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# Microbiological and biochemical aspects of inland Pecorino Abruzzese cheese

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## Abstract

Little is known on physicochemical and biochemical characteristics of “Pecorino” Abruzzese cheese in L'Aquila province, an artisanal cheese produced from ewe raw full-cream milk.

Three batches of inland “Pecorino” Abruzzese cheese were examined for microbiological, compositional, biochemical and sensory characteristics at the aim of isolating and storing in a bacterial collection, indigenous strain to preserve the microbial biodiversity present in this cheese, to a possible definition of a PDO. Cheese samples from three dairies, at different stages of production were collected and 148 colonies were characterized. Physicochemical assays, species-specific PCR and 16S rRNA gene sequencing revealed that the majority of the lactic acid bacteria (LAB) isolates were *Enterococcus faecium* and *En. faecalis*. They were highly prevalent, accounting for 48% of the isolates. The lactic microflora consisted of lactobacilli and lactococci from the species *Lactobacillus plantarum* (12.2%), *Lactobacillus brevis* (10.1%), *Lactococcus lactis* subsp. *cremoris* (11.5%), respectively.

Urea-PAGE electrophoresis showed extensive degradation of  $\alpha_{S1}$ -casein (CN) and moderate hydrolysis of  $\beta$ -CN. Formation of  $\gamma$ -CNs from  $\beta$ -CN were highlighted. RP-HPLC profiles of the ethanol-soluble and ethanol-insoluble fractions of the pH

4.6-soluble nitrogen showed only minor differences between the three farms: lower proteolysis in the soluble fraction than the insoluble.

Leucine, glutamic acid, lysine, valine were the free amino acids present at the highest levels in all the cheeses. Flavour and texture profile were characterized through a sensory analysis.

Keywords: Food Science, Microbiology

## 1. Introduction

“Pecorino” is a term used to define Italian cheeses made from 100% sheep’s milk and it has, in most cases, a protected designation of origin or PDO status. It is mostly produced in Central and in Southern Italy by a traditional procedure, characterized by a different ripening time ranging from 8 to 12 months (Di Cagno et al., 2003; Schirone et al., 2012).

Cheese ripening involves a complex series of biochemical events generating the characteristic taste, aroma and texture of each cheese variety (Sousa et al., 2001; McSweeney, 2004; Murtaza et al., 2014; Kraggeruda et al., 2008). The indigenous microbial content of cheeses, selected by the use of raw milk and the cheese-making environment and technology, may be considered to drive this process and may be one of the main factors that determine the typical features of a given cheese.

In recent years, several studies have been carried out on wild isolates from handicraft cheeses production without the addition of industrial starter cultures (Kirmaci et al., 2016). The microbial biodiversity of typical foods can be maintained by increasing our knowledge on the population of microorganisms present in cheeses produced using different methods and also in raw milk.

These studies may be also important to select new strains to be utilized as specific cultures in larger-scale production of traditional cheeses, to improve the manufacture of existing dairy products.

“Pecorino” Abruzzese is a traditional ewes’ milk cheese produced in Abruzzo (Central Italy). This cheese is produced under different conditions, which depend on manufacturing tradition and production scale. In general, “Pecorino” Abruzzese is semi-hard or hard cheese, made from raw or heat-treated milk, and often without the use of selected starter cultures. The particular flavour and typical organoleptic properties of “Pecorino” cheeses are associated with specific attributes of milk and the natural microbiota (Corroler et al., 1998). The artisanal cheeses have different and typical microbial population dynamics related to the production technology and geographic area of origin, with a microbiota quite heterogeneous (Dellaglio et al., 1995; Chaves-López et al., 2006; Serio et al., 2010; Schirone et al., 2011; Schirone et al., 2013).

Several studies on “Pecorino” Abruzzese cheese were done, and different kind of cheese were described such as “Pecorino” di Farindola, Canestrato di Castel del Monte on local farms of Abruzzo region or coastal area (Dellaglio et al., 1995; Chaves-López et al., 2006; Serio et al., 2010; Schirone et al., 2012; Schirone et al., 2013). However, no research has ever been performed on inland “Pecorino” Abruzzese cheese in L’Aquila province. This cheese is an artisanal one produced from ewe raw full-cream milk obtained from flocks grazing during the warm season highland pasture, without any industrial starter addition. The purpose of the present work was to monitor physico-chemical, biochemical and microbiological characteristics of a particular “Pecorino” Abruzzese cheeses produced in the province of L’Aquila. For this purpose three different farms located in the highland Abruzzi region, in the province of L’Aquila were investigated. Conventional and genotypic methods were used to identify indigenous bacterial isolates in order to characterize it, to preserve the manufacturing integrity, and to possibly lead to a definition of a PDO status.

## 2. Materials and methods

### 2.1. Cheese samples

Three ewe’s milk and three cheese samples were supplied by three farms located in the highland Abruzzi region, in the province of L’Aquila. They were made exclusively with ewe’s milk produced in the neighbouring area during the late spring. The sampling year was 2011. Flocks were kept outside pasturing in natural grassland. The cheese was made by heating raw milk (no more than 24–36 h old) at 36–38 °C. Natural whey culture “siero-innesto” was added. After 30 min paste lamb rennet was added, kept at 35 °C for 20–30 min and then put in wicker molds for 12 h to remove the whey. The cheeses were then salted with a cover of coarse salt. Cheese were ripening at 10–15 °C from 20 to 300 days at a relative humidity of 80–90%. Cheese samples were analyzed at 20, 60, 120, 210 and 300 days of ripening in cheese factory.

The samples were sent to our laboratory under refrigeration (4 °C) and immediately analyzed.

### 2.2. Isolation and counting of microorganisms

Samples (25 g) were diluted in 225 mL of 2% (w/v) sodium citrate solution and homogenized in a Stomacher Laboratory Blender 400 (PBI International Milan, Italy). Serial dilutions in quarter-strength Ringer's solution were performed and plated on specific media for viable counts. Aerobic mesophilic bacteria were counted on Plate Count Agar (Oxoid, Basingstoke, Hampshire, England) at 30 °C for 24–48 h, lactococci and streptococci were cultured on M17 Agar (Oxoid) containing 1% (w/v) lactose (Fluka Chimica, Milan, Italy), for 24–48 h in

anaerobic conditions; enterococci were aerobically cultured on Slanetz-Bartley Agar (Oxoid) at 37 °C for 48 h; mesophilic and thermophilic lactobacilli on MRS Agar (Oxoid) acidified to pH 5.4 with acetic acid, at 30 °C and 44 °C, respectively, for 48 h under anaerobic conditions using the Gas-Pack anaerobic system (AnaeroGen; Oxoid) (Schirone et al., 2011; Fortina et al., 2003). Citrate-fermenting bacteria were grown on MRS with the addition of 2% calcium citrate (Panreac) incubated at 30 °C for 48 h in aerobiosis. Yeast were grown on Yeast Peptone Dextrose Agar (YPD; 1% w/v yeast extract, 2% w/v peptone, 2% glucose and 2% w/v agar; Oxoid) supplemented with chloramphenicol (150 mg/L) at 25 °C for 72–96 h. To determine the *Enterobacteriaceae*, Violet Red Bile Glucose Agar (VRBGA) (Oxoid) was utilised, and dishes were incubated for 24 h at 37 °C (Schirone et al., 2013).

