Quality evaluation and discrimination of semi-hard and hard cheeses from the Marche region (Central Italy) using chemometric tools

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ABSTRACT

Twenty-nine semi-hard and hard cheeses from the Marche region (Central Italy) made from either raw or pasteurised ewes’, goats’ and cows’ milk were analysed for pH, water activity, gross composition, bacterial ecology, and volatile compound profile. Two chemometric tools were used to analyse the available data: principal component analysis (PCA) for preliminary exploratory data analysis, and partial least square discriminant analysis (PLS-DA) for cheese classification by type of milk, treatment (raw versus pasteurised), and origin. PCA and PLS-DA of the outcomes allowed the cheeses to be grouped according to type of milk (cows’ versus ewes’ or goats’), and treatment (raw versus pasteurised), whereas no clear separation between Protected Designation of Origin, niche, and specialty cheeses from similar productions or industrial competitors was attainable. Among the variables, the free fatty acid profile showed a high potential for the discrimination of goats’ milk cheeses.

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1. Introduction

Pecorino and caprino are the terms commonly used to identify semi-hard or hard cheeses produced throughout Central and Southern Italy with ewes’ and goats’ milk, respectively, whereas caciotta refers to cheeses manufactured from cows’ milk or a blend of cows’ and ewes’ milk. Ripening of these three cheese varieties is carried out for periods varying in length, under either uncontrolled conditions or defined regimes of temperature and humidity. In the Marche region (Central Italy), an ample assortment of these cheeses is manufactured, mostly without a “designation of origin”, at industrial, semi-industrial and artisan plants. Except for Caciotta di Urbino, the sole Protected Designation of Origin (PDO) cheese, whose production must comply with strict regulations (Gazzetta Ufficiale No. 208, 6th September 1995), these cheeses can be made with either raw or pasteurised milk, using either natural or commercial starter cultures (Babini et al., 2010). For pecorino cheese, wild herbs, such as basil, sweet marjoram, thyme, and nutmeg may be added to the cheese milk or the rennet to add flavour to the curd (Aquilanti et al., 2007a). In long-ripened pecorino cheeses, olive oil may be used to oil the rind and hence to avoid the formation of surface fissures. For a few speciality and niche pecorino cheeses, maturation is performed in wooden barrels, chests, buckets, tubs, or earthen jars lined with layers of walnut leaves or in pits dug into tuffaceous rock, whereas for specialty caprino and caciotta cheeses, vegetable rennet, consisting of fig latex or aqueous extracts of dried flowers from the cardoon varieties *Cynara cardunculus* or *Cynara scolymus* is used instead of ovine rennet for milk coagulation (Aquilanti et al., 2011). As a consequence of the considerable differences in the cheese-making technologies, a wide variety of pecorino, caprino, and caciotta cheeses from the Marche region are available on the local, regional and even national market under the same designations.

In raw milk cheeses, usually produced without the use of a commercial starter culture and according to artisan procedures that require the intervention of experienced cheese makers, fermentation and ripening are carried out by a heterogeneous microbial population, which includes starter lactic acid bacteria (LAB), able to rapidly convert lactose into lactic acid, and non-starter LAB (NSLAB), which mainly contribute to the development of cheese flavour and aroma (Aquilanti, Zanini, Zocchetti, Osimani, & Clementi, 2007b). Although the consumption of dairy products made from raw milk is assumed to be a higher health risk for the potential occurrence of human pathogens, namely *Escherichia coli*, *Salmonella* spp., *Staphylococcus aureus*, *Brucella melitensis*, and *Listeria monocytogenes* (West, 2008), raw milk cheeses are generally perceived by consumers as food products with premium quality and higher flavour intensity (Colonna, Durham, & Meunier-Goddik, 2011; Muir, Banks, & Hunter, 1997). By contrast, pasteurised milk cheeses, which are generally produced by large-scale dairy
industries with modern technologies under carefully controlled conditions, are claimed to have a less intense sensory profile (Beuvier et al., 1997; Colonna et al., 2011; Muir et al., 1997), due to killing of much of the indigenous microbiota of the raw milk and replacement with a few selected starter strains (Psoni, Tzanetakis, & Litopoulou-Tzanetaki, 2006).

Over the last decade, there has been an increasing demand for artisan and even home-made cheeses manufactured with raw milk according to traditional procedures strictly linked to the territory, and characterised by unique features that make them worthy of being protected and distinguished from similar products manufactured on an industrial scale. This consumer trend has led to a great deal of interest in the definition of cheese safety, quality, and typical characteristics, the establishment of objective and verifiable variables to qualitatively and quantitatively evaluate the intrinsic cheese features, and the implementation of analytical strategies for the safeguard of public health and for consumer protection.

To the authors knowledge, until now only a few studies have been focused on the comparative evaluation of large numbers of traditional Italian cheeses belonging to the same variety (Coda et al., 2006; Di Cagno et al., 2007) to differentiate cheeses, make correlations between selected technological parameters and specific cheese traits, and identify potential markers for cheese authentication.

In this 3-year study, 29 different pecorino, caprino, and caciotta cheeses produced with either raw or pasteurised milk at artisan dairy farms or industrial plants mainly located in the Marche region were analysed for physico-chemical properties, gross composition, bacteriological, ecological, and volatile profile. The objective of the study was to check the possibility of identifying objective and verifiable variables to evaluate cheese quality and possibly differentiate PDO, speciality or niche cheeses from similar productions or industrial competitors, on the basis of cheese variety (pecorino, caciotta, caprino), cheese-making technology (use of raw versus pasteurised milk, ripening conditions, use of particular ingredients), or production area.

