the manufacture of Pecorino Romano cheese. *Scotta-fermento* is a natural culture of LAB obtained through incubation of the residual whey from the manufacturing of Ricotta (Bottazzi and Ledda, 1967).

Although different thermophilic LAB species have been isolated from the *scotta-fermento* (Bottazzi and Ledda, 1967; Arrizza, 1972), the number of microorganisms present in this substrate is still largely unknown. However, the microbial community is likely to be scarce because of the thermisation treatment applied (at about 80-90°C) for the production of Ricotta. As a consequence, the fermentative phase of Pecorino Romano is poor and the control of microorganisms causing spoilage is very limited. To counteract the activity of such microflora responsible for spoilage, massive amounts of sodium chloride are employed by the producers, which can reach values up to 8.7% in the Pecorino Romano ripened for 12 months (Di Cagno *et al*., 2003). The isolation of such halophilic microorganisms as micrococci and yeasts, from the cheese surface is a direct consequence of this procedure (Deiana *et al*., 1997).

The aim of this work was to investigate the lactic microflora associated with Pecorino Romano cheese and to evaluate the effects of selecting autochthonous LAB cultures on the microbiological and physicochemical characteristics of the cheese during ripening. The experimental Pecorino Romano cheeses were compared with cheeses made, using the traditional cheese making technique.

MATERIALS AND METHODS

Isolation and Identification of LAB from Pecorino Romano

Thermised milk and cheeses at different ripening stages were collected from three producers of Pecorino Romano cheese. Milk and cheeses (n= 3 from each producer) were brought to the laboratory in isotherm containers and analysed upon the arrival.

Ten gram samples (milk or cheese) were homogenized using 90 mL of 0.1% (w/v) sterile peptone water (Oxoid, Milan, Italy) in a Stomacher Lab Blender 80 (PBI, Milan, Italy) for 2 min to obtain a 1:10 dilution. Further decimal dilutions were carried out in a similar way. From each dilution, 0.1 mL was spread-plated in triplicate on the surface of M17 (Terzaghi and Sandine, 1975) and MRS (De Man *et al*., 1960) agar plates. Plates were incubated in anaerobiosis for 48 hours at 37°C.

All isolates were tested for Gram reaction, catalase activity and morphology. Gram-positive and catalase-negative rods were presumptively identified according to the method and criteria of Kandler and Weiss (1986). In particular, the following tests were carried out: growth capability at 15°C and 45°C in MRS broth after incubation for 5 and 2 days respectively; gas production from glucose in MRS broth (without citrate) in Durham tubes; NH_3 production from arginine as described by Kandler and Weiss (1986). Carbohydrates fermentation capability was assayed in glucose-free MRS broth using bromocresol purple (0.04 g L^{-1}) as a pH indicator. Lactose, sucrose, galactose, mannose, maltose, melezitose, melibiose, and raffinose were tested as

[DOR: 20.1001.1.16807073.2011.13.4.13.2]

carbon sources. These were separately added to the medium, as filter sterilized solutions, to give a final concentration of 1% (w/v).

The presumptive assignation of the isolates into a *Lactobacillus* spp. was further verified by using API 50 CH test galleries (bioMerieux, Marcy l'Etoile, France).

Gram-positive and catalase-negative cocci were presumptively identified following Bridge and Sneath (1983) and Manero and Blanch (1999).

In particular, the following tests were carried out: growth ability at 10°C and 45°C in M17 broth after incubation for 5 and 2 days respectively; survival after heat treatment at 60.5°C for 30 minutes; growth capability in M17 broth at pH 9.6 and in the presence of 4.0 and 6.5% NaCl following incubation at 30°C for 2 days; esculin hydrolysis; arginine decarboxylase activity and reduction of methylene blue. Carbohydrates fermentation capability was assayed in lactose-free M17 using bromocresol purple (0.04 g L^{-1}) as a pH indicator. Lactose, *L*(-) xylose, sorbitol, melezitose, melibiose, D(-)arabinose, and D(+)cellobiose were tested as carbon sources. These were separately added to the medium, as filter sterilized solutions to give a final concentration of 1% (w/v).

The presumptive assignment of the isolates into a *Streptococcus* or *Enterococcus* spp. was further verified by using API 20 STREP test galleries (bioMerieux, Marcy l'Etoile, France).

Technological Characterization of Autochthonous LAB

The technological characterisation of LAB for cheese-making attributes involved single and mixed strain cultures. Growth kinetic and acidifying activity were evaluated according to the followings. Growth ability was assessed by inoculating each LAB culture in sterile whole ewes'milk at a rate of about 10^6 cells mL^{-1}. Viable counts of LAB were determined after 0, 2, 4, 6, and 24 hours of incubation at 42°C on M17 and MRS agar plates (Oxoid, Milan, Italy). The acidity (expressed as % of lactic acid) was determined at each stage of time by titrating 10 mL of the inoculated whole ewes' milk with 10N NaOH (phenolphthalein used as indicator).

Preparation of the Experimental Starter Culture and *Scotta-fermento*

The experimental mixed strain starter culture was prepared in the laboratory by mixing selected cocci (*Streptococcus thermophilus*) and lactobacilli (*Lactobacillus helveticus* and *L. delbrueckii* subsp. *lactis*) in a ratio of 3:1:1. All single cultures were preliminary grown separately in sterile ewes' milk at 42°C for 12 hours, then mixed and used to inoculate a suitable volume of sterile ewes' milk that was finally employed for the experimental cheese-making trials. In particular, each experimental trial was carried out using approximately 3,000 L of ewe's milk and the final starter culture being added at a 1% level (30 L). Before inoculation, a sample of the starter culture was collected to determine the viable LAB number which was always around $9\ log_{10}$ cfu mL^{-1}. Therefore, about $6\ log_{10}$ cfu of the starter culture were present per mL of inoculated milk. Such a number of LAB, added as starter culture, is usually sufficient to assure a suitable fermentative process (Pisano *et al.,* 2007).

The *scotta-fermento* was prepared at each dairy unit as follows: the *scotta* (which is the residual whey from the manufacturing of Ricotta) was initially incubated at 40°C to favour the development of the naturally present thermophilic LAB until the acidity reached 15-20 Soxhlet-Henkel (°SH). Aliquots of this natural LAB culture (1-1.5%) were used to inoculate fresh *scotta* which was then incubated at 45°C for 24 hours or until the acidity reached 30°SH. The *scotta-fermento* was added to the milk in control trial at the 0.4% level. Such an amount is slightly higher than that

[DOR: 20.1001.1.16807073.2011.13.4.13.2]

traditionally applied *i.e.*, between 0.2 and 0.25% (Bottazzi and Ledda, 1967).

Cheese-making and Sampling Procedure

Cheese-making trials were carried out at three dairies in Sardinia. To reduce the variation among batches the same lamb rennet paste (strength 1:10.000) was used and the rules for PDO Pecorino Romano cheese production were always carefully followed. At each experimental dairy unit, experimental and control cheese-making trials were carried out using 3,000 L each:

1. Experimental trial (E): Thermised (65°C for 17 s) ewes'milk + 1% experimental starter culture.
2. Control trial (C): Thermised (65°C for 17 seconds) ewes' milk+0.4% *scotta-fermento*.

