

Proteolysis and Microstructure of Piacentinu Ennese Cheese Made Using Different Farm Technologies¹

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ABSTRACT

The aim of this study was to provide the biochemical and structural characterization of Piacentinu Ennese cheese and to evaluate the impact of different farm technologies on cheese proteolysis and microstructure. Fifteen cheeses were manufactured according to traditional technology, i.e., from raw milk and farmhouse rennet in the absence of starter culture. Pasteurized milk, commercial rennet, and starter were used for production of 20 nontraditional cheeses. Proteolysis in Piacentinu Ennese cheese was monitored during a 2- to 10-mo ripening time. Low rates of overall proteolysis were observed in cheese, as percentages of total N soluble at pH 4.6 and in 12% trichloroacetic acid were about 11.40 and 8.10%, respectively, after 10 mo of age. Patterns of primary proteolysis by urea-PAGE showed that α_s -caseins were degraded to a larger extent than were β -caseins, although a considerable amount of both caseins was still intact after 10 mo. Reversed phase-HPLC analysis of the cheese peptide fractions showed a slow decrease in the levels of hydrophobic peptides coupled to increasing levels of hydrophilic compounds as the cheese aged. The structural characteristics of Piacentinu Ennese cheese were evaluated by scanning electron microscopy after 2, 4, and 6 mo of age. The micrographs showed a sponge-like structural network with a well-distributed system of empty spaces, originally occupied by whey and fat. The microstructure changed during cheese ripening to become more compact with cavities of smaller size. Farm technology significantly affected cheese proteolysis and microstructure. Nontraditional cheeses had higher levels of pH 4.6-soluble N and showed a larger hydrolysis of α_s -casein fractions by urea-PAGE analysis than did traditional cheeses.

Large differences between cheese-types also concerned the patterns of secondary proteolysis. Nontraditional cheeses had higher levels of 12% trichloroacetic acid-soluble N and showed larger proportions of free amino acids and hydrophilic peptides in the HPLC profiles of the corresponding 70% ethanol-soluble N fraction than traditional cheeses. Nontraditional cheeses also had a more open structure with a coarser and less continuous appearance than did traditional cheeses. A large amount of variability in cheese proteolysis and structure within nontraditional treatment reflected farm-dependent changes in manufacturing conditions related to the use of various types of rennet and starter.

Key words: Piacentinu Ennese cheese, proteolysis, microstructure, farm technology

INTRODUCTION

Piacentinu Ennese is a hard-pressed ewes' milk cheese of Sicily, exclusively produced in the homonymous province of Enna (Italy) in the amount of 35 tonne/yr. The traditional technology and tools used for cheese manufacture are almost equivalent to those for the Protected Denomination of Origin Pecorino Siciliano cheese (Gazzetta Ufficiale della Comunità Europea, 1996); the only distinctive difference is related to the addition of saffron to the milk before coagulation. This spice confers the cheese with a bright yellow color and potentially different sensory and health properties from other types of Pecorino cheeses (D'Auria et al., 2004). Piacentinu Ennese is traditionally made using raw milk, farmhouse rennet, and no starter culture; some producers use pasteurized milk, commercial rennet, and starter in the cheese production. The main purpose of this nontraditional methodology is to provide acceptable safety and standard levels of quality in cheese according to the increasing attention by the European Community policies toward food hygiene and safety aspects. Pasteurization of milk before cheese manufacture is intended to reduce microbial loads and eliminate pathogens and most of the spoilage microorganisms that may be present in milk (Grappin and Beuvier,

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1997). However, milk pasteurization is also known to adversely affect the development of many sensory properties of cheese. Generally, pasteurized milk cheeses ripen slower and develop a less intense and diversified flavor than raw milk cheeses in part because of the lower contents of free AA (FAA), fatty acids, and volatile compounds (McSweeney et al., 1993; Beuquier et al., 1997; Grappin and Beuquier, 1997). Milk pasteurization also affects cheese texture, giving rise to an open structure with numerous and irregular cavities that is less firm and more fracturable compared with that of raw milk cheeses (Creamer and Olson, 1982; Lau et al., 1991; Buffa et al., 2001). These differences in cheese quality have been ascribed to the deactivation of the indigenous microflora in pasteurized milk cheeses. McSweeney et al. (1993) observed that the less intense flavor in pasteurized milk compared with raw milk Cheddar cheese was due to the lower number and less diversified community of nonstarter lactic acid bacteria (NSLAB), such as mesophilic lactobacilli, in the former. The biodiversity of the native milk microflora is indeed accepted to give raw milk cheeses specific sensory properties and is considered to be a distinctive aspect of traditional cheeses. Replacing the native milk microflora by the standard culture starters may result in standardization of these attributes and loss of individual cheese personality.