For each sample 20 colonies were randomly selected and purified by streaking and then submitted to microscopic examination, Gram staining, catalase test. Isolates were finally stored at –80 °C.

According to regulation No. 2073/2005 (European Commission, 2005) we evaluated, in raw milk, the presence of food pathogens like *Staphylococcus aureus* coagulase positive, *Listeria monocytogenes* and *Salmonella spp* (ISO 6888–1:1999 (ISO, 1999); ISO 11290–1:1996 (ISO, 1996); ISO 6579:2002 (ISO, 2002).

### 2.3. Phenotypic characterization of isolates

After microscopic examination with phase contrast microscope (Leica), the ability of Gram-positive, catalase-negative, nonmotile rod-shaped bacteria to grow in MRS broth at 15 °C for 10 days and at 45 °C for 48 h was assessed. Pure cultures were subcultured on MRS broth and identified according to Schillinger and Lücke (1987).

The capability of cocci to grow in M17 broth at 10 °C for 10 days, and at 40 °C and 45 °C for 24–48 h was also assessed, enabling us to distinguish between mesophilic and thermophilic bacteria.

Lactic acid isomer produced was evaluated by the method of Von Krush and Lompe (1982). Growth in the presence of 2%, 4% and 6.5% of NaCl (in M17 and MRS) were also tested in microtiter plates as previously described (Parente et al., 1997).

Further biochemical characterization was carried out using API 50CH galleries (API System, BioMérieux, Montalieu Vercie, France) following the manufacturer's recommendations.

Results of phenotypic analysis were coded as 0 and 1, respectively, for negative and positive tests to obtain a binary data matrix used as data entry for SMC (Simple Matching Coefficient) (Sokal and Michener, 1958). The similarity data matrix obtained allowed the grouping of isolates based on their similarity to reference strains. An arbitrary similarity threshold of 0.85 was defined. Statistical analysis was carried out by Statistica 5.5 software modules (Stat Soft Inc., Tulsa).

Finally the isolates were identified using morphological, cultural and biochemical tests according to Kandler and Weiss (1986), Mundt (1986a), Mundt (1986b).

## 2.4. Genotypic characterization of isolates

Genomic DNA was prepared as described by De Los Reyes-Gavilán et al. (1992) using 1.5 mL of overnight cultures added to MRS broth and incubated at 37 °C. DNA concentration and purity was estimated spectrophotometrically by Nanodrop ND-2000 spectrophotometer (Thermo Scientific).

Distinction among *Lactobacillus plantarum*, *Lactobacillus pentosus*, and *Lactobacillus paraplantarum* was performed by amplification of the *recA* gene. These species are genotypically closely related and show highly similar phenotypes (Torriani et al., 2001). The primers utilized were paraF, pentF, planF and pREV (Torriani et al., 2001). Multiple PCR assay was performed as described by Torriani et al. (2001).

For the identification of *Lactobacillus brevis* the primers BrevI and BrevII binding to the V1 variable region of the 16S *rRNA* gene, were those described by Guarneri et al. (2001). The PCR mixtures and amplification programs were described by Guarneri et al. (2001).

For genetically differentiating between *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* primers Y1 and Y2 were utilized to amplify a 348-bp fragment from the 16S *rRNA* gene (Ward et al., 1998). The PCR was performed and programmed as described by Ward et al. (1998).

*Enterococcus faecalis* primers targeted the *ddl* gene (E1 and E2), which encodes the D-Ala: D-Ala ligase and produces a 941 DNA base pair product upon amplification (Dutka-Malèn et al., 1995). Primers for *Enterococcus faecium* (EM1A and EM1B), which produce a 658 DNA base pair product upon amplification, were used (Cheng et al., 1997; Harwood et al., 2004).

The other primers utilized in this study and their corresponding specificities were: the St1 and St2 sequences of the *lacZ* gene in *Streptococcus thermophilus* (Lick et al., 1996); the casei, para, rham and Y2 sequences of the 16S *rRNA* gene in *Lactobacillus casei*, *Lactobacillus paracasei* and *Lactobacillus rhamnosus* (Ward and Timmins, 1999); 16, Lc and Ls sequences of the 16S/23S *rRNA* gene spacer

region in *Lactobacillus curvatus* and *Lactobacillus sakei* (Berthier and Ehrlich, 1998).

PCR was performed in a Gene Amp 2400 PCR System (Perkin-Elmer) using the published parameters for the various genes unless otherwise stated. 10 µL of the PCR products were subjected to electrophoresis and the gel were photographed under UV transillumination. A Gene-Ruler™ DNA ladder mix (MBI Fermentas, Germany) was used as a size marker (Fortina et al., 2003).

The amplified Y1-Y2 product was microdialysed against water on Millipore type VS filters, with 0.025 mm pores and digested with *Mbo*II (12U) and *Cfo*I (5U) for 4 h at 37 °C. The resulting fragments were separated by electrophoresis.

The isolates phenotypically ascribed to the LAB group were verified by using primers Y1 and Y2 as described by Aquilanti et al. (2006). Reference strains were used in each reaction. The reference strains used in this study are listed in Table 1.

## 2.5. Compositional analysis

Fresh milk samples were analyzed for fat, protein, and lactose by infrared analysis (Programme d'Analyse des Troupeaux Laitiers du Québec) using a Milko Scan (model: Foss 4000, Foss Food Technology, Denmark) calibrated for sheep milk. Dry matter, fat and casein were determined according to the procedure of the Association of Official Analytical Chemists (AOAC, 1999); dry matter was obtained by using the oven method while the fat content was determined by Soxhlet method using diethyl ether (AOAC, 1999).

Cheese samples were analysed for moisture and pH as described by IDF (1989) and ISO (2004) Total protein and water-soluble N were determined by the Kjeldahl method (IDF, 1993). Each analysis was performed in triplicate.

## 2.6. Assessment of proteolysis

Both pH 4.6-soluble and -insoluble fractions of the cheeses were prepared by the method of Kuchroo and Fox (1982), modified by Sousa and McSweeney (2001).