Milk samples (10 mL) were collected from the vat after the addition of the experimental starter culture or *scotta-fermento*. Curd samples (10 g) were collected at moulding, while cheese samples were collected after 1, 8, 60, 150 and 240 days of ripening. Cheese samples (10 g) comprised portions of paste under the crust and in the internal part of the cheese. All the samples were carefully homogenized in 90 ml sterile Ringer's solution for 2 minutes in a Stomacher Lab Blender 80 (PBI, Milan, Italy) prior to microbiological and physicochemical analyses.

Microbiological Analyses

The following microbial groups were enumerated in experimental and control cheeses as follows: total colony count, on Plate Count Agar (Oxoid, Italy) at 37°C for 48 hours; mesophilic and termophilic streptococci, on M17 Agar (Terzaghi and Sandine, 1975), at 22°C for 72 hours and 45°C for 48 hours, respectively, in anaerobic conditions (Gas-Pack; Oxoid, Italy); mesophilic and termophilic lactobacilli, on MRS Agar (Oxoid, Italy) acidified to pH 5.4 at 22°C for 72 hours and 45°C for 48 hours, respectively, in anaerobic conditions (Gas-Pack; Oxoid, Italy); heterofermentative LAB in MRS broth with Durham tubes, 37°C for 24 hours (MPN method); staphylococci on Baird Parker Agar (Oxoid, Italy) supplemented with Egg Yolk Tellurite Emulsion (Oxoid, Italy) at 37°C for 48 hours; yeasts on Yeast Potato Dextrose Agar (YPDA), containing 1% w/v yeast extract, 2% w/v dextrose, 2% w/v peptone, 1.5% w/v agar, pH 4.5, at 25°C for 48 hours; faecal coliforms in Brilliant Green Bile Broth (Oxoid, Italy) at 44°C for 48 hours (MPN method); spores of sulphite-reducing clostridia, following heat treatment (80°C for 10 minutes) of the samples and inoculation on Differential Reinforced Clostridial broth (DRCM) (Oxoid, Italy), incubation at 37°C for 48 hours in anaerobic conditions (MPN method).

Physicochemical Analyses

Physicochemical analyses of experimental and control cheeses included: determination of total solids (IDF, 1982), ash (IDF, 1964), fat (IDF, 1986), NaCl (IDF, 1988) and pH (Balestrieri and Marini, 1996). Water activity (aw) was assessed using a Decagon Aqualab Cx-3 Water Activity System (Decagon Device Inc., Pullman, WA, USA), while total (TN), non-protein (NPN) and water-soluble nitrogen (WSN) were determined by Kjeldahl according to Bütikofer *et al*. (1993). All the results were expressed as g 100 g^{-1} of cheese (except for pH and water activity)

Lactose and lactic acid were determined by HPLC (St-Gelais *et al*., 1991). The instrument consisted of a quaternary pump (Ti-series), auto-sampler, degasser system and HP 1050 thermostated column compartment (Hewlett-Packard Co., Wilmington, DE, USA) with Waters 410 refraction index detector (Water Associates, Milford, MA, USA). A Supelcogel C-610H column (300x7.8 mm; Sigma-Aldrich Co., St. Louis, MO, USA) with Supelguard pre-column (50x4.6 mm; Sigma-Aldrich Co.)

[DOR: 20.1001.1.16807073.2011.13.4.13.2]

was used. The Mobile phase was 0.0049 N H_2SO_4 with a flow of 0.8 mL min^{-1}, 40°C. Data acquisition was carried out using HP CHEMSTATION Rev. A.06.03 software (Hewlett-Packard Co., Wilmington, DE, USA).

Free amino acids (FAAs) were extracted from the cheese as described by Aristoy and Toldrà (1991). Identification and quantification were carried out by an HP 1050 HPLC with an HP 1046A fluorescence detector (Hewlett-Packard Co., Wilmington, DE, USA) and HP Chemstation Rev. A.06.03 software (Hewlett-Packard Co.). Instrumental analytic conditions of derivation and quantification were as described by Gratzfeld-Hüesgen and Schuster (1999).

Free fatty acids (FFAs) were extracted from the cheese and determined through gas chromatography according to De Jong and Badings (1990). The procedure was also extensively described by Madrau *et al*. (2007).

Data Analysis

For each cheese-making trial all the microbial and chemical determinations were carried out in triplicate on 3 randomly selected experimental and control cheeses at each dairy. The results are expressed as mean values±standard deviation. Significant differences between mean values (experimental *vs.* control) were evaluated for each sampling time by One-way analysis of variance (One-way ANOVA) followed by a Student's *t*-test. Differences were considered significant at $P< 0.05$.

RESULTS AND DISCUSSION

Isolation and Selection of Autochthonous LAB from Pecorino Romano Cheese

A total of 169 Gram (+) and catalase (-) LAB cultures were isolated from the ewe's milk and Pecorino Romano cheese. Rods (*n*= 96) were separated in two different groups:

1. Rods growing at 45°C but not at 15°C, not fermenting pentoses and gluconate, and not producing gas from glucose were presumptively considered as homofermentative lactobacilli.

2. Rods growing at 15°C, fermenting pentoses, and not producing gas from glucose were presumptively considered as facultative heterofermentative lactobacilli.

Cocci (*n*= 73) were also separated into two groups:

1. Cocci growing at 45°C, but not at 10°C, on media containing 4.0% NaCl, at pH 9.6, not reducing 1% methylene blue, surviving after a heat treatment at 60.5°C for 30 min, not hydrolysing arginine and not decarboxylating esculine were presumptively considered as streptococci.

2. Cocci growing at 10°C and at 45°C, on media containing 6.5% NaCl, at pH 9.6, and hydrolysing esculine were presumptively considered as enterococci.

The further characterization of LAB isolates based on the utilization of different sugars (not shown) allowed a preliminary identification of the following species: *Lactobacillus delbrueckii* subsp. *lactis*, *Lb. helveticus* and *Lb. casei* subsp. *casei, Streptococcus thermophilus, Enterococcus faecium* and *E. faecalis.*

LAB species identification was further confirmed by determining the carbohydrate fermentation patterns through the API 20 STREP and API 50 CHL test galleries.

The identity of LAB cultures isolated from thermised milk and Pecorino Romano cheese are reported in Table 1. *Streptococcus thermophilus*, often recovered from the cheese-making environments (Marino *et al*., 2003), was the predominant species. This was likely due to its higher resistance, compared to other LAB, to heat-treatments (Catzeddu *et al*., 1995) commonly employed in the Pecorino Romano manufacturing (milk thermisation at 68°C and curd cooking at 48°C). The thermophilic rods *Lactobacillus delbrueckii* subsp. *lactis* e *Lb. helveticus* were isolated

[DOR: 20.1001.1.16807073.2011.13.4.13.2]

Table 1. LAB species isolated from milk, curd and Pecorino Romano cheese during ripening.