The influence of pasteurization on proteolysis of cheese from cow milk (Lau et al., 1991; McSweeney et al., 1993; Beuquier et al., 1997; Skeie and Ardö, 2000), ewe milk (Ordóñez et al., 1999; Albenzio et al., 2001), and goat milk (Trujillo et al., 2002) has been largely described. These studies have shown that little consistency exists, among different cheese varieties, in relation to the influence of milk pasteurization on profiles of cheese proteolysis. Grappin and Beuquier (1997) reviewed the literature, concluding that pasteurization had a negligible and variable effect on primary proteolysis, dependent on the type of cheese. Its specific impact on casein hydrolysis is often partially or totally concealed by the influence that different manufacturing parameters and physicochemical characteristics, related to different cheese varieties, may exert on the activity of proteases. Otherwise, the negative impact of milk pasteurization on the levels of FAA and small peptides, obtained by affecting the populations and the activity of peptidases from raw milk flora, has been clearly demonstrated. In Cheddar cheese, the contribution of NSLAB to cheese ripening has been shown to be mainly at the level of FAA formation and cheese flavor intensification, whereas starter bacteria make a greater contribution to secondary proteolysis (Lane and Fox, 1996; Lynch et al., 1996, 1997). Changes in the type of rennet used for milk coagulation may potentially introduce

further variability in proteolysis profiles of cheeses. Traditional manufacturing of Piacentinu Ennese makes use of farmhouse rennet as a coagulant, which is obtained by macerating abomasa from young lambs in salted brine. Commercial rennet paste is instead used in nontraditional cheese production. Commercial coagulants are generally characterized with various enzymatic composition and clotting activities; therefore, they may produce different extents of cheese proteolysis. Prieto et al. (2004) reported significantly higher contents of both pH 4.6-soluble N and 12% TCA-soluble N in Leòn raw milk cheese made using commercial rennet compared with the cheeses made using farmhouse rennet.

Horne et al. (2005) recently showed that Piacentinu Ennese cheeses made using nontraditional technology clearly had different volatile fingerprints, both in numbers and types of volatile organic compounds present, from those made using traditional means. However, to date, little attention has been paid to proteolysis and microstructure of these cheeses. The objective of this study was to provide the biochemical and structural characterization of Piacentinu Ennese cheese and to evaluate the impact of different farm technologies on cheese proteolysis and microstructure during a 2- to 10-mo ripening time.

MATERIALS AND METHODS

Cheese Sampling

Piacentinu Ennese cheeses ($n = 35$) were collected from 7 farms in the province of Enna (Italy). Fifteen cheeses were produced according to traditional technology by coagulating raw milk via farmhouse lamb or kids' rennet in absence of starter cultures. Twenty cheeses were produced according to nontraditional technology, i.e., from pasteurized milk using various types of thermophilic or mesophilic starter cultures and commercial lamb or kids' rennet paste, depending upon farm. Details of cheese manufacture according to traditional and nontraditional technologies are reported and compared in Figure 1. After production, all cheeses were transported to the Experimental Dairy Plant of *CoRFi-LaC* to be aged 2 to 10 mo under the same conditions.

Compositional Analysis

Grated cheese samples were analyzed as follows: total N by the Kjeldahl method (IDF, 1993), fat by the Gerber method (IDF, 1997), and NaCl by the Volhard method (AOAC, 2000). Nitrogen soluble in an acetate buffer at pH 4.6 (**pH4.6SN**) and in 12% TCA (**TCASN**) were determined (Bynum and Barbano, 1985) and expressed as a percentage of the total N content. All of