2. Materials and methods

2.1. Bacterial and yeast reference strains

Three reference strains were used for the construction of a denaturing gradient gel electrophoresis (DGGE) ladder, namely Lactobacillus brevis NCIMB 8664, Lactococcus lactis subsp. lactis DSM 20729, and Streptococcus thermophilus DSM 20617. These were obtained from the “National Collection of Industrial Marine and Food Bacteria” (NCIMB, Aberdeen, UK, www.ncimb.com) and the “Deutsche Sammlung von Mikroorganismen und Zellkulturen” (DSMZ, Braunschweig, Germany, www.dsmz.de).

2.2. Cheese sampling

Twenty-nine cheeses belonging to semi-hard or hard cheese categories were collected; these included 12 pecorino, 8 caprino, and 9 caciotta, cheeses. Nineteen cheeses made with raw milk were obtained directly from artisan dairies located throughout the Marche region, while the remaining ten cheeses, produced on a large scale (mostly in the region) with pasteurised milk, were purchased at retail level from supermarkets located in the district of Ancona (Marche, Italy). All cheeses were commercially ripe at the point of sampling; for each cheese, three wheels were collected.

For artisan cheeses, a questionnaire was compiled to examine the following aspects of the cheese-making process: type of cheese milk and other ingredients, type of starter cultures, coagulation agent, conditions and length of ripening. For industrial cheeses, the sole data reported on the label were recorded (Table S1, Supplementary material).

Each of the 29 cheeses under study was associated to a unique code including the reference to the type of milk used, either raw (r) or pasteurised (p), and the cheese variety, namely pecorino (pe), caprino (cp), or caciotta (ca). In more detail, the following cheeses were sampled and analysed: two pecorino cheeses ripened in pits dug into tuffaceous rock (r_8pe and p_3pe); one typical lemon-shaped pecorino cheese (cacio in forma di limone) manufactured by adding grated lemon peel on the cheese rind (p_4pe); one typical pecorino cheese (pecorino in botte) aromatised with local wild herbs and ripened in wooden barrels (r_7pe); two industrially produced pecorino cheeses (p_1pe and p_2pe); two pit caprino cheeses aged for approximately three months in pits dug into tuffaceous rock (r_1cp, and r_3cp); one short-ripened caprino cheese (r_2cp); three caprino cheeses produced at family-run dairy farms (r_4cp, r_5cp and r_6cp); two industrially produced caprino cheeses (p_1cp and p_2cp); one Cassiotta di Urbino PDO cheese (p_3ca) and one niche caciotta cheese made with a blend of cows’ and ewes’ milk coagulated with an aqueous extract of C. cardunculus dried flowers (p_4ca), both produced in the Montefeltro area; two niche caciotta cheeses produced at a small-scale dairy farm by adding extra-virgin olive oil from the local Raggia olive variety to cows’ milk prior to coagulation (r_2ca and r_5ca); three caciotta cheeses produced with a blend of cows’ and ewes’ milk (r_1ca, r_3ca, and r_4ca); two industrially produced caciotta cheeses (p_1ca and p_2ca).

All the cheeses were sampled when ready for commercialization; they had a cylindrical shape with a diameter of approximately 14–20 cm, a height of 7–10 cm, and a weight of 1–3 kg. Ripening time varied greatly, ranging from 2 wk to 8 m (Table S1, Supplementary material).

Cheese wheels were divided into three portions: one portion was immediately subjected to compositional analyses and viable counting while the other two were stored at –20 °C prior to DNA extraction and solid phase micro extraction coupled with gas chromatography (SPME-GC), respectively. For each portion, at least two independent measurements were made on samples taken from two different sections, namely under the rind, at approximately 1 cm, and at the core, at approximately 4 cm; the results were expressed as means of replicates ± standard deviations.

2.3. Physico-chemical analyses

The pH measurements were carried out using a pH-meter equipped with a solid electrode (HI2031, Hanna Instruments, Padua, Italy) which was inserted directly into the sample. The water activity (aw) was measured with an AWWIN electric hygrometer-indicator (International PBI, Milan, Italy).

2.4. Compositional analyses

The NaCl content was determined using the Volhard method (Reis et al., 2000), whereas total nitrogen and protein content was assessed according to the Kjeldahl method as previously reported by Malacarne, Formaggioni, Franceschi, Sumner, and Mariani (2006). The lipid fraction was extracted using petroleum ether and, after solvent evaporation, the fat content was determined gravimetrically. Detection and quantification of fat rancidity were assessed through the application of the Kreis test and the determination of the peroxide index (Martillotti, Antongiovanni, Rizzi, Santi, & Bittante, 1987), respectively.

2.5. Microbiological analyses

2.5.1. Viable counts

Microbiological analyses were carried out in the laboratory in accordance with the UNI CEI EN ISO/IEC 17025 European Standard.
Ten grams of each cheese sample were homogenised in 90 mL of sterile aqueous citrate 2% (w/v) solution in a Stomacher apparatus (400 Circulator, International PBI) for 2 min at 260 rpm. Serial dilutions were prepared in the same diluent and inoculated in duplicate by surface spreading on specific solid media. The following microorganisms were counted: (i) lactobacilli on MRS agar (Oxoid, Milan, Italy) incubated in sealed jars with the Anaerogen system (Oxoid) at 37 °C for 48 h; (ii) lactococci and thermophilic cocci on M17 agar (Oxoid), incubated at 22 °C for 48 h and at 45 °C for 24 h, respectively. Both media were added with cycloheximide to inhibit the growth of eumycetes (Osimani et al., 2009). The results of viable counts were expressed as means of the log of colony forming units (cfu) per gram of sample ± standard deviations.