Isolated species	Milk	Curd	Ripening time (Days)			
			5	30	90	240
Streptococcus thermophilus	3	10	12	10	15	1
Lactobacillus casei subsp. *casei*	1	0	0	1	3	2
Lactobacillus helveticus	2	6	10	12	5	1
Lactobacillus delbrueckii subsp. *lactis*	2	4	12	9	8	2
Lactobacillus spp.	2	3	4	3	3	1
Enterococcus faecalis	1	1	1	2	2	1
Enterococcus faecium	2	1	2	3	3	3

from both thermised milk and cheese at different ripening stages. Their ratio during ripening was quite constant. Thermophilic lactobacilli are a common microbial component of the *scotta-fermento* (Gobbetti and Di Cagno, 2002a) and *siero-fermento* which are natural cultures used for the production of hard paste cheese from ewe's (Albezio *et al*., 2001) and cow's milk (Cocconcelli *et al*., 1996; Lazzi *et al*., 2004). *Lactobacillus casei* subsp. *casei* was recovered in lower numbers from thermised milk and cheese at 30 days of ripening (Table 1). *Lactobacillus casei* subsp. *casei* is most commonly isolated from raw ewes' milk cheeses (De Angelis *et al*., 2001; Randazzo *et al*., 2006; Mangia *et al*., 2008) and is often used as adjunct starter culture to accelerate cheese ripening, to produce desirable flavours, and to avoid possible cheese defects caused by adventitious non starter LAB (Khedid *et al*., 2009).

Enterococcus faecium and *E. faecalis* were recovered in milk and during the entire cheese ripening thus confirming their superior ability to grow under stress conditions such as high temperature and high sodium chloride concentrations (Giraffa, 2003). Enterococci can play a significant role in cheese ripening since they are involved in the proteolysis process and in the production of aromatic compounds. For instance, *Enterococcus faecalis* is of the capacity to ferment citrate as the sole carbon source producing aromatic compounds such as diacetyl and acetaldehyde (Moreno *et al*., 2006).

In order for LAB starters to properly function, some attributes are of utmost importance: LAB starters should be able to outnumber the natural microflora and rapidly acidify the milk hence influencing the structural and rheological properties of cheese. For this reason, LAB isolates belonging to the species most frequently recovered from milk and cheese, i.e. *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *lactis* and *Lb. helveticus*, were further characterized to ascertain their growth ability as well as acidifying activity (data not shown). Based on these parameters, *Streptococcus thermophilus* L1, *Lactobacillus delbrueckii* subsp. *lactis* Ld10 e *Lb. helveticus* Lh5 were found to be the most suitable cultures when used either as single or mixed strains (Tables 2 and 3). For this reason they were used in the experimental starter culture preparation. *St. thermophilus* L1 grew faster compared to lactobacilli, producing, after 4 hours of incubation, 0.53% of lactic acid. However, the extent of lactic acid produced by this latter strain after 24 hours of incubation (0.85%) was lower than that produced after 24 hours by *Lactobacillus delbrueckii* subsp. *lactis* Ld10 (0.91%) and *Lb. helveticus* Lh5 (1.75%). These findings are in agreement with previous investigations carried out by different authors (Sagdic *et al*., 2002; Bottazzi and Ledda, 1967). Mixed cultures of *St. thermophilus* L1 and *Lb. delbrueckii* subsp. *lactis* Ld10 showed an increased acidification ability compared to the respective single cultures, starting from 4 hours of incubation (Table 2). However, this

[DOR: 20.1001.1.16807073.2011.13.4.13.2]

Table 2. Growth and acidification kinetics of Single (S) and Associated (A) *Streptococcus thermophilus* L1 and *Lactobacillus delbrueckii* subsp. *lactis* Ld10 strains (Average of three batches).

Time (h)	L1 (S) Log cfu mL^{-1}	L1 (A) Log cfu mL^{-1}	Ld10 (S) Log cfu mL^{-1}	Ld10 (A) Log cfu mL^{-1}	Lactic acid L1 (S) %	Lactic acid Ld10 (S) %	Lactic acid (A) %
0	6.10	6.26	6.42	6.00	0.17	0.17	0.17
2	7.40	7.38	7.70	6.83	0.26	0.17	0.25
4	8.64	8.62	7.41	7.36	0.53	0.38	0.60
6	8.10	8.98	7.81	8.28	0.61	0.42	0.79
24	8.34	7.79	8.26	8.00	0.85	0.91	1.62

Table 3. Growth and acidification kinetics of Single (S) and Associated (A) *Streptococcus thermophilus* L1 and *Lactobacillus helveticus* Lh5 strains (Average of three batches).

Time (h)	L1 (S) Log cfu mL^{-1}	L1 (A) Log cfu mL^{-1}	Lh5 (S) Log cfu mL^{-1}	Lh5 (A) Log cfu mL^{-1}	Lactic acid L1 (S) %	Lactic acid Lh5 (S) %	Lactic acid (A) %
0	6.10	6.26	6.34	6.41	0.17	0.17	0.17
2	7.40	7.00	6.76	7.25	0.31	0.22	0.14
4	8.64	7.45	7.24	7.67	0.53	0.55	0.22
6	8.10	8.18	8.71	7.52	0.61	1.20	0.85
24	8.34	8.00	7.95	8.12	0.85	1.75	1.42

was not the case for the mixed cultures of *St. thermophilus* L1 and *Lb. helveticus* Lh5, since *Lb. helveticus* in single culture was able to produce a significantly higher level of lactic acid (1.75%) after 24 hours of incubation. This superior acidifying ability of the *Lb. helveticus* single culture is inconsistent with previous findings (Badis *et al*., 2004; Sanna *et al*., 2005). More generally, the acidifying ability of *Lb. helveticus* seems more strain dependent than species dependent and this can explain the isolation of good and poor acidifying strains.

Microbiological and Physicochemical Properties of Control and Experimental Pecorino Romano Cheese

Microbiological parameters of experimental *vs.* control cheeses are reported in Table 4. Globally, the total microbial counts were significantly higher ($P< 0.05$) in experimental cheese as compared to the control for all the sampling stages (except for 8 and 240 days of ripening).

In both control and experimental cheeses the highest presence of thermophilic cocci and lactobacilli was recorded after one and sixty days of ripening, respectively. The number of thermophilic LAB in experimental cheese was substantially higher as compared to control cheese suggesting a good suitability of the starter culture. On this account, thermophilic cocci showed a significant growth rate their number being within the order of *9.70 Log* cfu g^{-1} past one day. The number of thermophilic cocci in the experimental cheese was always significantly higher ($P< 0.05$) in comparison with control until 60 days of ripening. A possible poor presence of lactic acid bacteria within the *scotta-innesto* (that was used in control cheese-making trials) led to the proliferation of such spoilage microrganisms as faecal coliforms whose number increased significantly ($P< 0.05$), compared to the experimental cheese, up to the 8^{th} day of ripening reaching, values

[DOR: 20.1001.1.16807073.2011.13.4.13.2]

of 460 MPN per g of cheese. Such a large number of coliforms may be responsible for significant cheese holes (Mangia *et al*., 2008). The evolution of the mesophilic microflora was similar for both cheese-making trials. Mesophilic LAB reached the highest numbers after 1 day of ripening since the number of mesophilic lactobacilli was likely influenced by heat treatment of milk. Although several of these microorganisms may be able to survive at pasteurization temperatures, milk thermisation (68°C per 15-17 seconds) can reduce their subsequent growth rate. The finding that the mesophilic microflora of Pecorino Romano manufactured from raw milk is more abundant than thermophilic species supports this hypothesis (Di Cagno *et al*., 2003). Globally, the number of lactic acid bacteria in the curd and in the young experimental cheeses reflected a good fermentative ability since lactose was readily metabolized during cheese-making and was no more detectable in cheeses at one day of ripening. This led to a more rapid and significant ($P< 0.05$) accumulation of lactic acid and to a pH drop in the experimental cheeses (Table 5). Eight days past ripening, these differences were not significant.