Protein content (total N x 6.38) of the pH 4.6-soluble fraction was determined by the macro-Kjeldahl method (IDF, 1964). Ethanol (70%)-soluble and -insoluble sub-fractions of the pH 4.6-soluble extracts were prepared according to the method described by Shakeel-Ur-Rehman et al. (1998). Urea-polyacrylamide gel electrophoresis (Urea-PAGE) of the pH 4.6-insoluble fraction of each of the cheeses, at 20, 60, 120, 210 and 300 days of ripening, was performed using the procedure of Andrews (1983) as modified by Shalabi and Fox (1987). Gels were stained directly with Coomassie Brilliant Blue G250, as described by Blakesley and Boezi (1977).

**Table 1.** Reference strains utilized as controls for identification of lactic acid bacteria isolated from “Pecorino” Abruzzese cheese (DSMZ is Deutsche Sammlung von Mikroorganismen und Zellkulturen, ATCC is American Type Culture Collection; <sup>T</sup> is: Type strain).

Species	Culture collection: reference strain
<i>Lactobacillus casei</i>	DSMZ: 20011 <sup>T</sup>
<i>Lactobacillus acidophilus</i>	DSMZ: 20079 <sup>T</sup>
<i>Lactobacillus delbrueckii lactis</i>	DSMZ: 20072 <sup>T</sup>
<i>Lactobacillus delbrueckii delbrueckii</i>	DSMZ: 20074 <sup>T</sup>
<i>Lactobacillus delbrueckii bulgaricus</i>	DSMZ: 20081
<i>Lactobacillus helveticus</i>	ATCC: 15807
<i>Lactobacillus plantarum</i>	DSMZ: 20174
<i>Lactobacillus paracasei</i>	DSMZ: 4905
<i>Lactobacillus paracasei</i>	DSMZ: 5622
<i>Lactobacillus brevis</i>	DSMZ: 20054
<i>Lactobacillus fermentum</i>	DSMZ: 20052
<i>Lactobacillus sakei</i>	DSMZ: 20017
<i>Lactobacillus curvatus</i>	DSMZ: 20019
<i>Lactobacillus pentosus</i>	DSMZ: 20314
<i>Lactobacillus graminis</i>	DSMZ: 20719
<i>Lactobacillus zaeae</i>	DSMZ: 20178
<i>Lactococcus lactis cremoris</i>	DSMZ: 20069 <sup>T</sup>
<i>Lactococcus lactis lactis</i>	DSMZ: 20481 <sup>T</sup>
<i>Lactococcus lactis hordniae</i>	DSMZ: 20450
<i>Leuconostoc mesenteroides mesenteroides</i>	DSMZ: 20343 <sup>T</sup>
<i>Leuconostoc mesenteroides cremoris</i>	DSMZ: 20346 <sup>T</sup>
<i>Leuconostoc mesenteroides dextranicum</i>	DSMZ: 20484 <sup>T</sup>
<i>Leuconostoc lactis</i>	DSMZ: 20202 <sup>T</sup>
<i>Pediococcus acidilactici</i>	DSMZ: 20333 <sup>T</sup>
<i>Pediococcus pantosaceus</i>	DSMZ: 20336 <sup>T</sup>
<i>Enterococcus faecium</i>	DSMZ: 20477 <sup>T</sup>
<i>Enterococcus faecalis</i>	DSMZ: 20478 <sup>T</sup>
<i>Weissella confuse</i>	DSMZ: 20196
<i>Weissella viridescens</i>	DSMZ: 20410
<i>Streptococcus thermophilus</i>	DSMZ: 20479

Peptide profiles of the ethanol-insoluble and-soluble fraction of pH 4.6 were determined according to the method described by Sousa and McSweeney (2001) by RP-HPLC, (Varian Associates Inc., Walnut Creek CA, USA). The samples

were prepared and then eluted with acetonitrile gradient as described by Sousa and McSweeney (2001). Eluates were monitored at 214 nm. Total free amino acids (FAA) were determined by the trinitro-benzene-sulphonic acid (TNBS) assay (Polychroniadou, 1988). Individual free amino acids were determined using the method described by Fenelon et al. (2000).

## 2.7. Assessment of lipolysis

Individual free fatty acids were determined according to the method of De Jong and Badings (1990).

Identification and quantification of the volatile components in elutes were achieved on a Varian gas-chromatograph (Model Star 3400CX), equipped with an autosampler (Varian 8200CX), coupled to a Varian FID detector interfaced with Star Chromatography Workstation 5.0 software for system control and data acquisition (Varian Analytical Instruments, Harbor City, California, USA). The free fatty acids were resolved on a WCOT fused silica capillary column (25 m x 0.32 mm, Chrompack) coated with FFAP-CB for Free Fatty Acid Analysis (DF 0.3, Varian). Analyses for each batch of cheese were carried out in triplicate.

## 2.8. Sensory evaluation

Using a 7 point hedonic scale (1 = poor to 7 = excellent), an experienced taste panel of 10 evaluators evaluated the three “Pecorino” Abruzzese samples for flavour and texture. Estimates for each batch of cheese were carried out in triplicate.

## 2.9. Statistical analysis

A Principal Component Analysis (PCA) of physico-chemical and microbiological data was performed by XLSTAT utilizing Pearson correlation index, in order to quickly identify the correlations between variables based on the characteristics of similarity/dissimilarity. Data were organized in the form of a matrix with  $n$  rows representing the objects (farms) and  $p$  columns representing variables (physico-chemical and microbiological data). The results of PCA are “loadings” graphs: they are two-dimensional graphs in which the variables are represented in the space of the first two principal components that together explain a significant proportion of the data variance (Benfeldt and Sorensen, 2001).

Phylogenetic analysis was conducted using Gel Compar II program (version 5.1, Applied Maths, Kortrijk, Belgium) by the UPGMA method.



### 3. Results and discussion

#### 3.1. Microbiological characteristics

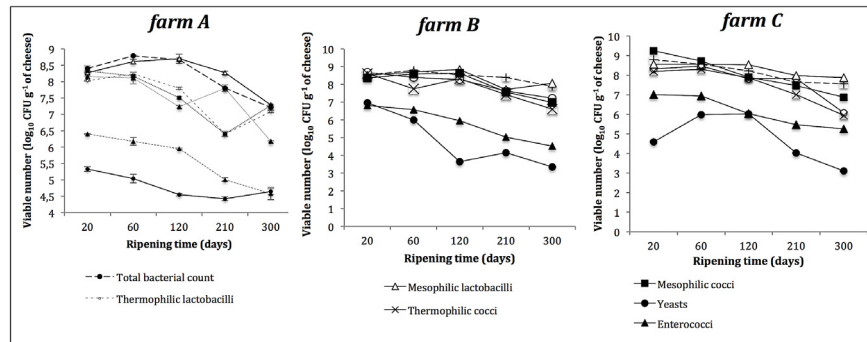
The microbial count of the raw milk samples is shown in Table 2. All the samples were within the European Union directive (EU regulation 2073/2005); pathogens such as *Listeria monocytogenes*, and *Staphylococcus aureus* and *Salmonella* spp. were not detected in all the batches.