2.5.2. Polymerase chain reaction-denaturing gradient gel electrophoresis

2.5.2.1. Bacterial DNA extraction from cheeses and bulk cells. The bacterial DNA was extracted directly from the cheese samples using the method described by Rantsiou, Urso, Dolci, Comi, and Coccoli (2008). Bulk cells were prepared as described by Ercolini, Moschetti, Blaiotta, and Coppola (2001). Briefly, colonies grown on MRS and M17 dilution plates, showing either confluent colonies or a number of colonies ranging from 30 to 300, were suspended in 1.5 mL of a sterile aqueous 1% (w/v) peptone solution, harvested with a sterile pipette, and transferred into 2-mL microtubes. To minimise the effect of differences in the cell concentration, bulk suspensions were standardised at 1 McFarland unit. Three hundred microlitres of each suspension underwent DNA extraction using the method described by Hynes, Ferretti, Gilmore, and Segarra (1992). The DNA quantity and purity were assessed by optical readings at 260, 280 and 234 nm, respectively, using a UV−Vis Spectrophotometer (Cary 1E Varian, Varian Medical Systems, Cernusco, Milan, Italy).

2.5.2.2. DNA amplification. The PCR primers U968-f (5′-AAC GCG AAG AAC CTG AC-3′) and L1401-r (5′-GGG TGT GGA CAA GCC CC-3′) were used to amplify the V6−V8 regions of the bacterial 16S rRNA gene (Randazzo, Torriani, Akkermans, De Vos, & Vaughan, 2002). The forward primer had at its 5′ end the additional 40-nucleotide GC-rich sequence (GC clamp) described by Muyzer, De Wall, and Uitterlinden (1993). One hundred and twenty nanograms of template DNA were amplified in a 25 μL reaction volume containing 0.625 μL of Taq DNA polymerase (EuroTaq, CelBio, Milan, Italy), 1 × reaction buffer, 3.0 mM MgCl₂, 0.2 μM dNTPs, and 0.1 μM of each primer. The amplification reactions were performed in a thermal cycler (Myycler, Bio-Rad, Milan, Italy) using the following cycling program: initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, elongation at 72 °C for 40 s, and a final elongation at 72 °C for 30 min. Five microlitres of each PCR product were analysed by electrophoresis in 1.5% (w/v) agarose gel, containing 2.5% (v/v) of an aqueous ethidium bromide solution (10 mg mL⁻¹), at 4 V cm⁻¹ (constant voltage) for 45 min, using a molecular weight standard (GeneRuler™DNA Ladder Mix, Fermentas, M-Medical, Milan, Italy). Gels were photographed under UV light (λ 320 nm) with a digital camera.

2.5.2.3. Denaturing gradient gel electrophoresis and sequencing of selected bands. The Dcode universal mutation detection system (Bio-Rad) was used for DGGE analysis of 20 μL aliquots of the PCR products. Electrophoresis was performed in a 0.8-mm polyacrylamide (8%, w/v):acylamide–bisacrylamide (37.5:1) gel with a denaturant gradient from 50.0 to 70.6% (100% denaturant was 7 M urea plus 40%, w/v, formamide) increasing in the direction of electrophoresis. Gels were subjected to a constant voltage of 130 V for 8 h at 60 °C; after electrophoresis, they were stained for 20 min in 1.25% TAE buffer (50 mM Tris–HCl, 25 mM acid acetic, 1.25 mM EDTA, pH 8.0) added with 0.01% of an aqueous ethidium bromide solution (10 mg mL⁻¹), rinsed in distilled water, and photographed under UV light with a digital camera.

The DGGE bands to be sequenced were excised from the gels with sterile pipette tips. The DNA was eluted with 50 μL TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0), and incubated overnight at 4 °C. Six microlitres of the elute from each DGGE band were re-amplified with the forward primer devoid of the CG-clamp, purified with micro-columns (GFX-PCR-DNA and Gel Band purification kit, GE Healthcare, Milan, Italy), and sent to M-Medical/Eurofins (Milan, Italy) for sequencing. To determine the closest known relatives of the partial 16S rRNA gene sequences, searches were performed in the GenBank public data library (www.ncbi.nlm.nih.gov) with the Blast search tools (Altschul, Gish, Miller, Myers, & Lipman, 1990). Only the alignments with an identity percentage ≥97% and an E-value ≥0.05 were taken into consideration for identification of the closest known relatives.

2.6. Solid phase micro extraction-gas chromatography with flame ionisation detector

Thirteen standard compounds (99% purity, Fluka, Sigma–Aldrich, Milan, Italy) were used. These included ethyl acetate, acetone, ethanol, acetic acid, and the following free fatty acids (FFAs): butyric (C4:0), caproic (C6:0), enantic (C7:0), caprylic (C8:0), pelargonic (C9:0), capric (C10:0), undecanoic (C11:0), undecenoic (C11:1) and lauric (C12:0) acid. For each compound, a pure standard stock solution was prepared in n-hexane at a final concentration of 150 μg mL⁻¹ and stored at −20 °C until use. These solutions were analysed both separately and in mixtures at different ratios to determine their specific retention time.

Cheese samples were finely ground and 3 g of each sample were added with deionised water (1 mL), placed in 15-mL glass vials sealed with PFTE/silicone rubber septa (Supelco, Bellefonte, PA, USA), and homogenised by vortex mixing for 3 min.