Yeasts were recovered in low number only from milk and curd. These microorganisms can play a significant role during cheese ripening. Deiana *et al*. (1984) showed that Pecorino Romano manufactured by use of *Debaryomyces hansenii* was characterized by improved sensory features likely because of the hydrolytic activity of the yeasts on proteins and fats that eventually led to an accelerated ripening. In other studies the same yeast species was employed as a starter microorganism to inhibit butyric clostridia thus preventing the late cheese swelling (Fatichenti *et al*., 1983). Staphylococci and clostridial spores were absent, or present at very low numbers in both experimental and control Pecorino Romano cheeses.

Physicochemical parameters of experimental and control cheeses are presented in Table 5. No significant differences were observed, except for lactic acid and pH: total solids (TS) and ash increased during ripening while total fat content increased until 60 days of ripening and remained constant afterwards. The slow increase of the ripening index (the WSN to TN ratio) can be attributed to the high NaCl concentration detected after two months of ripening for both experimental and control cheeses. This may clearly have had a negative influence on the rate of proteolysis during ripening (Di Cagno *et al*., 2003). The NPN/TN ratio that reflects the evolution of nitrogenous compounds, increased constantly up to the 5th month of ripening likely indicating the accumulation of free amino acids (FAAs) until this time (Table 6). This finding is different from those from other studies concerning ripened cheese manufactured from sheep milk in which FAAs content globally increase throughout the entire ripening (Izco *et al*., 2000; Mangia *et al*., 2008). After the 5th month FAAs content of experimental and control Pecorino Romano cheeses decreased. It should be mentioned that the NaCl content of Pecorino Romano is much greater than that present in the above mentioned cheeses and this could have influenced the proteolysis and FAAs release. Freitas and Malcata (1996) reported that the degradation of beta-casein by chymosin was either strongly or completely inhibited by 5% (w/w) and 10% (w/w) NaCl, respectively.

In all Pecorino Romano cheeses (experimental and control) leucine, isoleucine, valine, phenylalanine, tyrosine and lysine were the most abundant FAAs at 150 days of ripening, representing approximately 50% of total amino acids detected in both experimental and control cheeses (Table 6). These amino acids have been reported by different authors as the most abundant in other cheeses from sheep milk (Barcina *et al*., 1995; Freitas *et al*., 1998). After 150 days of ripening the content of several FAAs such as glutamic acid and lysine decreased, with the latter decreasing from 58.98 to 9.44 mg per 100 g (average contents between E and C cheeses). A similar trend for lysine was observed in

[DOR: 20.1001.1.16807073.2011.13.4.13.2]

Table 4. Evolution of microbial groups (Log cfu g^{-1} or mL^{-1}) in Experimental (E) and Control (C) Pecorino Romano cheese. For each sampling time asterisks denote significant differences between Experimental (E) and Control (C) cheese according to Student's *t*-test (P< 0.05).

	Ripening time													
	Milk		Curd		1 day		8 days		60 days		150 days		240 days	
Microbial groups	E	C	E	C	E	C	E	C	E	C	E	C	E	C
Total Colony count	5.54	5.54	6.92*	5.41	8.43*	7.84	8.33	8.30	8.98*	7.85	7.34*	5.48	6.38	6.18
Mesophilic lactobacilli	2.95	2.95	6.28*	5.11	8.45*	7.81	7.02*	8.30	7.47*	8.81	7.11	7.51	6.85*	5.88
Thermophilic lactobacilli	4.01	4.01	6.11	6.74	8.18	8.41	8.48*	7.90	9.36*	8.90	8.95*	7.43	7.90*	5.89
Mesophilic streptococci	4.26	4.26	4.20	4.11	6.43	5.70	6.70	7.38	7.36*	6.09	6.00	6.48	6.85*	4.96
Thermophilic streptococci	4.70	4.70	6.94*	5.78	9.70*	8.89	8.69*	7.00	7.13*	6.05	6.94	6.53	5.30	5.00
Heterofermentative LAB[a]	28	23	<3	11	11	11	<3	<3	<3	<3	<3	<3	<3	<3
Feacal coliforms[a]	<3	<3	9	20	<3*	20	<3*	460	<3	<3	<3	<3	<3	<3
Staphylococci	<1	<1	3.63	4.40	2.19	3.11	2.60	3.11	2.95	2.68	<1	<1	<1	<1
Sulphite–reducing Clostridial spores[a]	<3	<3	<3	<3	4	<3	<3	<3	<3	<3	<3	<3	<3	<3
Yeasts	1.36	1.36	1.60*	2.95	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1

[a] MPN method.

Table 5. Evolution of physicochemical parameters of Experimental (E) and Control (C) Pecorino Romano cheese (Mean value±Standard deviation). Within each sampling time asterisks denote significant differences between Experimental (E) and Control (C) cheese according to Student's *t*-test (P< 0.05).

		NaCl	TS[a]	Ash	Fat	Lactose	Lactic acid	WSN/TN[b]	NPN/TN[c]	pH	a_W
Curd	E	0.10 ± 0.00	53.63 ± 1.89	2.55 ± 0.13	25.35 ± 0.65	1.44 ± 0.05	0.33* ± 0.09	0.15 ± 0.00	0.03 ± 0.00	6.27* ± 0.22	0.97 ± 0.01
	C	0.10 ± 0.00	52.10 ± 4.51	2.55 ± 0.35	24.98 ± 0.38	1.47 ± 0.06	0.30 ± 0.12	0.14 ± 0.00	0.04 ± 0.00	6.34 ± 0.17	0.98 ± 0.00
1 day	E	0.10 ± 0.00	59.98 ± 0.19	2.78 ± 0.15	29.05 ± 1.50	0.00 ± 0.00	1.68* ± 0.03	0.20 ± 0.02	0.04 ± 0.00	4.97* ± 0.11	0.98 ± 0.00
	C	0.10 ± 0.00	60.20 ± 0.79	2.88 ± 0.26	28.85 ± 1.63	0.00 ± 0.00	1.64 ± 0.05	0.17 ± 0.00	0.04 ± 0.00	5.04 ± 0.09	0.98 ± 0.00
8 days	E	1.25 ± 0.17	62.43 ± 0.85	3.55 ± 0.29	30.10 ± 1.06	0.00 ± 0.00	1.58* ± 0.10	0.19 ± 0.00	0.08 ± 0.00	5.18 ± 0.18	0.95 ± 0.00
	C	1.05 ± 0.35	63.43 ± 2.00	4.15 ± 0.64	29.68 ± 1.51	0.00 ± 0.00	1.53 ± 0.10	0.19 ± 0.00	0.07 ± 0.00	5.13 ± 0.03	0.96 ± 0.01
60 days	E	5.03 ± 0.15	67.73 ± 1.51	7.38 ± 0.73	31.60 ± 1.45	0.00 ± 0.00	1.50 ± 0.12	0.27 ± 0.01	0.12 ± 0.01	5.39 ± 0.09	0.88 ± 0.02
	C	4.60 ± 0.29	67.90 ± 2.38	7.23 ± 0.49	31.03 ± 1.40	0.00 ± 0.00	1.50 ± 0.12	0.25 ± 0.03	0.09 ± 0.02	5.43 ± 0.03	0.88 ± 0.00
150 days	E	5.53 ± 0.21	68.03 ± 1.47	8.13 ± 0.49	30.73 ± 1.14	0.00 ± 0.00	1.55 ± 0.06	0.29 ± 0.02	0.13 ± 0.01	5.41 ± 0.14	0.86 ± 0.00
	C	5.16 ± 0.21	68.34 ± 0.65	8.06 ± 0.72	30.89 ± 1.07	0.00 ± 0.00	1.56 ± 0.05	0.27 ± 0.02	0.11 ± 0.00	5.40 ± 0.02	0.86 ± 0.01
240 days	E	5.70 ± 0.93	70.40 ± 0.35	8.48 ± 0.78	30.23 ± 0.47	0.00 ± 0.00	1.61 ± 0.07	0.29 ± 0.04	0.13 ± 0.02	5.38 ± 0.23	0.83 ± 0.01
	C	5.95 ± 1.29	70.20 ± 0.92	8.48 ± 1.24	30.90 ± 0.73	0.00 ± 0.00	1.54 ± 0.07	0.28 ± 0.03	0.12 ± 0.02	5.35 ± 0.23	0.83 ± 0.02

