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Biogenic amine content and microbiological profile of Pecorino di Farindola cheese

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ABSTRACT

"Pecorino di Farindola" is a traditional ewes' milk cheese produced in the Abruzzo region (Italy) and ripened for a minimum of 90 days. The main objective of this research was to characterize the microbiological and chemical composition of this cheese, manufactured in ten dairy farms during the winter cheese-making season (December through March). By using classical enumeration system on specific media variability was observed in the viable numbers of aerobic mesophilic bacteria, enterobacteria, coagulase-negative staphylococci, yeasts, enterococci, mesophilic and thermophilic lactobacilli, lactococci and thermophilic streptococci. *Salmonella* spp., *Listeria monocytogenes* and *Escherichia coli* O157:H7 resulted to be absent in all the samples. Among compounds possibly impacting on human health, the isomer cis-9, trans-11 conjugated linoleic acid (CLA), was determined in high levels in all samples, ranging from 9.2 to 12.7 mg/g fat. Great diversity was also found in biogenic amine contents with a relevant presence of tyramine in all the cheeses. This work represents the first study on Pecorino di Farindola cheese and could contribute to deepen the knowledge on its microbiological and biochemical features, focusing on hygiene and consumer health aspects.

1. Introduction

In Italy there is a large production of Pecorino cheeses (approximately 53.727 t) of different varieties, mostly without a "designation of origin," which have typical characters and originates from a delimited geographical area (Coda et al., 2006). Pecorino di Farindola is a traditional variety of cheese made from ewes' milk, produced in the Abruzzo region, in the east side of the Gran Sasso mountain (National Park of Gran Sasso, Italy). This cheese shows unique organoleptic characteristics derived from the environmental conditions and manufacturing practices already existing in the production area. The cheese is extremely valued in the local market and can be sold as short-time ripened cheese (ripening time 3 months) with a soft texture and a thin yellow rind or as a long-time ripened cheese (ripening time up to 12 months) with a harder texture, a more piquant and intense flavor. Traditional protocol for Pecorino di Farindola cheese making is shown in Fig. 1. It is manufactured mostly in artisan plants according to local traditions, by using raw milk and pig rennet without the addition of natural or commercial starter cultures. Adventitious microorganisms, represented mainly by non-starter lactic acid bacteria that derived from raw milk or from the dairy environment and surfaces of equipment used in cheese manufacture, play the most important role in cheese during ripening not only for sensory characteristics, but also with respect to safety hazards, as reported for other cheeses (Berthier et al., 2001; Coda et al., 2006; Pintado et al., 2008). As a consequence Pecorino di Farindola cheese exhibits considerable variability in the quality. The microbiota plays a major role in the development of the organoleptic characteristics of the cheese but it can also be responsible for the accumulation of undesirable substances, such as biogenic amines (BA). The presence of these compounds is often unavoidable in fermented foods (Stratton et al., 1991; Suzzi and Gardini, 2003; Pintado et al., 2008). Nevertheless, their presence can cause several problems for susceptible consumers, such as nausea, respiratory distress, hot flushes, sweating, heart palpitation, headache, bright red rash, oral burning, hyper- or hypotension, whose intensity is dependent on quantitative and qualitative differences (Stratton et al., 1991; Ladero et al., 2010a). Many factors contribute to the presence and accumulation of BA, such as availability of free amino acids, pH, a_w, salt-in-moisture level and temperature, redox potential, bacterial density, and synergistic effects between microorganisms (Joosten, 1988; Joosten and van Boekel, 1988; Stratton et al., 1991; Galgano et al., 2001; Gardini et al., 2001; Pinho et al., 2001) and, primarily, the presence of microorganisms. The presence of relevant amounts of BA in

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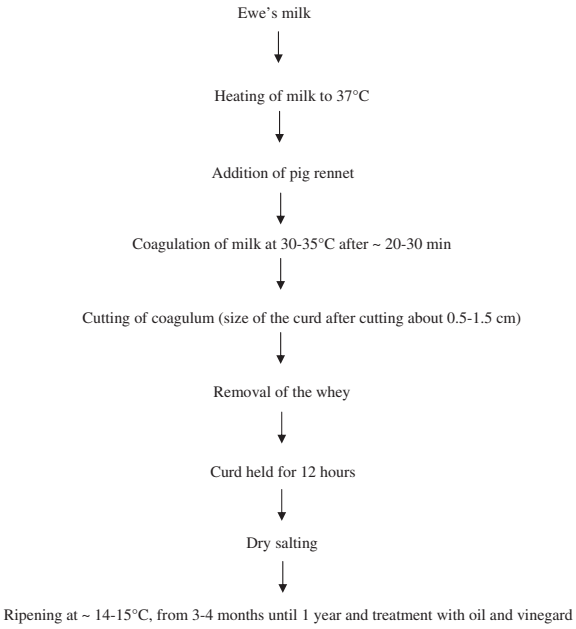

Fig. 1. Protocols for the manufacture of Pecorino di Farindola.

cheeses has been documented recently (Valsamaki et al., 2000; Galgano et al., 2001; Pinho et al., 2001; Martuscelli et al., 2005; Pintado et al., 2008; Ladero et al., 2009; Mercogliano et al., in press). In these studies the quantitative and qualitative accumulation of such compounds in cheese was reported as extremely variable and dependent on several factors. However, in many cases, the accumulation of BA has been attributed mainly to the activity of the non-starter microbiota, even if an indirect role of the starter lactic acid bacteria (LAB) can be hypothesized. In fact, the peptidases released after starter LAB lysis could be essential to provide precursor amino acids.

Another important aspect that regards the consumer health is the presence of conjugated linolenic acids (CLA), that are compounds derived from biohydrogenation of linolenic acid, present in milk or elaborated by microorganisms during fermentation (Sieber et al., 2004; Ogawa et al., 2005). CLA have attracted attention in recent years for their anti-carcinogenic properties (Ip et al., 1991) and other physiological properties, such as the improving of immune response (Cook et al., 1993).