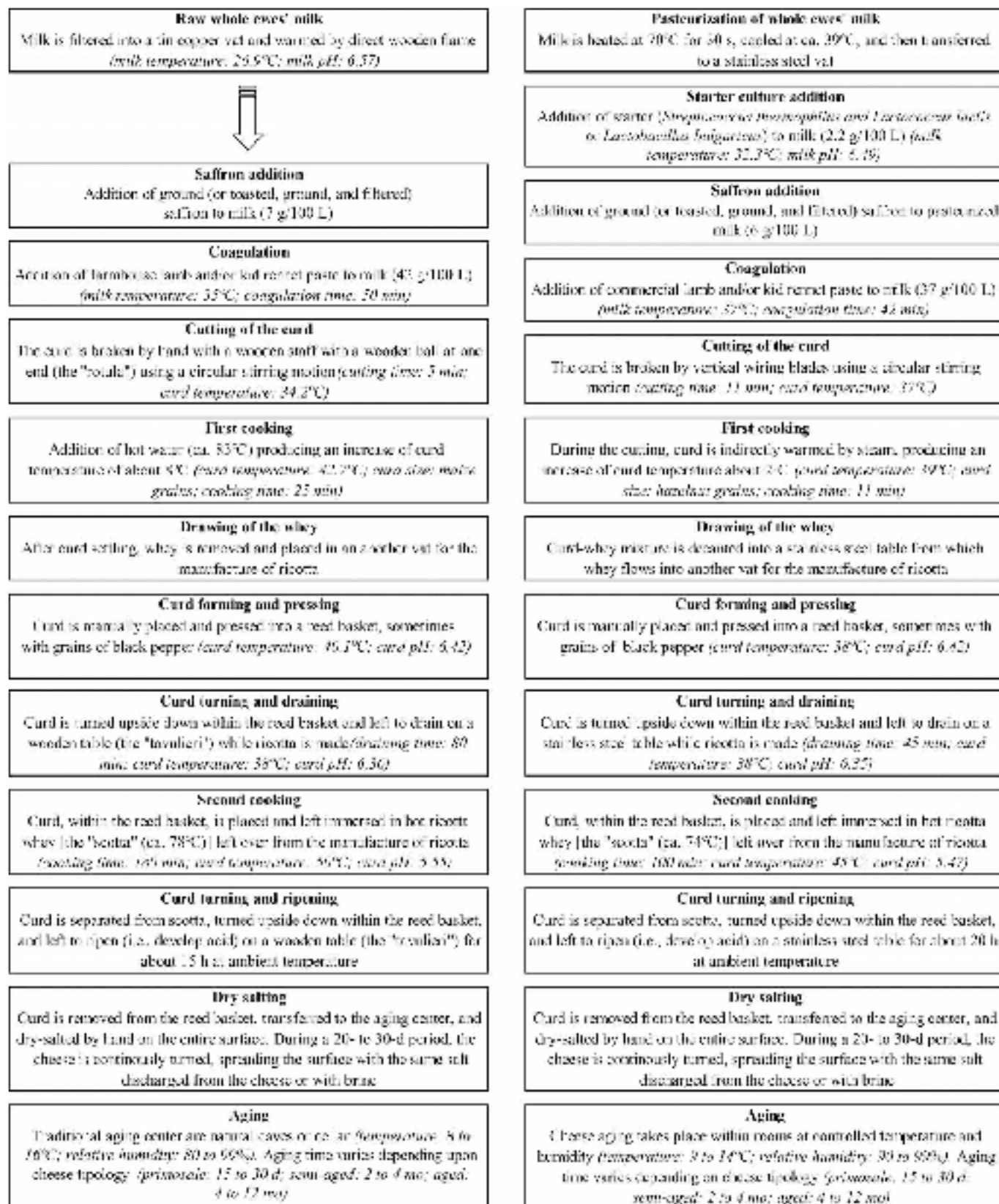


Figure 1. Protocol of Piacentinu Ennese cheese manufacture according to the traditional (left) and nontraditional (right) technology.

these analyses were made in duplicate. Total solids were determined in 4 replicates using a forced-air, oven-drying method at 100°C for 24 h (AOAC, 2000).