Vials were incubated at 60 °C for 40 min in a thermostatic bath (Bio-Rad) to establish equilibrium between headspace and sample. An SPME device equipped with a fibre assembly coated with an 85 μm polyacrylate (PA) film (Supelco) was used to sample the cheese volatile compounds. The SPME fibre was inserted into the vial through the septum and exposed to the headspace. Extraction of volatiles was performed at 60 °C for 30 min. Prior to use, the fibre was conditioned by performing two blank injections, at a temperature of 270 °C, to remove any possible contaminants. After the extraction step, the analytes were thermally desorbed from the fibre into the injection port operating at 230 °C in the splitless mode. A Shimadzu gas chromatograph model GC-2014 (Milan, Italy) equipped with a flame ionisation detector (FID) was used for the separation of analytes. Chromatographic separation was performed on a DB-WAX column (J&W Scientific, Agilent Technologies, Milan, Italy), 30 m × 0.25 mm i.d., 0.25 μm film thickness. The column temperature was held at 40 °C for 2 min during injection; it was then increased by 5 °C min⁻¹ to 230 °C, which was held for 10 min (total program time, 50 min) (Pinho et al., 2001). Nitrogen was used as carrier gas at a constant flow of 1.47 mL min⁻¹. Cheese volatiles were identified by matching their retention times with those of the pure standard compounds.

2.7. Statistical analyses

The data collected were subjected to one-way analysis of variance (ANOVA), along with the Tukey Kramer honestly significant
difference (HSD) using JMP v10 software (Cary, NC, USA) to determine the presence of significant differences among cheeses belonging to the same variety. Both principal component analysis (PCA) and partial least square discriminant analysis (PLS-DA) were also carried out, using SIMCA-P v11.5 software (UMETRICS, Vicenza, Italy), to check the possibility of identifying variables able to discriminate cheeses on the basis of variety, cheese-making technology (use of raw versus pasteurised milk, ripening conditions, use of particular ingredients), or production area.

3. Results and discussion

3.1. Physico-chemical and compositional analyses

The univariate analysis of variance revealed the occurrence of significant differences in pH and $a_w$ values among the 29 cheeses analysed (Table S1, Supplementary material). As expected, within each cheese variety, the lowest pH values were measured in pasteurised milk cheeses manufactured with commercial starter cultures, whose main activity is the early conversion of lactose into lactic acid, whereas significantly lower $a_w$ values were found in the cheeses with the longest maturation time.

Mean values of NaCl were in the range 0.46–3.51% (w/w); salt concentration is undoubtedly a significant parameter for both the microbiological and the physico-chemical characteristics of cheeses since it affects $a_w$, the composition of the microbial population and the enzymatic activities, especially proteolysis (Beresford, Fitzsimons, Brennan, & Cogan, 2001). The considerable variability in salt levels found in the cheeses analysed might feasibly be associated with differences in salting methods, which have not been considered in this study.

The raw protein content mean values ranged from 10.7 to 25.9% (w/w), 9.8 to 30.0% (w/w), and 14.6 to 31.9% (w/w) whereas fat values were within the ranges usually measured in cheeses under pecorino, and caprino cheeses, respectively. For both parameters, mean values of NaCl were in the range 0.46–3.51% (w/w); salt concentration is undoubtedly a significant parameter for both the microbiological and the physico-chemical characteristics of cheeses since it affects $a_w$, the composition of the microbial population and the enzymatic activities, especially proteolysis (Beresford, Fitzsimons, Brennan, & Cogan, 2001). The considerable variability in salt levels found in the cheeses analysed might feasibly be associated with differences in salting methods, which have not been considered in this study.

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3.2. Microbiological analyses

The PCR-DGGE approach first proposed by Ercolini et al. (2001) for the investigation of Southern Italian whey starter cultures was used to explore the microbial ecology of the cheeses. To that end, the bacterial DNA was extracted directly from both the cheeses and the bulk cells harvested from selected MRS and M17 dilution plates incubated at 37, and 22 or 45 °C, respectively. Despite the great potential of such an analytical approach for the disclosure of the microbial diversity of cheeses, to the authors’ knowledge, it has so far only rarely been exploited in dairy microbiology (Ercolini, Hill, & Dodd, 2003; Ercolini, Mauriello, Blaiotta, Moschetti, & Coppola, 2004; Ercolini et al., 2001; Van Hoorde, Verstraete, Vandamme, & Huys, 2008).

When the composition of the 29 bacterial communities was comparatively evaluated, a high variability was seen even within the same cheese variety (Table 1), although the two starter species usually used as dairy starter cultures, namely *Str. thermophilus* and *Lc. lactis* ssp. lactis, occurred in most of the cheeses analysed. This latter finding is in agreement with those of other studies revealing the major role played by these two microorganisms during fermentation (Beresford et al., 2001; Cogan et al., 1997; Randazzo, Pitino, De Luca, Scifo, & Caggia, 2008; Randazzo, Vaughan, & Caggia, 2006) and even early ripening (Rantsiou et al., 2008).