Microbial counts for batches A, B and C are reported in Fig. 1. A great variation in number and distribution of the different microbial groups was observed in all farms, with a high presence of aerobic mesophilic bacteria, followed by mesophilic and thermophilic lactobacilli, as observed in different “Pecorino” Abruzzese cheeses and reported by Schirone et al. (2013). Mesophilic lactobacilli were the predominant group of LAB in “Pecorino” Abruzzese cheese 20 days after manufacture, followed by streptococci and lactococci. The cheeses therefore contain a complex microbial population of cocci and rods from raw milk, selected by the cheese-making environment and technology.

During ripening, microbial counts decreased by only 1–3 log units from the early stage of maturation, for all groups examined. Presumptive thermophilic lactobacilli and streptococci gradually decreased in numbers to 7 log CFU/g in two of the three cheese batches and to 6 log CFU/g in the other one. They were less abundant than mesophilic bacteria, accounting for 31.5% of the microbial population at the end of ripening. In “Pecorino” Abruzzese, *Enterobacteriaceae* can be detected after 20 days of ripening at levels of 4 log CFU/g and are still present (2 log CFU/g) after 60 days. *Enterobacteriaceae* are not detected in the final product due to their gradual decrease during cheese ripening. At the end of ripening (300 days) yeasts were present at values ranging from about 3 to 4 log CFU/g. As reported by

**Table 2.** Cell numbers ( $\log_{10}$  CFU/mL) of the principal presumptive microbial groups found in raw milk. Average data from each batch analyzed in triplicate (mean  $\pm$  S.D.).

Microbial group	Farm A	Farm B	Farm C
Total bacterial count	5.62 $\pm$ 0.07	5.51 $\pm$ 0.24	5.40 $\pm$ 0.05
Thermophilic lactobacilli	4.83 $\pm$ 0.03	4.36 $\pm$ 0.03	4.23 $\pm$ 0.10
Mesophilic lactobacilli	5.04 $\pm$ 0.01	4.47 $\pm$ 0.01	4.44 $\pm$ 0.03
Thermophiliccocci	5.18 $\pm$ 0.08	4.54 $\pm$ 0.06	4.85 $\pm$ 0.04
Mesophiliccocci	5.61 $\pm$ 0.07	4.63 $\pm$ 0.01	5.12 $\pm$ 0.05
Yeasts	4.30 $\pm$ 0.06	5.14 $\pm$ 0.10	–
Enterococci	4.35 $\pm$ 0.06	4.51 $\pm$ 0.07	3.61 $\pm$ 0.01



**Fig. 1.** Trend curves of the different microorganisms during ripening of the cheeses in the three farm samples (farm A, farm B, farm C). In the Y axis: viable bacteria numbers in cheese samples ( $\log_{10}$  CFU  $g^{-1}$ ) are reported. CFU is Colony Forming Units.

different authors, the presence of yeast has been associated with the secondary microflora of a wide variety of cheeses. In most cases, however, their contribution to cheese ripening is unclear (Jakobsen and Narvhus 1996; Schirone et al., 2011).

At 20 days a large number of presumptive enterococci (about 7 log CFU/g) were detected in all batches examined. They represent a typical microflora of ewe milk and play an important role in the late ripening of several cheese varieties (Aquilanti et al., 2006). In “Pecorino” cheese enterococci constitute a relevant part of the cheese microbiota as reported by Schirone et al. (2012). Gelsomino et al. (2002) demonstrated that enterococci survive and grow in the hidden corners of the milking machine and in the bulk tank, thus directly contaminating the milk. From the milk, the enterococci are transferred into the cheese. They constitute part of the normal food microbiota and play an important role in the manufacture of typical cheeses from Mediterranean countries (Ogier and Serror, 2008).

### 3.2. Phenotypic identification of strain

About 2000 colonies were collected on the basis of phenotypic characteristics. All colonies were grown on specific media to characterize them and observed at the microscope.

Microscopic examination of the 2000 isolates showed that 32% of colonies corresponded to rods or rod-shaped bacteria, with the remaining 68% colonies corresponding to cocci.

81.4% of the cocci microscopically identified were able to grow at 10 °C, 80% grew in the presence of 6.5% NaCl, and 90% gave a blackening reaction in media containing esculin. We found that 60.7% of rod-shaped microscopically identified grew at 15 °C, 70.4% grew at 45 °C, and only 14.3% produced gas from glucose. These last bacteria were therefore considered to be obligate heterofermentative lactobacilli. All isolates were Gram positive, catalase-negative.

None of the strains metabolized citrate, in contrast with previous reports for another “Pecorino-type” cheese, “Pecorino” Romano (Bottazzi et al., 1971). This finding is consistent with those of Dellaglio et al. (1995) for “Pecorino” Abruzzese, suggesting that the absence of such bacteria is an ecological characteristic of this cheese.

Based on the results of this preliminary biochemical analysis, a total of 147 colonies were chosen for further genotypic analyses.

Only 93 of 147 isolates could be phenotypically identified. Since phenotypic tests give information on the biochemical and metabolic traits of LAB not reliable enough for the identification of these microorganisms, these data were utilized only for preliminary grouping of isolates. Most of the isolates were identified as *Enterococcus faecium* (27 isolates) and *Enterococcus faecalis* (26 isolates); the remaining were grouped into 12 clusters and 5 were only identified at the species level as *Lactobacillus* spp.

### 3.3. Genetic identification of strain

The genetic identification of the strains isolated from the three batches is shown in Table 3. Very often the most accurate results are obtained when both phenotypic and genotypic aspects are utilized to classify them and this approach was used in this work.

Molecular characterization of the isolates showed that *E. faecium* and *E. faecalis* were highly prevalent, accounting for 48% of the isolates. The lactic microflora consisted of lactobacilli: 12.2% of the isolates belonged to *Lb. plantarum* and 10.1% to *Lb. brevis*. Within *Lactococcus* species, *Lc. lactis* subspecies *cremoris* appeared dominant (11.5%), we found that 7.4% of isolates corresponded to *L. lactis* subsp. *lactis*. Only one or two isolates were identified as *Str. thermophilus*, *Lb. casei*, *Lb. pentosus*, *Lb. paracasei* and *Lb. delbrueckii* subsp. *lactis*, *Lb. curvatus*, *Lb. graminis*, *Lb. zaeae*. *Lb. curvatus*, *Lb. graminis*, *Lb. paracasei*, *Lb. zaeae* and *Leuconostoc mesenteroides* were recovered in only one of the farm. *Lb. brevis*, *Lb. pentosus*, *Lb. delbrueckii* subsp. *lactis* and *Lb. casei* were recovered in two farms.