Information on the microbiota, chemical characteristics and safety of Pecorino di Farindola cheese is rather limited. In particular, data on BA and CLA content in this cheese are not available. It is therefore interesting, both for scientific and public health purposes, to investigate the content of BA after ripening. The objective of this research effort was to characterize microbiological profiles as well as BA and CLA content of Pecorino di Farindola cheeses manufactured in ten dairy farms.

2. Methods

2.1. Samples of Pecorino di Farindola

Ten batches of Pecorino di Farindola cheese, one from each dairy farm, were manufactured during the winter season (December through March) and ripened for 90 days. All farms were in Pescara and Teramo Provinces (Abruzzo Region, Italy), in which the Consortium of Pecorino di Farindola is geographically located. The cheeses were produced following traditional manufacture as reported in Fig. 1. All cheeses were analyzed at the end of ripening.

2.2. Microbiological analyses

Samples (10 g) of cheese were diluited in 90 ml of a sodium citrate (2% w/v) solution and homogenized with a Stomacher Lab-Blender 400 (Steward Medical, London, UK) for 1 min. Serial dilutions in sterile peptone water (0.1% w/v) were plated in duplicate on selective media to enumerate the following microorganisms: aerobic mesophilic bacteria on Plate Count Agar (PCA; Oxoid, Milan, Italy) at 30 °C for 2 days; mesophilic and thermophilic lactobacilli on MRS agar (Oxoid), acidified to pH 5.4 with acetic acid, at 30 °C and 45 °C respectively for 2 days in anaerobic conditions using the Gas-Pack anaerobic system (AnaeroGen; Oxoid); lactococci and thermophilic streptococci on M17 (Oxoid), containing 1% (w/v) lactose (Fluka Chimica, Milan, Italy), at 30 °C and 45 °C respectively, for 2 days in anaerobic conditions; coagulase-negative staphylococci Baird-Parker agar with egg yolk tellurite enrichment (Oxoid) at 37 °C for 24 h; enterococci on Slanetz-Bartley agar (Oxoid) at 37 °C for 48 h; *Enterobacteriaceae* on Violet Red Bile Glucose Agar (VRBGA; Oxoid) at 37 °C for 24 h. Yeasts were grown on Yeast Peptone Dextrose Agar (YPD; 1% w/v yeast extract, 2% w/v peptone, 2% w/v glucose and 2% w/v agar; Oxoid) supplemented with chloramphenicol (150 mg/l) at 25 °C for 3–5 days. The presence of *Salmonella* spp., *Listeria monocytogenes* and *Escherichia coli* O157:H7 was determined according to standard methods (Association Française de Normalisation, 1997; International Organization for Standardization, 1998, 2002).

2.3. Chemical analyses

A radial slice of each cheese was taken at random, and used for chemical assays. The rind of each slice was carefully removed, and the rindless material was fully shredded. The determinations of ash, NaCl and total protein were done according to standard methods (AOAC, 2000a,b,c). Moisture was evaluated using an oven from Wtb Binder (Tuttlingen, Germany), at 100 °C. Total lipids were extracted according to the method of Folch et al. (1957). pH measurement was carried out on a sample (10 g) of cheese dispersed in 10 ml of deionized water using a pH meter MP 220 (Mettler, Toledo, Spain); water activity (a_w) was measured by an Aqualab instrument (Series 3, Decagon Devices, Inc., Pullman, Washington, USA). All determinations were performed in duplicate.

2.4. Biogenic amine determination

Determination of BA was carried out by acid extraction and derivatization using the methods of Eerola et al. (1993) and Moret and Conte (1996), as reported by Martuscelli et al. (2005).

2.5. HPLC analysis

The chromatographic system consisted of a Spectra System P4000 pump, a Spectra System AS3000 autosampler, a Spectra System UV1000 UV/VIS detector (ThermoFinningan Italia Spa, Rodano, Italy) and a personal computer running the ChromQuest for Windows chromatographic software (ThermoQuest Italia spa, Rodano, Italy). The sample (10 μl) was injected onto a C18 Spherisorb S3ODS2 (Waters spa, Vimodrone, Italy), equipped with a Spherisorb S5ODS2 guard column (Waters). The peaks were detected at 254 nm according to the elution programme as reported by Martuscelli et al. (2005).

2.6. Fatty acid analysis

Fatty acid methyl esters (FAME) were prepared by potassium methoxide-catalyzed transesterification as described by FIL-IDF, 1999. In particular 100 mg of fat were added with 2 ml of hexane

containing sodium sulphate anhydrous and then with 0.5 ml of 2 M KOH in methanol.

Analyses of FAME were carried out on a Fisons Mega II series GC equipped with a fused silica capillary column (SP 2560, 100 m × 0.25 mm i.d., 0.2 μm film thickness; Supelco, PA, USA). High-purity hydrogen was used as carrier gas at a linear velocity of 1 ml/min.

The temperature program was 70 °C for 4 min, that was first increased at a rate of 13 °C/min to the temperature of 175 °C, then at a rate of 5 °C/min to the final temperature of 215 °C. Injector and flame ionization detector (FID) temperature were 250 °C and 260 °C, respectively. The injector was operated in split mode (split ratio 1:20). The system was controlled by a Chrom Card software. Qualitative analysis was performed on the basis of the retention times of peaks in samples and peaks of fatty acid methyl esters solutions (FAME mix C4-C24; Sigma–Aldrich, Milan, Italy). Quantification of the fatty acid cis-9, trans-11 CLA was carried out using methyl-nonadecanoate as internal standard.