Except for pH and water activity the remaining parameters are expressed as g 100 $g^{-1}$of cheese.
[a] Total solids; [b] Water soluble nitrogen/Total nitrogen, [c] Non-protein nitrogen/Total nitrogen.

Table 6. Changes in the free amino acids (FAAs) composition in Experimental (E) and Control (C) cheese during ripening (Mean value of three batches expressed as mg 100 g^{-1} of cheese). Within each sampling time asterisks denote significant differences between Experimental (E) and Control (C) cheese according to Student's *t*-test (P< 0.05).

FAA	Ripening time											
	Curd		1 day		8 days		60 days		150 days		240 days	
	E	C	E	C	E	C	E	C	E	C	E	C
ASP	0.41*	0.19	2.92	2.24	4.78*	2.21	1.89	1.58	5.34	4.71	0.54*	1.05
GLU	1.81	1.15	4.79	5.01	9.73*	17.45	9.49*	27.64	29.08*	34.25	7.00*	14.43
ASN	0.24	N.D.	2.78	1.50	17.26*	9.18	19.90	18.99	24.96	21.73	14.15	14.51
SER	0.18	0.11	3.07	3.17	9.15	6.66	10.23	8.40	15.62	15.45	7.80	8.08
GLN	0.36	0.23	2.92	2.39	15.98	11.71	19.14	19.45	27.93	27.50	9.17	11.63
HIS	0.48	0.45	2.69	2.60	6.59	4.66	7.52	5.32	12.73	11.58	9.62	11.98
GLY	0.50	0.27	1.44	1.03	3.37	2.07	5.81	4.71	9.33	8.61	4.56	5.29
THR	0.14	N.D.	2.43	2.31	7.85	5.54	8.12	8.29	15.17	13.09	6.21	7.45
ALA	2.03	1.64	3.49*	6.47	12.22	11.94	14.53	15.43	19.41	17.97	16.11	15.45
ARG+GABA	N.D.	N.D.	4.93*	0.58	21.73*	11.54	22.21*	3.44	40.75*	14.18	24.02	N.D.
TYR	0.85	0.61	4.89*	3.69	17.91	12.55	62.53	61.15	60.89	56.10	47.24	54.33
CYS-CYS	1,95	2.11	2.71	3.10	4.44	3.55	6.15	6.62	8.33	9.42	N.D.	N.D.
VAL	0.65*	0.31	10.87	7.04	36.40	25.44	61.96	53.88	70.54	61.63	75.41	70.37
MET	1.59	3.59	4.27*	2.86	11.27	9.13	23.52	23.47	29.56	29.41	42.78	41.64
TRP	N.D.	N.D.	0.90	0.78	1.71	1.17	3.13	2.75	3.79	3.05	4.58	4.72
PHE	0.16	0.18	4.71*	2.95	21.71*	11.11	60.52	51.69	63.59	54.93	74.30	64.10
ILE	0.20*	0.09	4.59	3.50	17.03	11.68	42.27	40.10	70.65	63.25	92.73	86.33
ORN	N.D.	N.D.	3.28	4.99	5.07*	12.74	4.24*	13.15	6.42*	13.56	5.99	6.31
LEU	0.91*	0.44	10.09*	6.71	40.26*	24.55	96.58	85.09	108.89*	83.23	153.24*	134.22
LYS	0.33*	0.67	8.37	7.63	30.56	24.48	39.75	34.37	59.33	58.64	8.19	11.69
PRO	1.51	1.10	10.93	10.02	23.75	16.58	39.83	29.96	58.32	50.38	80.10	76.58
Σ FAA	14.29	13.12	97.06	80.55	318.74*	235.92	559.31	515.44	740.60*	652.66	683.70	640.13

Fiore Sardo cheese after 90 days of ripening although this cheese is substantially different from Pecorino Romano, being manufactured with raw milk and characterized by uncooked curd and a mesophilic microflora (Mangia *et al*., 2008). The general decrease in FAAs during the last part of the ripening is difficult to explain since the content of FAAs in other ripened cheeses commonly increases during maturation (Freitas *et al*., 1998; Madrau *et al*., 2006; Barcina *et al*., 1995). These results also indicate that the cheese-making conditions may exert a notable, though indirect, influence on the degradation of nitrogenous compounds, through their effect on cheese microflora. Lactic acid bacteria can metabolize lysine and other FAAs to produce aromatic compounds (Tavaria *et al*., 2002) whereas several authors showed the influence of different technological parameters on lysine (Marino *et al*., 2003) and FAAs (Marino *et al*., 2008) decarboxylation. Proline was detected in all Pecorino Romano cheeses and interestingly its concentration was significantly higher than that detected in Pecorino Romano cheese manufactured using raw milk (Di Cagno *et al*., 2003).

Overall the amino acid profile of experimental and control cheeses did not change significantly with the exception of arginine (ARG)+gamma-aminobutyric (GABA), glutamic acid and leucine. The first two amino acids (ARG+GABA) were found at higher levels in the experimental cheese as compared to the control ($P< 0.05$), whereas the opposite was true ($P< 0.05$) for the glutamic acid starting from 8 days of ripening. Leucine content was significantly ($P< 0.05$) higher in experimental *vs.* control cheeses throughout the whole ripening time (Table 6). These differences in the FAAs content are likely due to a different enzymatic activity of the selected starter culture compared to the control (*scotta-fermento*).