Assessment of Proteolysis

The pH 4.6-insoluble N of the cheeses was prepared as described by Fallico et al. (2004). These fractions were analyzed by urea-PAGE using a Protean Ixi vertical slab gel unit (BioRad Laboratories Ltd., Watford, Herts, United Kingdom) according to the method of Andrews (1983). The gels were stained using a modification of the method of Blakesley and Boezi (1977) with Coomassie Brilliant Blue G250. Cheese N fractions soluble (ESN) and insoluble (EIN) in 70% ethanol were prepared from pH4.6SN according to Kuchroo and Fox (1982). Freeze-dried aliquots of the ESN and EIN cheese extracts were analyzed by reverse phase-HPLC (RP-HPLC) as described by Lynch et al. (1996).

Statistical Analysis

A 2-way nested ANOVA model was used to assess the effects of farm technology (traditional or nontraditional) and aging time (2, 4, 6, 8, and 10 mo) on 8 chemical variables that were treated as dependent variables. Farm technology and cheese age were treated as fixed main effects; farm was treated as a random effect and was nested within farm technology. When significant main effects were found ($P \leq 0.05$), specific mean differences were determined using Tukey's Honestly Significant Difference test. Univariate analyses were performed using procedures GLM in SAS v.8.2 (SAS Inst., Inc., Cary, NC).

Scanning Electron Microscopy

A cylinder, 6 cm in length \times 1 cm in diameter, was extracted from each cheese using a cork borer; three 1-mm thick slices were removed from approximately the center of each cylinder to give samples deriving from the medium zone of the cheese. A square portion (4 mm \times 4 mm) was then removed from the center of each round slice by a blade. Cheese samples were prepared for scanning electron microscopy (SEM) analysis according to the procedure of Rousseau (1988) with some modifications. Samples were fixed in 2.5% (wt/vol) glutaraldehyde in a 0.1 M sodium cacodylate buffer (Sigma-Aldrich, Steinheim, Germany) (pH 7.2) for 3 d at 4°C. Fixed samples were rinsed with the cacodylate buffer for 10 min at room temperature. This step was repeated 3 times. Dehydration was carried out using a graded ethanol (Fluka, Sigma-Aldrich) series (10, 25, 50, 75, 85, and 95%, vol/vol) for 10 min in each bath at

room temperature and then absolute ethanol for 1 h at 4°C. After these steps, absolute ethanol was removed, and new absolute ethanol was added for maintenance at room temperature. Samples were dried by the critical point method in CO₂ using a Polaron CPD 7501 (Polaron, Watford, UK) and then fractured into small pieces by a blade. Fractured dried samples were mounted on SEM aluminum stubs using a carbon adhesive (SPI Supplies, West Chester, PA) and then coated (ca. 18 nm thick) with gold-palladium in a Polaron SC7620 mini-sputter coater in argon medium. Cheese samples were examined in an JEOL JSM-5900-LV scanning electron microscope (JEOL, Tokyo, Japan) with a voltage of 15 kV and a working distance of 15 to 22 mm. Images were recorded at 1,600 and 3,300 \times magnification.

RESULTS AND DISCUSSION

Chemical Composition

Overall means for the gross composition of Piacentinu Ennese as functions of cheese age and farm technology, respectively, are shown in Table 1. Aging time had the largest impact on cheese composition, significantly ($P < 0.05$) affecting the changes in all chemical parameters except for the content of fat in DM. Mean values for this parameter ranged from 50.7 to 52.8%, approaching those reported for Idiazábal and Manchego cheeses and exceeding those for Roncal (Freitas and Malcata, 2000) and Canestrato Pugliese (Albenzio et al., 2001). Levels of protein and salt-in-moisture (S/M) significantly ($P < 0.05$) increased during cheese ripening as a result of the significant decrease in moisture content. The S/M levels of Piacentinu Ennese cheese at the end of the ripening (12.80%) were higher than those of Canestrato Pugliese (8.9%) and Fiore Sardo (5.6%) but were much lower than those of Pecorino Romano (24.5%); all cheeses ripened 12 mo (Di Cagno et al., 2003). Differences in S/M levels among these cheeses were mainly related to different dry salting times. The manufacture of Piacentinu Ennese cheese includes a dry salting for 20 to 30 d. The same process lasts only 4 to 6 d in Canestrato Pugliese and Fiore Sardo, but is more prolonged (30 to 60 d) in Pecorino Romano cheese (Medina and Nunez, 2004). Proteolysis in cheese is often measured by means of quantification of cheese pH4.6SN and TCASN buffers. The majority of pH4.6SN is made up of large- and medium-sized peptides, produced by the action of residual rennet and plasmin, but it also contains small-sized peptides, FAA, and their catabolites produced by microflora. Conversely, TCASN consists primarily of small peptides, FAA, and other minor nitrogenous compounds produced by both starter and nonstarter bacteria (Sousa et al., 2001). Both pH4.6SN