Members of the species *Leuconostoc mesenteroides* and *Lactobacillus casei/Lactobacillus paracasei/Lactobacillus rhamnosus* were also identified with a high frequency. For the microorganisms belonging to the *Lb. casei* group, the PCR-DGGE analysis of the 16S rRNA gene was not suitable for an

<table>
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<tr>
<th>Cheese variety</th>
<th><em>Str. thermophilus</em></th>
<th><em>Str. macedonicus</em></th>
<th><em>Lc. lactis</em> ssp. lactis</th>
<th><em>Lc. lactis</em> ssp. cremoris</th>
<th><em>Lc. raffinolactis</em></th>
<th>Lactococcus ssp.</th>
<th>Enterococcus ssp.</th>
<th><em>Lb. acidophilus</em></th>
<th><em>Lb. helveticus</em></th>
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<td>Caciotta</td>
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<td>pm (4)</td>
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<td>Pecorino</td>
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<td>(12 cheeses)</td>
<td>rm (8)</td>
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<td>pm (4)</td>
<td>1$^c$</td>
<td>3$^c$</td>
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<td>Caprino</td>
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<td>(8 cheeses)</td>
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<td>pm (2)</td>
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$^a$ PCR-DGGE analysis of the DNA extracted directly from the cheeses.

$^b$ PCR-DGGE analysis of the DNA extracted from the bulk cells harvested from the dilution plates (usually ~6) showing around 30 colonies.

$^c$ PCR-DGGE analysis of the DNA extracted from the bulk cells harvested from the dilution plates (usually ~1) showing >300 colonies.
unambiguous assignment to a definite species, as previously demonstrated by Chen, Wang, and Chen (2008) and Felis and Dellaglio (2007).

Several minority NSLAB, including homfermentative (Lactobacillus acidophilus, Lactobacillus helveticus) and hetero-
fermentative (Lactobacillus plantarum, Lactobacillus sakei, Lactobacillus curvatus, Lb. brevis, Lactobacillus fermentum) lactoba-
cilli, as well as carnobacteria, enterococci (Enterococcus faecalis, Enterococcus faecium, Enterococcus italicus), and lactococci (L. lactis ssp. cremoris, Lactococcus raffinolactis) were also found. As previ-
ously elucidated by De Angelis et al. (2001), NSLAB constitute a major part of the indigenous microbiota of cheeses, even when produced with thermophilic starters and represent one of the main fac-
tors in determining the typical features of cheeses. Except for caciotta, a higher percentage of NSLAB diversity was seen in raw rather than pasteurised and in long- rather than short-ripened cheeses, in line with what highlighted by McSweeney, Oltogallli, and Fox (2004).

In raw milk cheeses, the primary source of NSLAB is the cheese milk, whereas in pasteurised milk cheeses the occurrence of these bacteria is mainly ascribable to post-pasteurisation contaminations or to the presence of species surviving pasteurisation in a heat-
shocked state (Beresford et al., 2001).

The occurrence of enterococci in a few raw ewes’ milk cheeses was consistent with the detection of these microorganisms in other Italian raw ewes’ milk cheeses produced at farmhouses (Mannu & Paha, 2002) or small-scale dairy farms (Aquilanti, Dell’Aquila, Zannini, Zocchetti, & Clementi, 2006; Aquilanti et al., 2007a). These microorganisms were also found to be part of the dominant microbiota in typical Spanish and Greek raw goats’ and ewes’ milk cheeses (Scintu & Piredda, 2007). As previously elucidated, the potential vectors of adventitious microorganisms contaminating milk and cheese during cheese making and ripening are numerous and of various origin, including dirty animals, soiled udders, contaminated dairy utensils, and manufacturing equipment. Accordingly, a great variety of adventitious Gram-positive bacteria, including streptococci (Streptococcus bovis, Streptococcus macedonicus, Streptococcus saccharolyticus), staphylococci (Staphy-
ococcus pasteuri, Staphylococcus saprophyticus, Staphylococcus succinicus, Staphylococcus epidermidis, Staphylococcus xylosus, Staphylococcus equorum, Staphylococcus caseolyticus and Macro-
coccus caseolyticus, formerly Micrococcus caseolyticus), and even Gram-negative bacteria belonging to the family Enterobacteraceae (Hafnia alvei, E. coli, Enterobacter cloaceae, Serratia spp., and Shigella spp.) were sporadically found in raw rather than pasteurised milk cheeses (data not shown).

When the fingerprints obtained with the two PCR-DGGE approaches were comparatively evaluated, the following evidence emerged. Overall, the analysis of the cultivable communities allowed a significantly higher biodiversity to be disclosed, compared with the direct approach, although in some cases species not detected in the bulk cells were identified by analysing the DNA extracted directly from the cheeses (Table 1). These findings might be ascribed to the masking effect of the most abundant templates during PCR (preferential amplification) or to differences in the detectability threshold of targeted species (Ercolini, 2004). A further explanation for the culture-independent findings of species not detectable using culture-dependent techniques might be the amplification of nucleic acids extracted from dead but intact cells, which cannot be cultivated on selective solid media, as previously elucidated by Rantsiou et al. (2008).

Regarding the bulk cells analysis, although substrates and culture conditions commonly employed for the cultivation of different LAB groups were selected, microbial species, which did not fit their optimal development conditions, were also grown (data not shown), in line with the results previously obtained by other authors (Ercolini et al., 2001).

Overall the results of the PCR-DGGE analyses clearly demonstrated that either approach, taken singly, gives an incomplete picture of the real microbial diversity due to the introduction of bias. However, once combined, these approaches provide a detailed description of the cheese microbiota diversity. Similar conclusions were previously drawn from the analysis with the same PCR-DGGE approach of different fermented food products (Aquilanti et al., 2007c; Garofalo, Silvestri, Aquilanti, & Clementi, 2008).