The evolution of free fatty acids (FFAs) during the ripening of Pecorino Romano is presented in Table 7. In general, the FFAs content increased progressively during ripening especially in the experimental cheeses, even if no significant differences were detected among experimental and control cheeses. At the end of the ripening, oleic (C18:1), palmitic (C16:0), butyric (C4), stearic (C18:0), and myristic (C14:0) acids were the more abundant FFAs. Similarly, these FFAs were also the more abundant in other hard paste cheeses manufactured from sheep milk such as Fiore Sardo (Mangia *et al*., 2008) and Pecorino Sardo (Madrau *et al*., 2006). A notable content of butyric acid was detected during the entire ripening and this was likely due to the activity of the pre-gastric lipases contained in the lamb rennet paste (Mucchetti and Neviani, 2006) as well as to the activity of the LAB lipases that preferentially release short chain fatty acids (Prieto *et al*., 2002). Linoleic (C18:2) and linolenic acids (C18:3) were not detected during the first days of ripening and at 240 days they were present only in very small quantities. Moreover, their content in the experimental cheeses was slightly higher as compared to the control. Overall, the level of FFAs detected in Pecorino Romano at the end of the ripening was largely lower in comparison with other PDO cheeses from sheep milk (Gobbetti *et al*., 1999; Larráyoz *et al*., 1999; Fernàndez-Garcìa *et al.*, 2006). It is widely recognized that such different lipases as milk, microbial and rennet lipases are the main agents responsible for the lypolitic processes in cheese (Catalano *et al*., 1985). The reduced lypolisis in Pecorino Romano could be due to the influence of heat treatments (Driessen, 1989; Atasoy and Tűrkoğlu, 2008) and/or high NaCl concentration (Atasoy and Tűrkoğlu, 2008; Gobbetti *et al*., 1999) on the lipase activity. Altogether, these results indicate that both proteolytic and lypolitic activities in Pecorino Romano cheese are likely influenced by the NaCl content. Accordingly, Freitas and Malcata (1996) demonstrated that the FFAs and FAAs content of Picante cheese was globally higher in cheeses of a lower NaCl content.

[DOR: 20.1001.1.16807073.2011.13.4.13.2]

Table 7. Changes in the free fatty acid (FFAs) composition in Experimental (E) and Control (C) cheese during ripening (Mean value of three batches expressed as mg 100 g^{-1} of cheese). For each sampling time no significant differences were detected between Experimental (E) and Control (C) cheeses according to Student's *t*-test (P< 0.05).

	Ripening time											
	Curd		1 day		8 days		60 days		150 days		240 days	
FFA	E	C	E	C	E	C	E	C	E	C	E	C
C4	8.63	12.86	7.51	6.01	15.18	15.71	35.14	33.47	39.05	36.68	48.81	48.89
C6	2.64	5.68	5.87	2.74	5.19	5.45	13.14	11.53	18.26	13.45	19.39	21.63
C8	1.97	5.02	1.33	1.71	2.93	2.94	7.96	6.90	10.66	5.98	13.70	14.63
C10	8.14	20.35	10.93	8.99	14.05	13.34	28.73	22.31	36.44	27.57	38.62	44.73
C12	7.57	14.42	10.90	9.38	11.53	10.61	17.42	15.82	22.75	19.65	24.72	26.74
C14:0	6.39	9.66	9.96	8.29	12.27	12.25	22.88	21.58	30.99	25.14	36.87	41.04
C16:0	14.24	18.46	26.09	22.29	31.76	31.20	53.48	52.12	72.45	57.40	84.39	90.25
C18:0	15.61	17.41	20.41	18.06	21.94	19.48	27.71	26.02	32.31	29.40	40.11	36.41
C18:1	20.17	32.65	29.93	22.92	32.72	31.94	59.53	52.11	79.55	62.22	97.48	96.64
C18:2	N.D.	N.D.	N.D.	N.D.	11.72	11.35	14.63	9.72	17.15	13.89	22.59	20.85
C18:3	N.D.	N.D.	N.D.	N.D.	2.70	4.53	8.35	7.28	10.76	9.04	10.63	9.24
ΣFFA	85.35	136.49	122.92	100.38	161.97	158.79	288.96	258.86	370.35	300.42	437.30	451.03

CONCLUSIONS

The results obtained in this work revealed that the Pecorino Romano cheese microflora was mainly represented by thermophilic lactic species and that mesophilic LAB were present as well, though in lower numbers. The number of LAB in the fermentative phase and in the first part of the ripening stage was significantly higher in experimental cheeses, compared to control, thus suggesting the efficacy of the selected starter culture used for the manufacturing of Pecorino Romano on an industrial scale. After 60 days, cheese ripening proceeded slowly in either one of the experimental or control cheeses. Some technological peculiarities, such as the high NaCl concentration and the resulting low water activity (a_w) are likely responsible for the reduced lypolisis and proteolysis during ripening. Hence, the use of a reduced amount of NaCl in the Pecorino Romano cheese-making together with the use of autochthonous selected starter cultures will likely allow the improvement of the physicochemical features of this PDO cheese, while maintaining its traditional traits.

ACKNOWLEDGEMENTS

This work was financially supported by the Italian Government, Ministero dell'Università e della Ricerca Scientifica e Tecnologia (MURST), research Project "Microrganismi a Agenti Infettivi di Interesse Agro-Alimentare-cluster C06+07, biotecnologie Lattiero casearie".

REFERENCES

1. Albenzio, M., Corbo, M. R., Rehman, S. U., Fox, P. F., De Angelis, M., Corsetti, A., Sevi A. and Gobbetti, M. 2001. Microbiological and Biochemical Characteristics of Canestrato Pugliese Cheese Made from Raw Milk, Pasteurized Milk or by Heating the Curd in Hot Whey. *Int. J. Food Microbiol*., **67:** 35-48.
2. Aristoy, M. C. and Toldrà, F. 1991. Deproteinization Techniques for HPLC Amino Acid Analysis in Fresh Pork Muscle and Dry-cured Ham. *J. Agric. Food Chem*., **39**: 1792-1795.
3. Arrizza, S. 1972. Caratteristiche Microbiologiche Dello Scotta Fermento. *Sci. Tec. Latt. Cas*., **23**: 226-230.
4. Assolatte, 2004. *Associazione Italiana Lattiero-Casearia*. Milan, Italy. http://www.assolatte.it/
5. Atasoy, A. F. and Tűrkoğlu, H. 2008. Changes of Composition and Free Fatty Acid Contents of Urfa Cheeses (A White-brined Turkish Cheese) during Ripening: Effects of Heat Treatments and Starter Cultures. *Food Chem*., **110**: 598-604.
6. Badis, A., Guetarni, D., Moussa-Boudjemaa, B., Henni, D. E., Tornadijo, M. E. and Kihal, M. 2004. Identification of Cultivable Lactic Acid Bacteria Isolated from Algerian Raw Goat's Milk and Evaluation of Their Technological Properties. *Food Microbiol*., **21**: 343-349.
7. Balestrieri, F. and Marini, D. 1996. *Metodi di Analisi Chimica dei Prodotti Alimentari*. Monolite Publishers, Rome, Italy. PP?
8. Barcina, Y., Ibáñez, F. C. and Ordóñez, A. I. 1995. Evolution of Free Amino Acids during Idiazábal Cheese Ripening. *Food Control*, **6**: 161-164.
9. Bottazzi, V. and Ledda, A. 1967. Studio Sulla Microbiologia Dello Scotta-fermento Usato Nella Produzione del Pecorino "Romano". *Ann. Microbiol. Enzimol*., **17**: 41-53.
10. Bridge, P. D. and Sneath, P. H. A. 1983. Numerical Taxonomy of *Streptococcus*. *J. General Microbiol*., **129**: 565-597.
11. Bütikofer, M., Rüegg, M. and Ardö, Y. 1993. Determination of Nitrogen Fractions in Cheese: Evaluation of a Collaborative Study. *Lebensm. Wiss. Technol*., **26**: 271-275.
12. Catalano, M., De Felice, M. and Gomes T. 1985. Influenza Della Frazione Lipidica Sulla Qualità dei Formaggi. *Il Latte*, **10**: 936-943.
13. Catzeddu, P., Deiana, P., Blaiotta, G. and Usai, S. 1995. Ecologia Dello *Streptococcus Thermophilus* e Indagine sul Genotipo, Biodiversità Microbica. Aspetti Tassonomici, Biotecnologici e