Microorganisms are an essential component of most cheeses, playing important roles during cheese manufacture and ripening. During cheese ripening, starter culture and secondary microflora promote a complex series of biochemical reactions essential for the correct development of both flavour and texture. The secondary flora consists of a complex mixture of adventitious microorganisms – bacteria, yeast and moulds – which find their way into cheeses from ingredients or the environment, and are in general specifically associated with a particular cheese

**Table 3.** Genetic identification of lactic acid bacteria isolated from three farms based on polyphasic approach (<sup>a-</sup> is: Not detected).

Species	Farm A	Farm B	Farm C
<i>Lactobacillus brevis</i>	7A,10A,14A,19A, 20A,32A,46A	– <sup>a</sup>	2C,5C,14C,27C,32C, 34C,36C,40C
<i>Streptococcus thermophilus</i>	4A,23A	6B	21C
<i>Lactobacillus curvatus</i>	25A	–	–
<i>Lactobacillus graminis</i>	–	28B	–
<i>Lactobacillus pentosus</i>	42A	–	16C
<i>Lactobacillus plantarum</i>	1A,24A,29A,31A, 33A,41A,49A	1B,13B,27B,40B	3C,18C,20C,25C,33C, 42C,47C
<i>Lactococcus lactis</i> subsp. <i>lactis</i>	3A,17A,28A,44A	3B,20B,22B,36B,44B,17B	38C
<i>Lactococcus lactis</i> subsp. <i>cremoris</i>	6A,9A,13A,22A, 40A, 45A	2B,10B,16B,24B,25B,42B,47B	13C,19C,35C,48C
<i>Enterococcus faecium</i>	8A,12A,15A,18A, 26A,30A,34A,36A, 39A, 47A,50A	4B,5B,8B,11B,12B, 15B,19B,23B,26B, 30B,32B,33B,39B, 43B,46B,48B	1C,6C,10C,11C,23C, 24C,30C,37C,44C
<i>Enterococcus faecalis</i>	2A,5A,11A,16A, 37A, 38A,43A	7B,9B,14B,21B,29B,31B,34B,37B,38B, 41B,45B	4C,7C,8C,9C,15C,17C,22C,26C,28C,39C, 41C,43C,45C,49C
<i>Lactobacillus paracasei</i>	21A,48A	–	–
<i>Lactobacillus zeae</i>	35A	–	–
<i>Lactobacillus delbrueckii</i> subsp. <i>lactis</i>	–	35B	29C
<i>Lactobacillus casei</i>	–	18B	12C,31C,46C
<i>Leuconostoc mesenteroides</i>	27A	–	–

variety, making a significant contribution to the specific characteristics of that particular variety (Sousa et al., 2001).

This preliminary examination of the principal microbial species colonizing inland “Pecorino” Abruzzese cheese revealed that the lactic acid microflora of this cheese is composed of mesophilic cocci and enterococci, as reported for other typical Italian “Pecorinos”, such as “Pecorino” Umbro and “Pecorino” Sardo cheese (Gobbetti et al., 1997; Mannu et al., 1999).

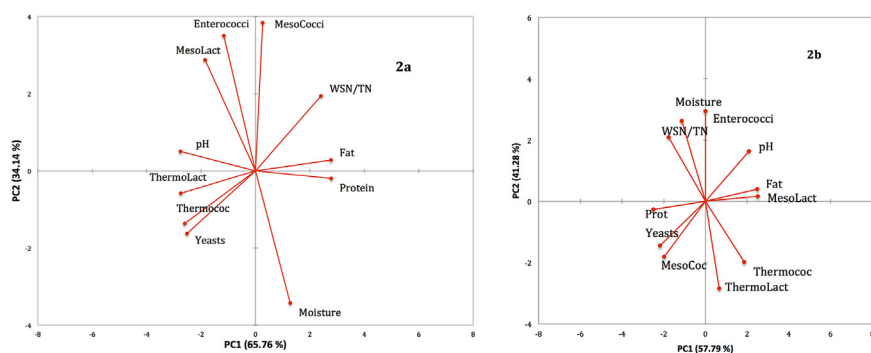
The “Pecorino” Abruzzese lactic acid microflora was also found to be rich in thermophilic bacteria, which accounted for 31.5% of the microbial population at the end of ripening, a percentage higher than reported in Canestrato Pugliese (10.6%), Fiore Sardo and “Pecorino” Romano (<1%) (Ortigosa et al., 1999), cheeses, all of which are ewes’ milk cheeses resembling “Pecorino” Abruzzese. This feature may be characteristic of this ewes’ milk cheese.

This resident microbiota is mostly composed by mesophilic lactobacilli that largely contribute to the determination of the typical flavour and texture of these traditional cheeses.

### 3.4. Physicochemical characteristics

Fig. 2a, b shows the PCA of the physicochemical and microbiological data at 20 days (Fig. 2a) and 300 days of ripening (Fig. 2b). The amount of variation explained by the first and second principal component represent 99,9% and 99,07% of the total variation, respectively. Aim of this analysis was to study the possible influence of physicochemical parameters on the microbial diversity of cheese.

The data on physicochemical characteristics of milk and “Pecorino” Abruzzese are shown in Table 4 and Table 5. The pH slightly increased during ripening and no relevant differences were detected among the different farms, with values included in the range of 5.27–5.91, respectively. Concerning the pH, its range is probably



**Fig. 2.** PCA based on physico-chemical and microbiological data at 20 (2a) and 300 days (2b) of ripening. Data are the means of three farms.

**Table 4.** Physicochemical characteristics of ewe's milk. Analyses were performed in triplicate (mean  $\pm$  S.D.).

	Farm 1	Farm 2	Farm 3
pH	6.58 $\pm$ 0.27	6.44 $\pm$ 0.32	6.62 $\pm$ 0.18
Dry matter (%)	19.34 $\pm$ 0.34	21.05 $\pm$ 0.26	18.02 $\pm$ 0.29
Fat (%)	6.97 $\pm$ 0.18	6.89 $\pm$ 0.21	7.57 $\pm$ 0.19
Lactose (%)	3.98 $\pm$ 0.38	4.58 $\pm$ 0.32	4.87 $\pm$ 0.36
Protein (%)	5.97 $\pm$ 0.11	6.37 $\pm$ 0.14	6.14 $\pm$ 0.18
Casein (% of protein)	77.85 $\pm$ 0.27	77.99 $\pm$ 0.34	78.58 $\pm$ 0.38

due to a combination of lactic acid metabolism and  $\text{NH}_3$  production related to the amino acid catabolism. Statistical analysis supports this hypothesis (Fig. 2a,b). This aspect has also been described in the literature (Mansour et al., 2008; Yvon

**Table 5.** Physico-chemical characteristics of "Pecorino" Abruzzese cheese during ripening. Analyses were performed in triplicate (mean  $\pm$  S.D.). Water Soluble Nitrogen/Total Nitrogen (WSN/TN).