2.7. Statistical analyses

The averages and standard deviations were calculated for each experimental parameter, pertaining to either the microbiological or the biochemical characterization. Descriptive statistics, analyses of variance (ANOVA) pairwise comparisons of mean values (following Tukey's test), and principal component analysis (PCA) were all performed using the statistical software STATISTICA for Windows (STAT. version 8.0, StatSoft Inc. Tulsa, OK, USA).

3. Results and discussion

3.1. Microbiological composition

Numbers of aerobic mesophilic bacteria, coagulase-negative staphylococci, enterococci, yeasts, mesophilic and thermophilic lactobacilli, lactococci and thermophilic streptococci in Pecorino di Farindola cheeses, manufactured in the ten dairy farms considered, are tabulated in Table 1. After preliminary morphological and biochemical tests, Gram positive and catalase negative rods and cocci were presumptively enumerated as LAB. One-way ANOVA was used as a basis to determine whether there were significant differences among the mean scores of each microbial group, across the ten samples. Results showed significant differences among the dairy farms, for all microbial groups. Tukey's post-hoc test was then employed to find out whether such differences, between each pair of dairy farms, were turn significant. High numbers of aerobic mesophilic bacteria after 90 days ripening from above 10^7 cfu/g to $>10^8$ cfu/g, were found in all cheeses. LAB dominated the adventitious microbiota prevailing in all cheeses. In general cell numbers of presumptive mesophilic and thermophilic lactobacilli varied from cheese to cheese such as those of the presumptive lactococci. The lowest values were found for thermophilic lactobacilli in all batches, whereas the highest value was found for thermophilic streptococci in cheese F8, showing above 10^9 cfu/g. Overall, in the early phase of manufacture non-starter lactic acid bacteria (NSLAB) are present at very low numbers, whereas during ripening they increase from approximately 2.0 log cfu/g in hygienically produced raw milk cheeses to ≥6.0 log cfu/g in ripened cheese (Berthier et al., 2001). NSLAB grow at low temperature, are acid-tolerant and tolerate the lack of fermentable carbohydrates, low pH, and a_w and the presence of bacteriocins, which make the environmental conditions very hostile during ripening. Coda et al. (2006) reported that many Italian Pecorino cheeses showed NSLAB values higher than 6.0 log cfu/g of at the end of ripening, with the only exception concerning Pecorino Umbro and Pecorino di Pienza cheeses, which were manufactured from pasteurized ewes' milk. As regard enterococci, in Pecorino di

Table 1
Microbiological composition of Pecorino di Farindola cheese from ten samples at 90 days of ripening (mean ± S.D.).[a]

Microbiological viability (log cfu/g cheese)	Samples										p
	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	
Aerobic mesophilic bacteria	7.43 ± 0.12bd	6.98 ± 0.03cd	6.99 ± 0.13cd	6.94 ± 0.11c	7.65 ± 0.31ab	7.90 ± 0.16ae	7.53 ± 0.36ab	8.35 ± 0.25e	7.98 ± 0.08ae	7.70 ± 0.24ab	<0.001
Coagulase-negative staphylococci	6.27 ± 1.34acd	5.87 ± 0.28abc	5.21 ± 0.07ab	5.12 ± 0.11b	2.11 ± 0.08e	6.20 ± 0.08acd	6.91 ± 0.14cd	6.99 ± 0.53d	3.59 ± 0.12f	5.49 ± 0.06ab	<0.001
Enterococci	7.67 ± 1.34a	6.50 ± 0.03bc	6.04 ± 0.10b	6.28 ± 0.03b	3.47 ± 0.13d	6.20 ± 0.28b	7.30 ± 0.22ac	8.21 ± 0.32a	7.57 ± 0.06a	7.59 ± 0.06a	<0.001
Yeasts	3.98 ± 0.12bcd	2.36 ± 0.49a	4.01 ± 0.09bcd	4.13 ± 0.31cd	3.37 ± 0.09be	4.38 ± 0.41d	2.56 ± 0.37a	2.87 ± 0.14ae	2.25 ± 0.21a	3.66 ± 0.30bc	<0.001
Mesophilic lactobacilli	6.96 ± 0.07c	6.11 ± 0.14a	7.01 ± 0.28c	7.26 ± 0.36c	7.27 ± 0.23c	6.07 ± 0.19a	5.81 ± 0.13ab	5.31 ± 0.38b	5.80 ± 0.14ab	5.71 ± 0.27ab	<0.001
Thermophilic lactobacilli	3.13 ± 0.17abc	3.15 ± 0.21abc	2.91 ± 0.27ac	3.87 ± 0.63bde	4.30 ± 0.57e	2.77 ± 0.21c	3.37 ± 0.23abcd	3.96 ± 0.35de	3.67 ± 0.18abde	3.66 ± 0.20abde	0.024
Lactococci	6.69 ± 0.02bc	6.36 ± 0.05b	6.36 ± 0.02b	7.27 ± 0.37a	5.17 ± 0.20d	7.18 ± 0.21a	7.19 ± 0.19a	7.16 ± 0.13a	7.15 ± 0.20a	7.00 ± 0.03ac	<0.001
Thermophilic streptococci	5.06 ± 0.07bc	5.31 ± 0.16cd	4.85 ± 0.33abc	4.24 ± 0.78a	4.52 ± 0.28ab	5.77 ± 0.08d	7.06 ± 0.20f	9.16 ± 0.06g	8.06 ± 0.20e	8.20 ± 0.16e	<0.001

[a] Probability p-value, obtained from ANOVA, is also listed. Means in rows without common letters (a–g) are significantly different ($p \leq 0.05$).