[DOR: 20.1001.1.16807073.2011.13.4.13.2]

Metodologici., 26-27 Ottobre, Reggio Emilia, Milan, Italy, Tecnos.
14. Cocconcelli, P. S, Parisi, M. G., Senini, L. and Bottazzi, V. 1996. Use a RAPD and 16S rDNA Sequencing for the Study of *Lactobacillus* Population Dynamic in Natural Whey Culture. *Lett. Appl. Microbiol*., **25**: 8-12.
15. De Angelis, M., Corsetti, A., Tosti, N., Rossi, J., Corbo, M. R. and Gobbetti, M. 2001. Characterization of Non-starter Lactic Acid Bacteria from Italian Ewe Cheeses Based on Phenotypic, Genotypic, and Cell Wall Protein Analysis. *Appl. Environ. Microbiol*., **67**: 2011-2020.
16. De Jong, C. and Badings, H. T. 1990. Determination of Free Fatty Acids in Milk and Cheese: Procedures for Extraction, Clean up, and Capillary Gas Chromatographic Analysis. *J. High Resol. Chromatogr*., **13**: 94-98.
17. Deiana, P., Fatichenti, F., Farris, G. A., Lodi, R., Mocquot, G., Todesco, R. and Cecchi, L. 1984. Metabolization of Lactic and Acetic Acids in Pecorino Romano Cheese Made with a Combined starter of Lactic Acid Bacteria and Yeast. *Le Lait*, **64:** 380-394.
18. Deiana, P., Rossi, J., Caredda, M., Cattina, A. P. and Smacchi, E. 1997. La Microflora Secondaria del Formaggio Pecorino Romano. *Sci. Tec. Latt. Cas*., **48(6)**: 487-500.
19. De Man, J. D., Rogosa, M. and Sharpe, M. E. 1960. A Medium for the Cultivation of Lactobacilli. *J. Appl. Bacteriol*., **23**: 130-135.
20. Di Cagno, R., Banks, J., Sheehan, L., Fox, P. F., Brechany, E. Y., Corsetti, A. and Gobbetti, M. 2003. Comparison of the Microbiological, Compositional, Biochemical, Volatile Profile and Sensory Characteristics of Three Italian PDO Ewes' Milk Cheeses. *Int. Dairy J*., **13**: 961-972.
21. Driessen, F. M. 1989. Heat-Induced Changes in Milk. *Bulletin of International Dairy Federation 238*. Brussels, Belgium: IDF.
22. Fatichenti, F., Bergere, J. L., Deiana, P. and Farris, G. A. 1983. Antagonistic Activity of *Debaryomyces hansenii* towards *Clostridium tyrobutyricum* and *Clostridium butyricum*. *J. Dairy Res*., **50**: 449-457.
23. Fernàndez-Garcìa, E., Carbonell, M., Calzada, J. and Nunez, M. 2006. Seasonal Variation of the Free Fatty Acids Contents of Spanish Ovine Milk Cheeses Protected by a Designation of Origin: A Comparative Study. *Int. Dairy J*., **16**: 252-261.
24. Freitas, A. C., Fresno, J. M., Prieto, B., Franco, I., Malcata, F. X. and Carballo, J. 1998. Influence of Milk Source and Ripening Time on Free Amino Acid Profile of Picante Cheese. *Food Control*, **9**: 187-194.
25. Freitas, A. C. and Malcata, F. X. 1996. Influence of Milk Type, Coagulant, Salting Procedure and Ripening Time on the Final Characteristics of Picante Cheese. *Int. Dairy J*., **6**: 1099-1116.
26. Giraffa, G. 2003. Functionality of *Enterococci* in Dairy Product. *Int. J. Food Microbiol*., **88**: 215-222.
27. Gobbetti, M., Lanciotti, R., De Angelis, M., Corbo, M. R., Massini, R. and Fox, P. 1999. Study of the Effects of Temperature, pH, NaCl, and a_w on the Proteolytic and Lipolytic Activities of Cheese-related Lactic Acid Bacteria by Quadratic Response Surface Methodology. *Enzyme Microb. Technol*., **25**: 795-809.
28. Gobbetti, M. and Di Cagno, R. 2002a. Cheese: Hard Italian Cheeses. In: "*Encyclopedia of Dairy Sciences*". (Eds.): Roginski, H., Fuquai, J. W. and Fox, P. F., Elsevier Science Ltd, London, PP. 378–385.
29. Gobbetti, M., Folkertsma, B., Fox, P. F., Corsetti, A., Smacchi, E., De Angelis, M., Rossi, J., Kilcawley, K. and Cortine, M. 1999. Microbiology and Biochemistry of Fossa (Pit) Cheese. *Int. Dairy J*., **9**: 763-773.
30. Gratzfeld-Huesgen, A. 1999. *Sensitive and Reliable Amino Acids Analysis in Protein Hydrolisates Using the Agilent 1100 Series HPLC*. Technical Note by Agilent Technologies, Publication Number 5968-5658E. Available from: http://www.agilent.com/chem
31. IDF. 1964. *Determination of the Ash Content of Processed Cheese Products*. Standard 27:1964. International Dairy Federation, Brussels, Belgium.
32. IDF. 1982. *Cheese and Processed Cheese: Determination of the Total Solid Content* (Reference Method). Standard 4A:1982. International Dairy Federation, Brussels, Belgium.
33. IDF. 1986. *Cheese and Processed Cheese Products: Determination of Fat Content.* Gravimetric Method (Reference Method).

[DOR: 20.1001.1.16807073.2011.13.4.13.2]