### 3.3. Volatile compound profile analyses

As highlighted by different authors, SPME-GC is fundamental for cheese characterisation, quality evaluation and its correlation with the area and methodology of production, and potentially for cheese authentication (Gioacchini, De Santi, Guescini, Brandi, & Stocchi, 2010; Pillonel, Ampuero, Tabacchi, & Bosset, 2003). Compared with other techniques, it is characterised by high sensitivity and simple sample preparation, which does not require the use of solvents. In this study, the analysis of the cheese volatile profile was mainly focussed on linear short- and medium-chain free fatty acids (FFAs) with <12 carbon atoms, which can be generated by three main biochemical pathways: lactose fermentation, proteolysis, and lipolysis (Curioni & Bosset, 2002). Each FFA is characterised by a distinctive odorous note (Pinho, Ferreira, & Ferreira, 2002) and

<table>
<thead>
<tr>
<th>Lb. casei group</th>
<th>Lb. brevis</th>
<th>Lb. sakei/curvatus</th>
<th>Lb. delbrueckii ssp. delbrueckii</th>
<th>Lb. plantarum</th>
<th>Lb. homoiocchi</th>
<th>Lb. fermentum</th>
<th>Lb. coryniformis</th>
<th>Lc. mesenteroides sp.</th>
<th>Carnobacterium sp.</th>
<th>No. of diverse species detected</th>
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</tbody>
</table>

(Continued next page)
these compounds have been reported to greatly contribute to the
flavour of aged Italian cheeses (Randazzo et al., 2008; Ziino, 
Condurso, Romeo, Giuffrida, & Verzera, 2005). Except for lauric 
acid, the whole range of volatile compounds considered in this 
study was identified in at least one cheese (Table 2). Significant 
differences in their relative abundance were found among the 29 
cheeses, even within the same variety. In more detail, significantly 
higher levels of caproic, caprylic, and capric acids were found in 
goats’ milk cheeses, and especially in the two raw milk products 
ripened for approximately three months in sealed pits dug into 
tuffaceous rock (\(r_{1cp}\) and \(r_{3cp}\)). Even among ewes’ milk cheeses, 
significantly higher levels of the above-cited FFAs plus enantio-
pelargonic, and undecenoic acid were found only in the ewes’ milk 
cheese ripened in underground pits (sample \(r_{8pe}\)). As shown in a 
previous investigation, FFAs are particularly abundant in cheeses 
ripened in pits compared to those ripened under conventional 
conditions (Giacchini et al., 2010). This latter finding might be 
ascribed to the intense lipolytic activity of the complex microbial 
population selected by the specific pit environment, which is 
characterised by high relative humidity and a decreased oxygen 
concentration (Gobbetti et al., 1999).

When pasteurised and raw milk cheeses were comparatively 
evaluated, the latter were generally characterised by higher volatile 
compound contents irrespective of the cheese variety. This finding 
is consistent with those of previous investigations, where pas-
teurised and raw milk cheeses were compared based on their 
sensory profile and flavour chemistry (Fernández-García, 
Carbonell, & Nuñez, 2002; Van Leuven, Van Caelenberg, & 
Dirinck, 2008). As previously elucidated by Codà et al. (2006), 
this difference might be at least partially ascribed to indigenous lipo-
protein lipases in milk which are almost completely inactivated 
by pasteurisation.

### 3.4. Multivariate analyses of variance

Data from physico-chemical (\(\text{pH}, \omega_w\)) composition (NaCl, raw 
protein, and lipid), microbiological (viable counts of presumptive 
lactobacilli, lactococci and thermophilic cocci; occurrence of LAB 
and NSLAB species assessed by PCR-DGGE) and volatile profile 
(FFAs identified by SPME-GC) analyses were subjected to PCA and 
PLS-DA.

PCA allows a linear combination of \(n\) parameters to be calculated 
to maximise the distance between the observables (in this case the 
29 cheeses) in a multidimensional space and to make group 
assignments. PLS-DA makes it possible to better resolve PCA 
groupings and hence separates classes of observations on the basis 
of their \(X\)-variables.

For each analysis, data were visualised through a “scores plot”, 
in which each point represents an individual observable and a 
“loadings plot”, in which the relationships among the variables 
responsible for the separation and clustering of the observables 
can be identified.

As recommended for variables with non-normal distribution 
(Davis & Goldsmith, 1986), \(\text{pH}, \omega_w\), NaCl, protein, lipid, and viable 
counts were subjected to log-transformation prior to both PCA and 
PLS-DA. All data were mean-centred and scaled. 

PCA carried out on 29 observables and 38 variables yielded 
a two principal components (PCs) model, explaining 32.5% of the 
variance (data not shown). Through the analysis of the \(t_1/t_2\) scores 
plot (Fig. 1a), caciotta, pecorino, and caprino cheeses could not be 
neatly separated. As far as the latter cheese variety is concerned, 
a great dispersion along the first principal component (PC1) was 
seen. This high variability might be explained by the significant 
differences that emerged in the caprino cheese volatile profiles, 
which in turn could be attributed to the remarkable differences in 
the conditions and length of the ripening for the cheeses analysed. 
By contrast, a much lower dispersion was seen for ewes’ and cows’ 
milk cheeses, thus suggesting a higher similarity between these 
cheeses, whose close proximity to the origin of the axes indicates 
that they have average properties. No neat separation by raw versus 
pasteurised milk or production area was seen. Similarly, no sepa-
ration between PDO (Casciotta di Urbino, Formaggio di Fossa di 
Sogliano), speciality (Pecorino in botta, Cacio in forma di limone) or 
niche (Caserotto, Novella) cheeses from similar productions or 
industrial competitors was observed. On the one hand, this finding 
might be explained by the need to implement the analytical 
approach proposed. On the other hand, these results could be due 
to the progressive introduction of innovations in traditional cheese-
making procedures, such as the use of pasteurised rather than raw 
milk (as verified for the two PDO cheeses, Cacio in forma di limone, 
and Novella) or commercial instead of natural starter cultures (as 
for Pecorino in botta and Caserotto), which might be responsible for 
a standardization of the intrinsic characteristics of the cheeses. In 
the latter case, there is a real risk of manufacturing cheeses that 
have, at least partially, lost their typical traits because of the 
establishment of a standardised production system which is quite 
unrelated to the environmental location and cheese-making 
traditions.