Farindola they were found at different but high viable numbers, ranging from 10^6 to $>10^8$ cfu/g in all cheeses, a part for sample F5 in which the enterococci were detected at 10^3 cfu/g. Such high levels of enterococci can have an active role during the whole ripening process, and in particular on BA formation (Martuscelli et al., 2005). Enterococci comprise a major part of the fresh cheese curd microbiota and in some cases they are the predominant microorganisms in the fully ripened product. Their levels in different cheeses depend on the extent of milk contamination and their survival and growth under the particular conditions of cheese manufacture and ripening. The persistence and dominance of enterococci during ripening has been attributed to their wide range of growth temperatures and their high tolerance to heat and salt (Galgano et al., 2001; Sarantinopoulos et al., 2001). The numbers of enterobacteria were lower than 10 cfu/g except for cheeses from batches F6 and F10, for which values of about 10^3 and 10^4 cfu/g were found, respectively (data not shown). The presence of *Enterobacteriaceae* after 90 days ripening can indicate a poor microbiological quality of the raw milk, and/or improper milk-handling and manufacturing practices. Nevertheless, the presence of enterobacteria at the end of ripening has been observed in ewes' milk Pecorino Abruzzese cheese (Martuscelli et al., 2005) as well as in similar cheeses (Freitas and Malcata, 2000). Viable coagulase-negative staphylococci showed a high variability too, ranging from 10^2 to 10^7 cfu/g.

Pathogens such as *Salmonella* spp., *L. monocytogenes*, and *E. coli* O157H:7 resulted absent in all the cheese batches.

Yeasts were present at values ranging from about 10^2 to 10^4 cfu/g. Similar values have been reported by Gardini et al. (2006) for Pecorino Crotonese and other Italian cheeses (Suzzi et al., 2000, 2001, 2003). Yeasts are associated with the secondary microflora of a wide variety of cheeses; however, in most cases, their contribution to cheese ripening is unclear (Jakobsen and Narvhus, 1996; Wyder and Puhan, 1999). The occurrence in cheese of some species of yeasts with high counts is attributable to their tolerance towards low pH, reduced water activity and high salt concentrations, as well as to their ability to grow at low storage temperature which characterizes the ripening environment (Ferreira and Viljoen, 2003).

3.2. Chemical characterization

Pecorino di Farindola is considered a semi-hard cheese. Overall, with the exception of batch F9 (21% of moisture) all the cheeses showed moisture values ranging from 34 to 51% (w/w) (Table 2). The range of moisture in our samples was higher than other Italian Pecorino cheeses that showed mean values from 35 to 38.2% (w/w) (Coda et al., 2006). Values for protein and fat in dry matter were 32.5–44.13% and 39.72–56.90% (w/w) respectively, whereas pH values ranged from 5.13 to 5.72. In particular batches F8 and F10 were characterized by the lowest values, pH 5.13 and 5.19, respectively. These values were in agreement with those (pH 4.68–5.80) indicated by other authors (Coda et al., 2006). In particular, Pecorino Sardo and Pecorino Umbro cheeses were characterized the lowest values, pH 4.68 and 5.05, respectively.

Large variability was also reported for ash and NaCl content as reflected by the significant *F*-values of ANOVA. Differences in NaCl content among the ten groups of cheeses may probably be ascribed to the distinct salting procedures. No significant correlations were found among the tested parameters and BA.

3.3. Biogenic amines

In general, the BA content of cheese can be extremely variable and depends on the type of cheese, the ripening time, the manufacturing process and the microorganisms present (Ordònez et al., 1998).

Table 2
Chemical characterization of Pecorino di Farindola cheese from ten samples at 90 days of ripening (mean ± S.D.).[a]

Chemical composition (%, w/w)	Samples										*p*
	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	
Moisture	47.0 ± 1.41a	39.0 ± 1.41c	46.5 ± 2.12a	34.0 ± 1.41g	49.0 ± 1.41ab	51.0 ± 0.0b	43.0 ± 0.0de	39.5 ± 0.0cd	21.0 ± 0.0f	51.0 ± 0.0b	<0.001
Protein in dry matter	34.35 ± 0.21a	35.80 ± 0.13d	38.82 ± 0.27b	38.65 ± 0.35b	41.5 ± 0.42g	40.48 ± 1.41f	37.28 ± 2.83e	44.13 ± 0.71h	32.5 ± 1.41c	34.72 ± 1.41a	<0.001
pH	5.62 ± 0.02ag	5.68 ± 0.01ab	5.68 ± 0.09ab	5.72 ± 0.02b	5.54 ± 0.06fg	5.43 ± 0.54e	5.25 ± 0.25d	5.13 ± 0.16c	5.47 ± 0.12ef	5.19 ± 0.37cd	<0.001
Fat in dry matter	42.90 ± 0.16a	46.04 ± 0.24e	44.68 ± 0.43d	47.05 ± 0.41f	43.09 ± 0.16a	39.72 ± 0.25b	40.67 ± 0.78c	42.82 ± 0.40a	49.88 ± 0.31g	56.90 ± 0.42h	<0.001
NaCl in dry matter	5.44 ± 0.01e	5.24 ± 0.04d	5.89 ± 0.02a	5.85 ± 0.04ab	5.94 ± 0.01b	6.22 ± 0.03c	6.36 ± 0.01g	6.29 ± 0.03c	6.47 ± 0.02h	5.65 ± 0.01f	<0.001
a_w	0.92 ± 0.01abc	0.93 ± 0.01acd	0.92 ± 0.01abc	0.89 ± 0.02b	0.95 ± 0.03ad	0.92 ± 0.02abc	0.95 ± 0.06ad	0.97 ± 0.01d	0.89 ± 0.02bc	0.94 ± 0.05ad	0.029
Ash content	4.8 ± 0.14ag	4.1 ± 0.14ab	5.16 ± 0.03ab	5.16 ± 0.1b	4.88 ± 0.04fg	5.64 ± 0.01e	4.54 ± 0.03d	5.25 ± 0.01c	6.11 ± 0.01ef	5.75 ± 0.03cd	<0.001

[a] Probability *p*-value, obtained from ANOVA, is also listed. Means in rows without common letters (a–h) are significantly different ($p \leq 0.05$).