Physico-chemical	Farm	Days of ripening				
		20	60	120	210	300
pH	A	5.27 $\pm$ 0.23	5.26 $\pm$ 0.11	5.35 $\pm$ 0.31	5.40 $\pm$ 0.11	5.53 $\pm$ 0.12
	B	5.48 $\pm$ 0.15	5.62 $\pm$ 0.21	5.72 $\pm$ 0.14	5.81 $\pm$ 0.19	5.87 $\pm$ 0.33
	C	5.38 $\pm$ 0.12	5.52 $\pm$ 0.17	5.66 $\pm$ 0.22	5.68 $\pm$ 0.15	5.91 $\pm$ 0.35
Moisture (%)	A	35.51 $\pm$ 0.25	30.26 $\pm$ 1.71	29.66 $\pm$ 1.92	27.78 $\pm$ 3.24	25.24 $\pm$ 3.36
	B	33.29 $\pm$ 0.82	27.39 $\pm$ 0.64	22.56 $\pm$ 0.48	22.71 $\pm$ 0.94	23.80 $\pm$ 1.24
	C	31.46 $\pm$ 0.61	30.85 $\pm$ 0.74	27.41 $\pm$ 0.25	25.84 $\pm$ 0.19	26.81 $\pm$ 0.34
Protein (%)	A	24.7 $\pm$ 0.3	26.7 $\pm$ 0.6	28.9 $\pm$ 0.4	29.9 $\pm$ 0.2	33.9 $\pm$ 0.6
	B	21.4 $\pm$ 0.4	24.7 $\pm$ 0.3	26.1 $\pm$ 0.4	27.9 $\pm$ 0.2	29.6 $\pm$ 0.6
	C	23.2 $\pm$ 0.7	22.9 $\pm$ 0.3	26.5 $\pm$ 0.2	28.7 $\pm$ 0.5	31.3 $\pm$ 0.7
Fat (%)	A	32.6 $\pm$ 0.8	34.1 $\pm$ 0.2	36.0 $\pm$ 0.4	36.9 $\pm$ 0.1	37.8 $\pm$ 0.3
	B	30.0 $\pm$ 0.6	36.8 $\pm$ 0.2	38.1 $\pm$ 0.5	38.1 $\pm$ 0.2	39.2 $\pm$ 0.9
	C	31.7 $\pm$ 0.2	33.2 $\pm$ 0.7	34.8 $\pm$ 0.1	36.1 $\pm$ 0.5	38.7 $\pm$ 0.5
WSN/TN (%)	A	21.4 $\pm$ 1.4	22.7 $\pm$ 0.9	26.1 $\pm$ 1.3	30.4 $\pm$ 0.7	37.6 $\pm$ 1.1
	B	16.1 $\pm$ 1.1	20.0 $\pm$ 1.6	23.7 $\pm$ 0.8	26.4 $\pm$ 1.4	31.1 $\pm$ 1.8
	C	22.1 $\pm$ 1.2	25.4 $\pm$ 1.2	28.4 $\pm$ 0.9	30.5 $\pm$ 1.3	39.7 $\pm$ 1.7

and Rijnen, 2001). As demonstrated, lactate resulting from the conversion of lactose by LAB and amino acids resulting from casein proteolysis and aminoacid degradation give rise to a pH increase. Aminoacid catabolism is a major process for flavour formation in cheese. The ability of LAB to degrade aminoacid to aroma compounds is highly strain-dependent.

In the statistical analysis (Fig. 2a, b) it is possible to observe that mesophilic LAB are positively correlated to pH from the beginning of the ripening (20 days).

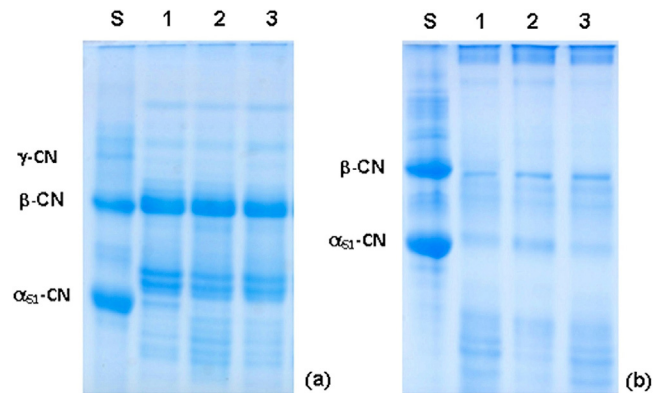
The levels of moisture for the “Pecorino” Abruzzese cheese decreased during ripening and varied from 35.51 to 23.80%. These values are consistent with those reported by Di Cagno et al. (2003) for “Pecorino” Romano, Fiore Sardo and Canestrato Pugliese, other hard ewes’ cheeses.

At the end of ripening (300 days) average value of protein and fats (Table 5) were  $33.9 \pm 0.6\%$ ,  $29.6 \pm 0.6\%$  and  $31.3 \pm 0.7\%$ , for the proteins and  $37.8 \pm 0.3\%$ ,  $39.2 \pm 0.9\%$  and  $38.7 \pm 0.5\%$  w/w for the fats, for the three cheeses examined respectively. These values were higher than those reported for other “Pecorino” cheeses (Di Cagno et al., 2003).

Based on the ratio of the % of water-soluble nitrogen/total nitrogen, (Table 5), farm B cheese greatly differed from the other two cheeses. “Pecorino” Abruzzese shows a high level of proteolysis: at the end of ripening the values of water-soluble N/total N (%) approached or exceeded those reported for Canestrato Pugliese (28.8%) and Fiore Sardo (24%) (Di Cagno et al., 2003), Parmesan (32–34%) and “Pecorino” Romano (20–28%) (Fox and Guinee, 1987), all known for their high level of proteolysis during ripening. Statistical analysis (Fig. 2a,b) shows that WSN/TN (Water Soluble Nitrogen/Total Nitrogen) is correlated at the beginning with mesophilic LAB and enterococci and at the end of ripening with enterococci. This is most likely due to an initial acidification of the substrate by LABs balanced during ripening, by a proteolytic activity of enterococci.