The evaluation of the \(p_1/p_2\) loadings plot (Fig. 1b) revealed 
that, as expected, variables giving similar information grouped 
together. Interestingly \(\omega_w\) and viable counts of presumptiv 
lactobacilli, lactococci and thermophilic cocci were positively corre-
lated, being positioned close together on the same side of the plot 
origin, while they were negatively correlated to FFAs. When 
clusters of variables were compared to clusters of cheeses, it was 
evident that the first PC (PC1) distinguished between cheeses from 
different ripening periods, irrespective of the cheese variety. 
Indeed, two groupings of observables were seen by considering 
ripening time, one positioned on the left side of the plot, including 
short-ripened cheeses (2–16 wk of maturation), and the other, 
positioned on the right side, including long-ripened cheeses (20– 
32 wk). Previous studies aimed at investigating changes in the 
microbiological and physico-chemical characteristics of traditional 
cheeses from the Mediterranean basin during maturation and 
ripening have clearly shown that higher microbial counts and \(\omega_w\) 
values (Arenas, González, Bernardo Fresno, & Tornadito, 2004; De 
Souza, Rosa, & Ayub, 2003; García Fontán, Franco, Prieto, 
Tornadito, & Carballo, 2001) and, conversely, lower contents of 
FFAs and other volatile compounds are found in short- rather than 
long-ripened cheeses (Freitas & Malcata, 1998; Pinho, Ferreira, & 
Ferreira, 2003).

To evaluate the possibility to better discriminate cheeses, each 
cheese variety was modelled separately by disjoint PCA models and 
the specificity of the three models was computed according to the 
DmodX criterion on the critical values (\(D_{crit}\) with 95% confidence 
interval (Eriksson et al., 2006). No additional information was 
gained from the evaluation of the three separated models (data not 
shown).

PLS-DA resulted in a two components model and explained 
28.7% of the variance; this analysis gave information which was 
substantially analogous to that of PCA. However, the \(t_1/t_2\) score plot 
allowed a better discrimination of the three cheese varieties to be 
accomplished (Fig. 2, panel a) within each cheese variety, a 
distinction between raw vs pasteurized milk cheeses could even 
be highlighted in the three dimensional visualization of PLS-DA 
scores plot (data not shown). In both PCA and PLS-DA score plots, 
two caprino cheeses (\(r_{1cp}\) and \(r_{3cp}\)) were positioned far from the 
other cheeses belonging to the same variety. It is worth noting that 
both these goats’ milk cheeses had been ripened for approximately 
three months in sealed pits dug into tuffaceous rock. From the
Table 2
Compounds detected in the SPME-GC analysis (in brackets the corresponding odor notes).

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Total area</th>
<th>Ethylacetate</th>
<th>Acetone/ETOH</th>
<th>Acetic acid (vinaigre sour, sharp)</th>
<th>Butyric acid (cheesy, rotten, sharp)</th>
<th>Caproic acid (puentig, blue, cheese, sour)</th>
<th>Caprylic acid (goaty, waxy, soapy, musty, rancid, fruity)</th>
<th>Pelargonic acid (waxy, rancid)</th>
<th>Capric acid (goaty, fatty)</th>
<th>Undecanoic acid (oily)</th>
<th>Undecenoic acid (fatty, fruity rosy)</th>
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<td>Caciotta cheese</td>
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<td>0b</td>
<td>0c</td>
<td>238 ± 103a</td>
<td>803 ± 346a</td>
<td>0a</td>
<td>937 ± 268a</td>
<td>0a</td>
<td>885 ± 229a</td>
<td>0a</td>
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<td>0a</td>
<td>1160 ± 464</td>
<td>65 ± 17a</td>
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<td>0a</td>
<td>212 ± 35b</td>
<td>0a</td>
<td>205 ± 52b</td>
<td>0a</td>
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<td>r_3ca 9</td>
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<td>0a</td>
<td>26 ± 6bc</td>
<td>47 ± 32b</td>
<td>46 ± 9b</td>
<td>0a</td>
<td>54 ± 18b</td>
<td>0a</td>
<td>48 ± 20b</td>
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<td>0b</td>
</tr>
<tr>
<td>r_4ca 10</td>
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<td>0b</td>
<td>20 ± 12b</td>
<td>0b</td>
<td>53 ± 2b</td>
<td>21 ± 8b</td>
<td>0a</td>
<td>30 ± 4b</td>
<td>0b</td>
<td>27 ± 4b</td>
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<td>r_5ca 8</td>
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<td>0b</td>
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<td>23 ± 5b</td>
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<td>0b</td>
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<td>199 ± 27d</td>
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<td>133 ± 34cfg</td>
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<td>0b</td>
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<td>57 ± 13gh</td>
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<td>0b</td>
<td>0b</td>
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<td>570 ± 13c</td>
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<td>580 ± 0b</td>
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<td>0b</td>
<td>224 ± 130a</td>
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<td>7386 ± 3214a</td>
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<td>2516 ± 1665b</td>
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<tr>
<td>r_5cp 6</td>
<td>492 ± 4</td>
<td>0a</td>
<td>0a</td>
<td>0a</td>
<td>104 ± 0</td>
<td>115 ± 0</td>
<td>0a</td>
<td>138 ± 2b</td>
<td>0a</td>
<td>139 ± 0b</td>
<td>0b</td>
</tr>
<tr>
<td>r_6cp 9</td>
<td>1612 ± 17</td>
<td>0a</td>
<td>0a</td>
<td>0a</td>
<td>79 ± 0</td>
<td>0b</td>
<td>0b</td>
<td>25 ± 1b</td>
<td>0b</td>
<td>27 ± 0b</td>
<td>0b</td>
</tr>
<tr>
<td>r_7cp 12</td>
<td>6299 ± 2946</td>
<td>17 ± 16</td>
<td>52 ± 39</td>
<td>108 ± 116</td>
<td>795 ± 404b</td>
<td>2306 ± 1177b</td>
<td>0b</td>
<td>1366 ± 744b</td>
<td>0b</td>
<td>750 ± 327b</td>
<td>0b</td>
</tr>
<tr>
<td>r_8cp 7</td>
<td>2627 ± 277</td>
<td>0b</td>
<td>0b</td>
<td>0b</td>
<td>76 ± 4</td>
<td>403 ± 4</td>
<td>1023 ± 75b</td>
<td>0b</td>
<td>638 ± 60b</td>
<td>0b</td>
<td>416 ± 123b</td>
</tr>
</tbody>
</table>