The Pecorino di Farindola cheeses examined confirmed this variability in the total content of BA ranging from 209.0 to 2393.0 mg/kg cheese (Table 3). ANOVA showed significant differences among contents of eight BA assayed for. Four cheeses contained more than 1000 mg/kg cheese of total BA. According to Taylor (1985), the threshold of risk is 100 mg/kg total amines of cheese, if ingestion is associated with such potentiating co-factors as amine oxidase-inhibiting drugs or alcohol, or else if there are pre-existing gastrointestinal diseases (Stratton et al., 1991). As all the cheeses studied had the same ripening time (90 days) the ageing of cheese, one of the most important factor affecting the BA formation, could be excluded. Tyramine resulted to be the BA present in the highest concentration in all the cheeses examined, representing in six samples more than 40% of the total amines. A high relationship between tyramine and total BA content in Pecorino di Farindola cheese was found ($R^2 = 0.9869$). The relevant incidence of tyramine in cheese manufactured from raw ewes' milk has been reported (Roig-Sagués et al., 2002; Martuscelli et al., 2005; Pintado et al., 2008; Ladero et al., 2010b). The original quality of the milk and the length of "ripening" or storage appear to be dominant factors in the production of BAs in cheeses (Novella-Rodriguez et al., 2002). The high amounts of tyramine found in Spanish traditional cheeses have been related to the high amounts of tyrosine present in the milk protein with respect to other amino acids (Roig-Sagués et al., 2002). At the contrary Pintado et al. (2008) reported that despite the high concentrations of the precursor amino acid tyrosine in cheeses from some dairies, they do not provide evidence of tyramine in their biogenic amine inventory. During fermentation and ripening, the environmental factors that affect the activity of decarboxylating enzymes may be more important than precursor availability. However, because the general trend in the BA content of all Pecorino di Farindola cheese is the relative high production of tyramine, the influence of proteolytic enzyme activity of pig rennet could be hypothesized. In fact, proteinases and peptidases released from the rennet are some of the principal proteolytic enzymes acting during cheese-making and ripening (Fox and Stepaniak, 1993). Nevertheless, no information on the properties and characteristics of pig artisanal rennet is found in the revised literature. Generally lamb rennet is still used to produce hard cheeses in parts of Italy, in the form of rennet pastes that are essential for the development of cheese flavor and to impart a sharp, "piccante" or "pecorino" flavor to the cheese (Santillo et al., 2007). The use of pig rennet is very ancient and in Italy (as probably in the world) it is only applied in the manufacture of Pecorino di Farindola cheese. Concerning other BA, high values of ethylamine were generally found, whereas high levels of putrescine were found in the cheeses F2, F7 and F9, and cadaverine in the cheese F3, F5, F6 and F10. High levels of putrescine and/or cadaverine have also been reported for Semicotto caprino cheese (Galgano et al., 2001). *Enterobacteriaceae* are generally considered as microorganisms with a high decarboxylase activity, particularly in relation to the production of cadaverine and putrescine (Suzzi and Gardini, 2003). After 90 days ripening these microorganisms were present at low number in all Pecorino di Farindola cheeses, a part F6 and F10 samples. An incorrect storage of raw materials, as well as an uncontrolled fermentation, can induce a proliferation of *Enterobacteriaceae*, which can release their decarboxylases in the early steps of production. The enzyme released, and not the microbial cells, is responsible for the BA accumulation and its action can continue also in the absence of viable cells (Bover-Cid et al., 2001). However, it is difficult to find a straight correlation between microbial counts and BA content in cheese, because amine-producing abilities of different strains of various bacteria differ widely (Halász et al., 1994).

Any food with free amino acids, especially tyrosine and phenylalanine, are subject to BA formation if poor sanitation and

Table 3
Biogenic amines contents of Pecorino di Farindola cheese from ten samples at 90 days of ripening (mean ± S.D.).[a]

Composition (mg/kg cheese)	Samples										*p*
	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	
Ethylamine	208.3 ± 2.7e	110.5 ± 3.6d	12.9 ± 2.5b	33.9 ± 1.8a	449.7 ± 3.5f	481.6 ± 3.4g	35.4 ± 0.1a	30.8 ± 2.2a	601.3 ± 2.7h	70.7 ± 1.5c	<0.001
Phenylethylamine	40.8 ± 0.8b	9.5 ± 2.1a	8.8 ± 1.0a	5.7 ± 0.4a	37.6 ± 2.3b	59.3 ± 1.3d	28.6 ± 1.1c	0.0 ± 0.0a	127.1 ± 10.0e	0.0 ± 0.0a	<0.001
Putrescine	57.0 ± 2.8f	297.7 ± 2.3h	24.0 ± 1.9b	39.2 ± 1.4d	30.4 ± 3.0c	51.0 ± 3.2e	394.1 ± 3.0i	9.9 ± 0.8a	284.0 ± 0.4g	11.7 ± 1.1a	<0.001
Cadaverine	26.8 ± 3.2a	46.2 ± 3.0b	137.1 ± 1.6f	48.3 ± 1.3b	176.1 ± 1.6g	276.1 ± 1.8h	26.8 ± 0.8a	91.3 ± 2.8d	65.9 ± 3.5c	111.2 ± 1.6e	<0.001
Histamine	5.8 ± 1.8bc	4.1 ± 0.8b	7.2 ± 0.6c	0.0 ± 0.0a	0.0 ± 0.0a	0.0 ± 0.0a	0.0 ± 0.0a	0.0 ± 0.0a	21.8 ± 3.5e	12.9 ± 1.1d	<0.001
Tyramine	428.3 ± 2.7f	273.5 ± 6.0e	174.8 ± 2.0d	120.3 ± 1.3c	558.1 ± 3.0h	826.6 ± 13.4i	499.6 ± 2.3g	52.3 ± 2.8a	1171.3 ± 1.4l	72.05 ± 1.6b	<0.001
Spermidine	93.3 ± 2.3a	50.4 ± 2.5b	52.0 ± 2.8b	76.0 ± 0.5e	96.3 ± 1.3a	143.9 ± 3.5g	96.4 ± 1.3a	25.0 ± 1.8c	121.6 ± 2.8f	36.3 ± 1.3d	<0.001
Total biogenic amines	860.0	792.0	417.0	323.0	1348.0	1839.0	1081.0	209.0	2393.0	314.9	

Spermine was not detected in all samples (<3 mg/kg cheese).
[a] Probability *p*-value, obtained from ANOVA, is also listed. Means in rows without common letters (a–l) are significantly different ($p \leq 0.05$).