Urea-PAGE of pH 4.6-insoluble and ethanol-soluble N fraction are shown in Fig. 3. Urea-PAGE of the pH 4.6-insoluble N fraction (Fig. 3a) showed that primary proteolysis appeared to proceed rather similarly in all samples. Common features were extensive degradation of  $\alpha_{S1}$ -casein (CN) and moderate hydrolysis of  $\beta$ -CN. Electrophoresis patterns of all samples were similar, although farm A (lane 1) showed different peptides of higher electrophoretic mobility in comparison with farm B (lane 2), and farm C (lane 3).

Formation of  $\gamma$ -CNs from  $\beta$ -CN was evident from the protein bands with the slowest electrophoretic mobility. The higher hydrolysis of  $\alpha_{S1}$ -CN than  $\beta$ -CN was found to be a characteristic also for other similar hard cheese, such as Canestrato Pugliese (Albenzio et al., 2001) or Fiore Sardo (Di Cagno et al., 2003). The geographical area of cheese making, season of production, ripening temperature



**Fig. 3.** Urea-PAGE of pH 4.6-insoluble (a) and ethanol-soluble (b) fractions of “Pecorino” Abruzzese cheeses. Lanes: S ovine Na-caseinate (standard), 1 (farm A), 2 (farm B), 3 (farm C) at 120 days of ripening.

and duration, effect of chymosin and type of dairy are all factors which influence the extent of proteolysis.

The urea-PAGE electrophoregram of pH 4.6-soluble N fraction (Fig. 3b) showed a proteolysis pattern different among the farms, particularly for the peptides with low electrophoretic mobility. Farm A sample was characterized by pronounced hydrolysis of  $\alpha_{S1}$ -CN and slight degradation of  $\beta$ -CN. Farm B showed lower hydrolysis both  $\alpha_{S1}$ -CN and  $\beta$ -CN but mostly for  $\beta$ -CN.

Electrophoresis patterns of farm C sample were similar to farm A with slight hydrolysis of  $\beta$ -casein (CN) and formation of  $\gamma$ -CN.

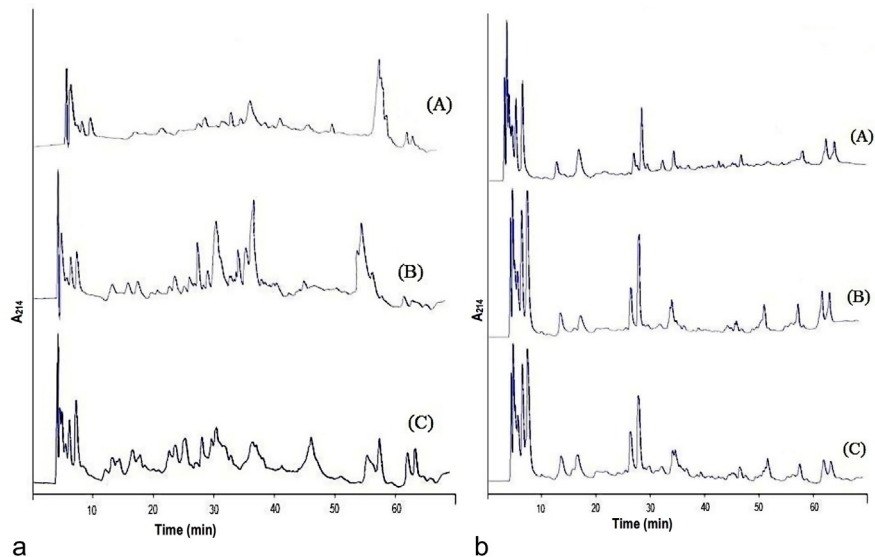
RP-HPLC peptide profiles of pH 4.6 ethanol-insoluble and soluble fractions are shown in Fig. 4.

Both fractions of all the samples showed a highly complex peptide profile. Chromatograms of the ethanol-insoluble fraction are shown in Fig. 4a. In this figure, samples A, B, C, showed rather complex peptide profiles and differing in the peak areas. In Fig. 4b some of the peptides present at high concentrations were eluted before 10 and 60 min. Peptides present at lower concentrations were eluted at the intermediate region (from 20 to 40 min).

Peptide profiles of the ethanol-soluble fraction are shown in Fig. 4b: the chromatograms show higher concentrations of peptides at 10 and 30 min, hence providing evidence for the presence of free amino acids and small peptides, at relatively high concentrations. These results show a lower proteolysis in the soluble fraction than the insoluble.

The total concentration of free amino acids (FAA) was similar for farm A and C ( $132 \pm 5.4$  and  $169.2 \pm 5.3$ , respectively), but differed from farm B which

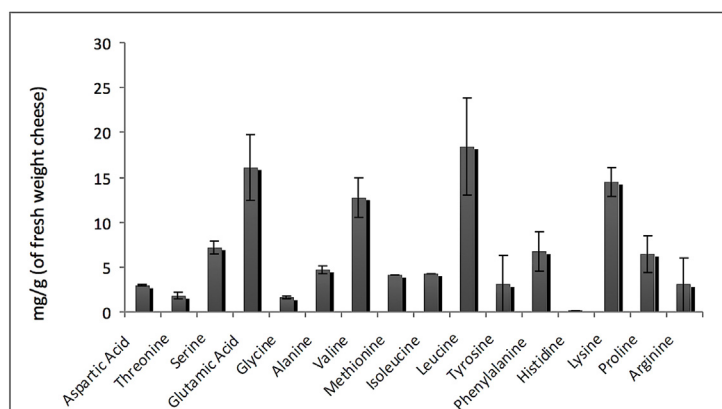




**Fig. 4.** (a) RP-HPLC chromatograms of the ethanol-insoluble fractions from farm (A), farm (B) and farm (C) of inland “Pecorino” Abruzzese cheeses at 120 days of ripening. (b) RP-HPLC chromatograms of the ethanol-soluble fractions from farm (A), farm (B) and farm (C) of inland “Pecorino” Abruzzese cheeses at 120 days of ripening.

amounted to  $73.6 \pm 5.1$  mg/g of fresh weight cheese. The composition of individual free amino acid present in the cheeses is shown in Fig. 5. Some amino acids, e.g., leucine, glutamic acid, lysine, valine were found at high levels in all three ewes’ milk cheeses.

Most of these amino acids were previously found to be present at high concentrations in several extra-hard Italian cheese varieties (Di Cagno et al., 2003).