* The number of peaks seen in the chromatograms together with the average peak area (× 10², arbitrary units) ± standard deviation (SD) of specific compounds. Superscript letters refer to one way analysis of variance (ANOVA) performed within each column and each cheese variety; different letters refer to significantly different values (P < 0.05).
loadings plot (Fig. 2, panel b), the variables acetic, butyric, enantic, caprilic, pelargonic, capric and undecanoic acids seem to be responsible for such a separation. This finding fully coincides with those of Gioacchini et al. (2010) who reported that pit cheeses are characterised by a high concentration of fatty acids, which in turn could be ascribed to the intense lipolysis exerted by the specific microbiota growing on the cheese rind during pit ripening and including coagulase-negative cocci (Fontana, Cappa, Rebecchi, & Cocconcelli, 2010) and eumycetes (De Santi et al., 2010).

The evaluation of the PLS-DA loadings plot (Fig. 2b) allowed a good understanding of the variables which contribute most to cheese clusterings. In more detail, *a*<sub>w</sub> was mainly responsible for the separation of caciotta cheeses, together with the occurrence of *Lb. helveticus*, *Lactobacillus delbrueckii* spp. *delbrueckii*, *Lb. sakei*/*Lb. curvatus*, *Lb. acidophilus*, *Lc. raffinolactis*, and *Lc. lactis* ssp. *cremonis*, which were exclusively found in the DGGE profiles of the cheeses belonging to this variety, and with the occurrence of *Lb. brevis*, which was found more frequently in the caciotta cheeses than in the other two cheese varieties (Table 1). As expected, caciotta cheeses were positioned on the extreme positive side of the PC1 axis, whereas pH, acetic acid and butyric, enantic, caprilic, pelargonic, and capric acids had high negative loadings (correlations) in the eigenvector of PC1 (Fig. 2b). This finding is explainable by the lower lipid content of cows’ milk with respect to ewes’ and goats’ milk (Engels, Dekker, de Jong, Neeter, & Visser, 1997) and to the shorter ripening time of cheeses belonging to this variety (Table S1, Supplementary material).

Finally, NaCl values and the occurrence of *Lb. plantarum*, *Lec. mesenteroides* and *Lactobacillus coryniformis* mostly contributed to the separation of pecorino cheeses. The first species seems to be particularly characteristic of this traditional Italian cheese, having been isolated from a number of pecorino cheeses produced in different Italian regions (Aquilanti et al., 2007a,b; De Angelis et al., 2001).

From the evaluation of the Variable Influence on Projection (VIP) plot of the PLS-DA model, all the variables considered had a good discriminatory power, being characterised by VIP values higher than 0.8 (data not shown), thus suggesting their appropriateness for the objective evaluation of the main intrinsic cheese characteristics.
4. Conclusions

The univariate analysis of variance in data referring to physico-chemical (pH, water activity), compositional (NaCl, protein, and lipid content), microbiological (viable counts of presumptive lactobacilli, lactococci and thermophilic cocci; occurrence of starter lactic acid bacteria and non-starter lactic acid bacteria species assessed by polymerase chain reaction-denaturing gradient gel electrophoresis) and volatile profile (free fatty acids identified by solid phase micro extraction coupled with gas chromatography). PLS-DA allowed a grouping of cheeses by type of milk (cows' versus ewes' or goats') and its treatment (raw versus pasteurised), whereas no clear separation between PDO, niche and speciality cheeses from similar productions or industrial competitors was attainable.

Among the variables considered, FFAs showed a high potential for the discrimination of goats' milk cheeses; a few compounds present in these cheeses (enalnic, pelargonic, capric, caprilic, acetie, butyric and undecanoic acids) seemed to be good candidates as markers of caprino cheeses ripened in pits dug into tuffaceous rock, according to a traditional technology.

The results preliminarily obtained in this study should encourage ongoing efforts to establish objective and verifiable variables to evaluate the intrinsic characteristics of traditional cheeses, to certify their specificity, to trace them to their production area, and possibly to differentiate PDO, speciality and niche cheeses from similar cheeses or industrially produced competitors for authentication purposes.

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