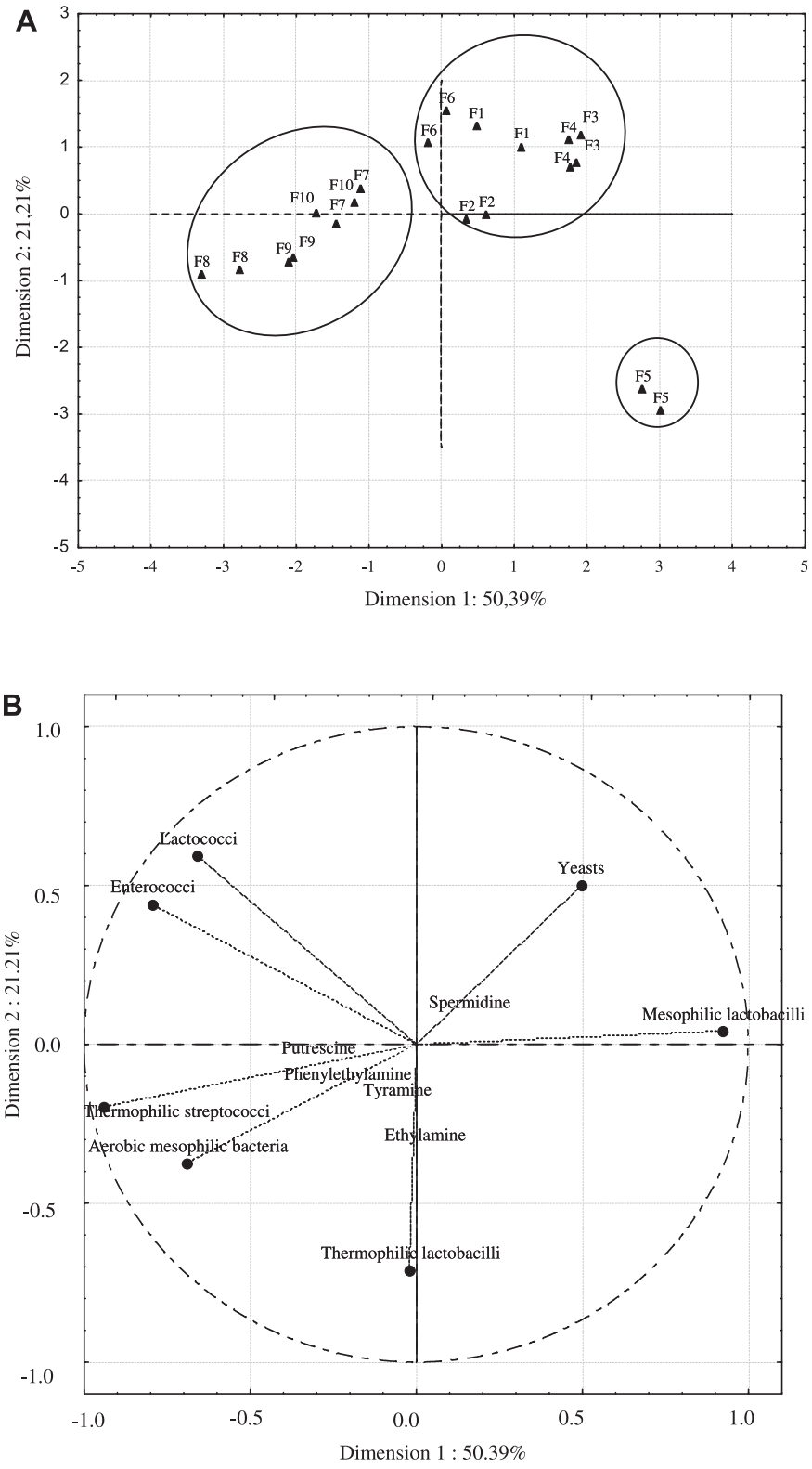

Fig. 2. Score plot (A) and loading plot (B) of the first and second principal components (PC) after PC analysis encompassing microbial group number (by name) and biogenic amine content (by name), for ten samples of Pecorino di Farindola cheese at 90 days of ripening.

Table 4
Fatty acid composition of ten samples Pecorino di Farindola cheese at 90 days of ripening (mean ± S.D.).[a]