Standard 5B:1986. International Dairy Federation, Brussels, Belgium.
34. IDF. 1988. *Cheese and Processed Cheese Products: Determination of Chloride Content* (Potentiometric Titration Method). Standard 88A:1988. International Dairy Federation, Brussels, Belgium.
35. Kandler O. and Weiss, N. 1986. Regular, Non-spore-forming Gram-positive Rods. In: *"Bergey's Manual of Systematic Bacteriology*". (Eds.): Sneath, P. H. A., Mair, N. S., Sharpe, M. E. and Holt, J. G., Williams & Wilkins, Baltimore, **2**: 1208–1234.
36. Khedid, K., Faid, M., Mokhtari, A., Soulaymani, A. and Zinedine, A. 2006. Characterization of Lactic Acid Bacteria Isolated from the One Humped Camel Milk Produced in Morocco. *Microbiol. Res*., **164:** 81-91.
37. Izco, J. M., Torre, P. and Barcina, Y. 2000. Ripening of Ossau-Iraty Cheese: Determination of Free Amino Acids by RP-HPLC and of Total Free Amino Acids by the TNBS Method. *Food Control,* **11**: 7-11.
38. Larráyoz, P., Martínez, M. T., Barrón, L. J. R., Torre, P. and Barcina, Y. 1999. The Evolution of Free Fatty Acids during the Ripening of Idiazábal Cheese: Influence of Rennet Type. *Eur. Food Res. Technol*., **210**: 9-12.
39. Lazzi, C., Rossetti, L., Zago, M., Neviani, E. and Giraffa, G. 2004. Evaluation of Bacterial Communities Belonging to Natural Whey Starters for Grana Padano Cheese by Length Heterogeneity-PCR. *J. Appl. Microbiol*., **96**: 481-490.
40. Madrau, M. A., Mangia, N. P., Murgia, M. A., Sanna, M. G., Garau, G., Leccis, L., Caredda, M. and Deiana, P. 2006. Employment of Autochthonous Microflora in Pecorino Sardo Cheese Manufacturing and Evolution of Physicochemical Parameters during Ripening. *Int. Dairy J*., **16**: 876-885.
41. Manero, A. and Blanch, A. R. 1999. Identification of *Enterococcus* spp. with a Biochemical Key. *Appl. Environ. Microbiol*., **65(10)**: 4425-4430.
42. Mangia, N. P., Murgia, M. A., Garau, G., Sanna, M. G. and Deiana, P. 2008. Influence of Selected LAB Cultures on the Evolution of Free Amino Acids, Free Fatty Acids and Fiore Sardo Cheese Microflora during the Ripening. *Food Microbiol.,* **25**: 366-377.
43. Marino, M., Maifreni, M. and Rondinini, G. 2003. Microbiological characterization of Artisanal Montasio Cheese: Analysis of Its Indigenous Lactic Acid Bacteria. *FEMS Microbiol. Lett*., **229(1)**: 133-140.
44. Marino, M., Maifreni, M., Bartolomeoli, I. and Rondinini, G. 2008. Evaluation of Amino Acid-decarboxylative Microbiota throughout the Ripening of an Italian PDO Cheese Produced Using Different Manufacturing Practices. *J. Appl. Microbiol*., **105**: 540-549.
45. Moreno, M. R. F., Sarantinopoulos, P., Tsakalidou, E. and De Vuyst, L. 2006. The Role and Application of Enterococci in Food and Healt. *Int. J. Food Microbiol*., **106**: 1-24.
46. Mucchetti, G. and Neviani, E. 2006. Il Formaggio. Microbiologia e Tecnologia Lattiero-casearia. ed. Tecniche Nuove, Milan, Italy, PP. 189-416.
47. Pisano, M. B., Fadda, M. E., Deplano, M., Corda, A., Casula, M. and Cosentino, S. 2007. Characterization of Fiore Sardo Cheese Manufactured with the Addition of Autochthonous Cultures. *J. Dairy Res*., **74**: 255–261.
48. Prieto, B., Franco, I., Prieto, J. G., Bernardo, A. and Carballo, J. 2002. Proteolytic and Lipolytic Changes during the Ripening of León Raw Cow's Milk Cheese: A Spanish Traditional Variety. Int. *J. Food Sci. Technol*., **37**: 661-671.
49. Randazzo, C. L., Vaughan, E. E. and Caggia, C. 2006. Artisanal and Experimental Pecorino Siciliano Cheese: Microbial Dynamics during Manufacture Assessed by Culturing and PCR-DGGE Analyses. *Int. J. Food Microbiol*., **109**: 1-8.
50. Sagdic, O., Arici, M. and Simsek, O. 2002. Selection of Starters for a Traditional Turkish Yayik Butter Made from Yoghurt. *Food Microbiol*., **19**: 303-312.
51. Sanna, M. G., Mangia, N. P., Garau, G., Murgia, M. A., Massa, T., Franco, A. and Deiana, P. 2005. Selection of Folate-producing Lactic Acid Bacteria for Improving Fermented Goat Milk. *Ital. J. Food Sci*., **2(17):** 143-154.
52. St-Gelais, D., Doyon, G., Rolland, J. R. and Goulet, J. 1991. Sugar and Organic Acid Concentrations during Ripening of Cheddar Cheese-like Products. *Milchwissenschaft*, **46**: 288-291.

[DOR: 20.1001.1.16807073.2011.13.4.13.2]

53. Tavaria, F. K., Dahl, S., Carballo, F. J. and Malcata, F. X. 2002. Amino Acid Catabolism and Generation of Volatiles by Lactic Acid Bacteria. *J. Dairy Sci*., **85**: 2462-2470.

54. Terzaghi, B. E. and Sandine, W. E. 1975. Improved Medium for Lactic Streptococci and Their Bacteriophages. *J. Appl. Microbiol*., **29**: 807-813.

خصوصیات میکروبیولوژی و فیزیکوشیمیایی پنیر پکورینو رومانو (Pecorino Romano) تولید شده با استارتر انتخابی

ن.پ. مانگیا، م. ا. مورگیا، ج. گاراو و پ. دیانا

چکیده

اثـر اسـتارتر اتوکتونـوس (authochthonous) تولیـد شـده از اسـترپتوکوس ترموفیلـوس (*Sterptococos thermophilos*)، لاکتوباسیلوس دلبروکی (*Lactobacillus delbruekli*) و لاکتوباسـیلوس هلوتیکـوس (*Lactobacillus helveticus*) بـر خـصوصیات میکروبیولـوژی و فیزیکوشیمیایی پنیر پکورینورومانو در مرحله رسیدن مورد بررسی قرار گرفت. مطلوبیت کـشت اسـتارتر آزمایشگاهی در سطح صنعتی این پنیر نیز ارزیابی شد. برای نمونه کنترل، از پنیر پکورینـو رومـانو تولیـد شده با اسکوتا فرمنتو (*Scotta fermento*) استفاده شد. فلور میکروبی لاکتیکی در پنیر آزمایـشگاهی در مقایسه با نمونه کنترل افزایش معنی دار است و این افزایش با کاهش قابل توجه میکروارگانیزم هـای فسادزا همراه بود. میزان آمینو اسیدهای آزاد مخصوصاً آرژنین، گاما آمینو بوتیریـک اسـید و لوسـین در پنیر آزمایشگاهی بیشتر بود. این اختلاف ها می توانـد در اثـر اخـتلاف فعالیـت آنزیمـی کـشت اسـتارتر انتخاب شده نسبت به کشت استارتر اسکوتا فرمنتو که بعنوان نمونه کنترل اسـتفاده شـده باشـد. بیـشترین اسید های چرب آزاد ارزیابی شده در هر دو پنیر در پایان مرحله رسیدن، اسـیدهای اولئیـک (C18:1)، پالمیتیک (C16:0)، بوتریک (C4)، استئاریک (C18:0) و میریستیک (C14:0) بودند. بطـور کلـی اختلاف معنی داری در میزان اسیدهای چرب آزاد برای نمونه آزمایشگاهی و کنترل مشاهده نـشد، اگـر چه معدل میزان این اسیدها در نمونه آزمایشگاهی نسبت به نمونه کنترل بیشتر بود. بایـد اضـافه کـرد کـه پنیر پکورینو رومانو در مقایسه با دیگر پنیرهای پی دی او (PDO) سـاردینی کمتـرین معـدل اسـیدهای چرب آزاد را داشت. غلظت بالای نمک طعام و فعالیت آبی کم پنیر پکورینو رومانو بر فعالیت آنزیمـی لیپاز و حتی بر لیپاز موجود در رنت موثر بود. علیرغم این موضوع نتایج نشان داد که اگـر رعایـت ریـزه کاری های لازم بشود کشت اسـتارتر انتخـاب شـده بـرای بهبـود خـصوصیات فیزیکـی و شـیمیایی پنیـر پکورینو رومانو مفید است.

[DOR: 20.1001.1.16807073.2011.13.4.13.2]