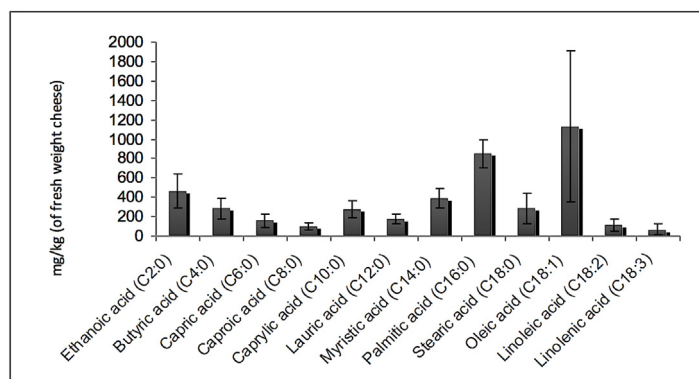


**Fig. 5.** Concentration of individual free amino acids of the three cheese batches (farms A, B, C) of “Pecorino” Abruzzese cheese at 120 days of ripening. Concentrations of individual free amino acids are expressed as mg/g (of fresh weight cheese). Average data from each batch analyzed in triplicate.

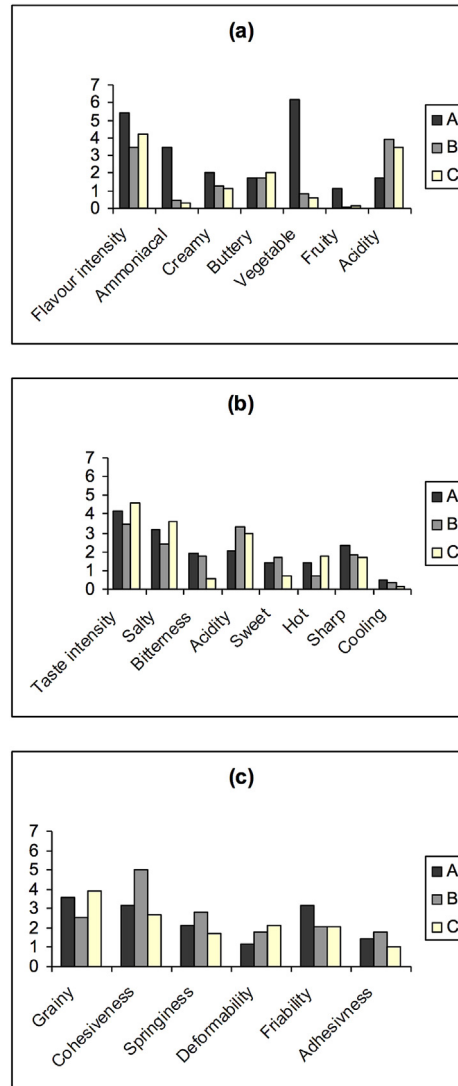
The total concentration of free fatty acids in all samples showed an average content of  $4310 \pm 1507$  mg/kg of fresh weight cheese. Individual fatty acids present in the cheeses were also analysed; the results are in Fig. 6. The carboxylic acid profile shows that among the short-chain fatty-acids the most abundant were ethanoic and butyric. Medium-chain acids (myristic and palmitic acid) and long-chain fatty acids (stearic, oleic, linoleic) were present at highly variable concentration. Most of the ethanoic acid found probably originated from the degradation of lactose by heterofermentative lactic acid bacteria. Another important aspect is the presence of linolenic and linoleic acids for their anti-carcinogenic properties at average content of 16.04 mg/g fat and 27.13 mg/g fat, respectively (Ip et al., 1991). Schirone et al. (2011) demonstrated the presence of a high content of conjugated linolenic acids (CLA) in the “Pecorino” of Farindola, another “Pecorino” from a different area in the Teramo province. The CLA are compounds derived from biohydrogenation of linolenic acid present in the milk or elaborated by microorganisms during fermentation. Among lactic acid bacteria *Lactobacillus plantarum* – present in the microbiota of inland “Pecorino” cheese – together with *Lactobacillus acidophilus* were reported as the greatest CLA producers (Kishino et al., 2002).

### 3.5. Sensory evaluation

Results of sensory evaluation of “Pecorino” Abruzzese cheeses, from different farms, at 120 days of ripening were shown in Fig. 7. Differences in lipolysis, and particularly in concentration of amino acids and proteolysis, seemed to strongly affect the flavour quality. Although all three cheeses received a medium-high score for all parameters considered, they were generally recognized for spicy, fruity and vegetal aromas taste that make the bouquet sumptuous and harmonious and with comfortable body and texture.



**Fig. 6.** Total fatty acid composition of the three cheese batches (farms A, B and C) at 120 days of ripening. Concentrations of fatty acids are expressed as mg/kg (of fresh weight cheese). Average data from each batch analyzed in triplicate.



**Fig. 7.** Sensory evaluation data for flavour (a), taste (b) and body and texture (c) of “Pecorino” Abruzzese cheeses from three cheese batches (farms A, B and C) at 120 days of ripening.

## 4. Conclusions

Inland “Pecorino” Abruzzese cheese is characterized by two relevant features, the ewe raw full-cream milk obtained from flocks grazing highland pasture and the use of lamb rennet.

The microflora plays a major role in cheese ripening and the selection of suitable strains would make possible for cheese makers to control or modify flavour development. However, the selection of strains for flavour improvement is difficult, due to the complexity of the flora and the interactions between individual components of the microflora and the cheese environment.

Analysis of the biochemical characteristics of milk and cheese made possible to characterize the environment of the inland “Pecorino” Abruzzese cheese to better understand the characteristics of the indigenous microbial population. By using a combination of phenotypic and molecular techniques, an effective species and strain differentiation was achieved, and the dominant bacterial species in these inland “Pecorino” Abruzzese cheeses were analyzed. From the evaluation of the microbial community, a remarkable heterogeneity emerged, probably due to the use of raw milk with no added starter. These results suggest that the wild bacterial population should be preserved by isolating, characterizing and keeping the highest number of strains and by protecting traditional productions. This is important either to preserve the typical organoleptic characteristics of this traditional unpasteurized milk cheese and also to select new strains as indigenous starter cultures for the dairy industry. This work represents the first study on inland “Pecorino” Abruzzese cheese and could contribute to deepen the knowledge of its microbiological and biochemical features. The data collected can be utilized, in particular, for characterization and a possible definition of a PDO status.

## Declarations

### Author contribution statement

Valeria Centi: Performed the experiments.

Federica Matteucci: Analyzed and interpreted the data.

Aldo Lepidi: Conceived and designed the experiments.

Maddalena Del Gallo, Claudia Ercole: Wrote the paper.

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### Competing interest statement

The authors declare no conflict of interest.

### Additional information

No additional information is available for this paper.

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