Fatty acids[c]	Samples										*p*
	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	
C4:0	30.0 ± 0.14e	35.5 ± 0.14g	36.5 ± 0.42h	21.9 ± 0.14b	13.5 ± 0.14c	20.9 ± 0.14a	21.1 ± 0.42a	21.3 ± 0.71ab	26.6 ± 0.14d	32.9 ± 0.14f	<0.001
C6:0	36.1 ± 0.92b	37.8 ± 0.07h	31.5 ± 0.42f	36.2 ± 0.49b	30.5 ± 0.21e	25.4 ± 0.14a	28.0 ± 0.42d	22.0 ± 0.71c	24.8 ± 0.07a	33.8 ± 0.14g	<0.001
C8:0	43.4 ± 1.34c	43.1 ± 0.21c	30.6 ± 0.35d	38.1 ± 0.49e	40.4 ± 0.35f	24.4 ± 0.49a	35.5 ± 0.71b	25.6 ± 0.28a	24.8 ± 0.14a	35.0 ± 0.14b	<0.001
C10:0	138.3 ± 0.64l	129.6 ± 0.21i	87.5 ± 0.42c	94.1 ± 0.85d	102.8 ± 0.14f	101.0 ± 0.14e	124.4 ± 0.28h	83.6 ± 0.07b	80.7 ± 0.07a	111.2 ± 0.49g	<0.001
C12:0	79.1 ± 0.64i	68.2 ± 0.21g	46.7 ± 0.42c	62.8 ± 0.85f	57.3 ± 0.14e	36.2 ± 0.14b	73.5 ± 0.28h	48.5 ± 0.07a	52.7 ± 0.07d	48.2 ± 0.49a	<0.001
C14:0	138.2 ± 0.78g	139.0 ± 0.71h	123.3 ± 0.14a	111.9 ± 0.64d	121.6 ± 0.21e	123.6 ± 0.85a	142.3 ± 0.07i	128.2 ± 0.07f	98.7 ± 0.21b	102.6 ± 0.49c	<0.001
C14:1c9	3.7 ± 0.49e	2.0 ± 0.21a	1.8 ± 0.07ab	1.3 ± 0.14c	1.8 ± 0.14ab	1.4 ± 0.21bc	3.6 ± 0.71e	2.2 ± 0.07ad	1.9 ± 0.21ab	2.5 ± 0.35d	<0.001
C15:0	15.3 ± 0.21ab	14.9 ± 0.07ab	15.5 ± 0.07b	21.4 ± 0.07e	14.6 ± 0.21a	14.8 ± 0.42ab	14.9 ± 0.07ab	15.3 ± 0.28ab	17.6 ± 0.07d	16.5 ± 0.21c	<0.001
C16:0	237.0 ± 0.14d	228.6 ± 0.28c	268.3 ± 0.07a	258.7 ± 0.71g	267.7 ± 0.35a	289.2 ± 0.07b	264.6 ± 0.21h	255.5 ± 0.07e	288.8 ± 0.49b	257.1 ± 0.42f	<0.001
C16:1c9	10.6 ± 0.21a	7.3 ± 0.28b	8.5 ± 0.57d	11.0 ± 0.28a	10.7 ± 0.28a	11.2 ± 0.35a	11.8 ± 0.42f	7.9 ± 0.21c	9.7 ± 0.21e	7.4 ± 0.28bc	<0.001
C18:0	65.2 ± 0.28b	85.8 ± 0.21d	89.8 ± 0.14a	95.7 ± 0.28g	92.8 ± 0.28f	102.6 ± 0.49h	68.8 ± 0.07c	116.9 ± 0.14i	87.4 ± 0.21e	88.8 ± 0.28a	<0.001
C18:1t11	20.7 ± 0.64c	31.1 ± 0.49a	23.7 ± 0.64d	34.8 ± 0.14h	27.0 ± 0.14f	17.8 ± 0.35b	24.9 ± 0.14e	30.8 ± 0.42a	28.1 ± 0.64g	30.5 ± 0.42a	<0.001
C18:1c9	143.8 ± 0.35c	137.7 ± 0.71a	186.4 ± 0.42g	158.3 ± 0.42d	169.2 ± 0.21e	192.8 ± 0.21i	142.3 ± 0.21b	190.4 ± 0.07h	200.6 ± 0.07l	178.8 ± 0.14f	<0.001
C18:1c11	3.2 ± 0.28a	3.0 ± 0.28a	3.8 ± 0.07b	3.5 ± 0.49ab	4.5 ± 0.49cd	4.7 ± 0.42cd	3.4 ± 0.28ab	3.4 ± 0.28ab	4.9 ± 0.42d	4.2 ± 0.14ce	<0.001
C18:2c9,c12	18.8 ± 0.07f	14.4 ± 0.07e	20.4 ± 0.14b	29.5 ± 0.07g	19.7 ± 0.57a	10.2 ± 0.14d	20.6 ± 0.21b	19.6 ± 0.35a	24.0 ± 0.07c	23.5 ± 0.14c	<0.001
C20:0	2.2 ± 0.14ad	1.9 ± 0.57d	4.1 ± 0.07c	2.4 ± 0.14ab	2.7 ± 0.14b	3.4 ± 0.14e	2.4 ± 0.28ab	3.6 ± 0.21e	4.6 ± 0.14c	4.2 ± 0.35c	<0.001
C18:3c9,12,15	3.8 ± 0.21e	8.8 ± 0.07c	10.2 ± 0.14ad	7.9 ± 0.28b	10.5 ± 0.14a	9.8 ± 0.21d	8.2 ± 0.07bc	10.6 ± 0.14a	11.9 ± 0.49g	11.3 ± 0.14f	<0.001
CLac9,t11	9.7 ± 0.07ab	10.5 ± 0.14c	9.8 ± 0.57bd	9.6 ± 0.21ab	10.7 ± 0.21c	9.7 ± 0.35ab	9.2 ± 0.07a	12.7 ± 0.00e	10.2 ± 0.14cd	9.4 ± 0.14bc	<0.001
C20:4c5,8,11,14	1.6 ± 0.14ac	1.3 ± 0.14c	2.7 ± 0.21bd	1.9 ± 0.07a	2.9 ± 0.07b	1.6 ± 0.07ac	1.7 ± 0.14a	2.4 ± 0.21d	2.7 ± 0.07bd	2.8 ± 0.14b	<0.001
Saturated	784.5b	784.1b	733.6f	743.1a	743.7a	741.2g	775.4h	720.3d	706.3c	730.2e	<0.001
Monounsaturated	181.9a	181.0a	224.1b	208.7d	213.1e	227.8f	185.8c	234.6g	245.1h	223.3b	<0.001
Polyunsaturated	33.8d	35.0e	43.0a	48.8b	43.7a	31.2c	39.6f	45.2g	48.8b	46.9h	<0.001
Short chain[b]	326.7i	314.1h	232.8c	253.0e	244.4d	207.9a	282.5g	200.9b	209.5a	261.0f	<0.001
Middle chain[b]	404.7b	391.7d	417.3a	404.3b	416.3a	440.0g	437.1f	409.0e	416.5a	386.0c	<0.001
Long chain[b]	268.8c	294.3e	350.7a	343.3g	339.8f	352.3 ab	281.3d	390.2i	374.2h	353.4b	<0.001

[a] Probability *p*-value, obtained from ANOVA, is also listed. Means in rows without common letters (a–l) are significantly different ($p \geq 0.05$).
[b] Short chain fatty acids (C4:0, C6:0, C8:0, C10:0, C12:0); medium chain fatty acids (C14:0, C14:1c9, C15:0, C16:0, C16:1c9); long chain fatty acids (C18:0, C18:1t11, C18:2c9,c12, C20:0, C18:3c9,12,15, CLac9,t11, C20:4c5,8,11,14).
[c] Fatty acids are expressed as mg/g fat.

low quality foods are used or if the food is subjected to temperature abuse or extended storage time.

In order to determine the most important factors causing variability PCA was carried out using as variables BA levels, viable numbers of aerobic mesophilic bacteria, enterococci, yeasts, mesophilic and thermophilic lactobacilli, lactococci and thermophilic streptococci. The PCA results are shown in Fig. 2, score plot (A) and loading plot (B); it can explain 71.6% of the total variance. Dimension 1 accounts for 50.39% of the variance; the negative segment of the loading plot for this dimension (B) is closely related to the levels of thermophilic streptococci, putrescine and phenylethylamine, whereas its positive counterpart is mainly related to mesophilic lactobacilli. Dimension 2 explained 21.21% of the variance; this dimension is negatively related with thermophilic lactobacilli, tyramine and ethylamine.

Then, in score plot (A) it is possible to distinguish three different cheese groups. The sample F5 is well differentiated from the other for the highest number of mesophilic and thermophilic lactobacilli. The cheeses comprising out from F7 to F10, were grouped on the basis of the highest number of thermophilic streptococci. Not significant correlations were found between single microbial groups and BA. Our results indicate that BA production in Pecorino di Farindola is a complex phenomenon which depends on several variables interacting with each other. It appears to be very important the role of pig rennet but also the study of isolates from different microbial groups could give important information to reduce the BA content in this particular Pecorino cheese.

3.4. CLA concentration in cheeses

In recent years, CLA have attracted strong interest in food science and medicine because some isomers are believed to have important physiological functionality. Biological activity has been attributed to two major isomers, cis-9, trans-11 and cis-12, trans-10 CLA (Banni et al., 2002; Belury, 2002; Pariza et al., 2001). The CLA content in cheese can be influenced by the geographical origin, because of the seasonal variations and the locally varying grass-fodder of free fed animals, as well as the initial CLA content of sheep milk, the ageing time, the temperature conditions of the cheese production and milk fermenting microorganisms (Sieber et al., 2004). In addition to the data on CLA, the composition of total fatty acids in Pecorino di Farindola cheeses was also investigated and the results are presented in Table 4. Saturated fatty acids were in relevant concentration in all the samples with an average content of 746.2 mg/g fat, followed by monounsaturated 212.5 mg/g fat and polyunsaturated 41.6 mg/g fat. Among the ten batches little variability was observed. As regard CLA content, cheeses showed an average content of about 10.15 mg/g fat, with highest value of 12.7 mg/g fat in sample F8. This high content of CLA in Pecorino di Farindola cheese may be probably related to the type of milk used for cheese production and animal feeding, in agreement with data reported by Prandini et al. (2007). Among 30 different cow cheeses, 8 goat cheeses and 12 ewe cheeses (Pecorino) they detected the highest amount of CLA in Fontina Valdostana, followed by Pecorino cheese. Fontina Valdostana is produced from raw milk from Valdostana cows bred in the highest Europe alpine pasture land such as Pecorino di Farindola from sheep bred in high Gran Sasso mountain pasture land. However the role of native microorganisms present in the microhabitat and during milk fermentation has to be considered relevant. Among lactic acid bacteria *Lactobacillus plantarum* and *Lactobacillus acidophilus* have been reported as the greatest CLA producer (Kishino et al., 2002). In the last decade several investigations on CLA bioconversion through common lactic acid bacteria and propionibacteria from dairy products were carried out (Sieber et al., 2004). Sample F8 with highest CLA content was characterized by the high number of lactic acid bacteria and thermophilic streptococci.

4. Conclusions

Pecorino di Farindola is characterized by two important features, the ewe raw full-cream milk obtained from animal feeding high pasture land (Gran Sasso mountain) and the use of pig rennet. Pecorino di Farindola cheeses showed high variability in microbiota counts and biogenic amine contents. The presence of high contents of BA in Pecorino di Farindola cheeses, such as for other Pecorino cheeses, could be related to the enzymatic activity of proteases derived from microorganisms, or from another origin (pig rennet) that is important from a qualitative point of view, i.e., in relation to the type of amino acids provided to the amino acid decarboxylating microbiota, in particular tyrosine. The bacteriological composition of milk could be critical to define the BA profile in cheese. Therefore, large amounts of BA in cheese could indicate unsuitability, from a hygienic point of view, of the milk used for cheese making. Moreover, results emphasize the need to control the indigenous bacterial population responsible for high production of BA and the use of competitive adjunct cultures is suggested. In addition, the presence of high CLA content in all the cheeses suggests the interest in carrying out further study on ewe raw milk composition used to produce Pecorino di Farindola cheese as well as on autochthonous microbiota able to convert linoleic acid to CLA, in order to select suitable strains potentially useful as starter cultures.

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