Table 1. Overall means (\pm SD) for the effects of aging time and farm technology on the gross composition of Piacentinu Ennese cheese¹

	Aging time (mo)					Farm technology	
	2 (n = 7)	4 (n = 7)	6 (n = 7)	8 (n = 7)	10 (n = 7)	Traditional (n = 15)	Nontraditional (n = 20)
pH	5.30 ^{ab}	5.26 ^b	5.32 ^{ab}	5.32 ^{ab}	5.37 ^a	5.28 \pm 0.17	5.34 \pm 0.14
Moisture, %	32.49 ^a	30.23 ^b	28.02 ^c	27.19 ^c	26.69 ^c	28.75 \pm 2.75	29.05 \pm 2.70
Protein, %	26.83 ^c	27.65 ^{bc}	28.80 ^{ab}	29.15 ^{ab}	29.67 ^a	29.11 \pm 1.85	27.90 \pm 1.92
Fat in DM, %	52.77	50.96	51.71	52.26	50.70	51.35 \pm 3.11	51.93 \pm 2.64
S/M, %	8.86 ^b	11.23 ^a	11.33 ^a	12.27 ^a	12.80 ^a	10.45 \pm 2.81	11.94 \pm 4.08
pH4.6SN/total N, %	7.87 ^b	11.35 ^a	9.62 ^{ab}	10.04 ^{ab}	11.40 ^a	8.16 \pm 1.68 ^b	11.48 \pm 2.20 ^a
TCASN/total N, %	3.90 ^c	5.31 ^b	5.59 ^b	5.97 ^b	8.10 ^a	4.15 \pm 1.25 ^b	6.99 \pm 1.95 ^a
TCASN/pH4.6SN, %	49.84 ^{bc}	46.60 ^c	57.14 ^{bc}	58.54 ^b	71.39 ^a	51.26 \pm 12.35	60.78 \pm 12.12

^{ab-c}Means sharing common subscripts were not significantly different ($P < 0.05$).

¹Protein = total N \times 6.38, S/M (salt-in-moisture) = $[(\text{NaCl})/(\text{moisture content})] \times 100$; pH4.6SN/total N = pH 4.6 acetate buffer-soluble N as a percentage of total N; TCASN/total N = 12% TCA-soluble N as a percentage of total N; and TCASN/pH4.6SN = 12% TCA-soluble N as a percentage of pH4.6 acetate buffer-soluble N.

and TCASN, expressed as a percentage of total N, significantly ($P < 0.05$) increased in Piacentinu Ennese as the cheese aged. However, both proteolysis indices were much lower compared with other ewes' milk cheese varieties, such as Canestrato Pugliese, Fiore Sardo, Pecorino Romano (Guinee and Fox, 1984; Di Cagno et al., 2003), Fossa (Gobbetti et al., 1999), and Idiazábal (Mendia et al., 2000), indicating low rates of overall proteolysis. The high levels of S/M characterizing Piacentinu Ennese cheese throughout aging (8.86% at 2 mo up to 12.80% after 10 mo) might have exerted a negative influence on the rate and extent of casein hydrolysis. Increasing S/M is known indeed to inhibit rennet and microbial activities in cheese for different reasons. Proteolysis of caseins, mainly of β -CN, by coagulant has been found to be significantly reduced by 5% S/M and completely inhibited in the presence of 10% S/M (Fox and Walley, 1971). Otherwise, the enzymatic activities of microflora are limited by a lowering of water activity resulting from the decrease in moisture associated to increasing salt (Beresford and Williams, 2004). Among the cheese varieties compared, only Pecorino Romano had higher levels of S/M than Piacentinu Ennese cheese at similar stages of ripening (Guinee and Fox, 1984; Di Cagno et al., 2003). Finally, the ratio of TCASN to pH4.6SN, measuring the "depth" of cheese proteolysis, significantly ($P < 0.05$) increased with the age of Piacentinu Ennese as well. This finding suggested that large- and medium-sized peptides, produced by primary action of chymosin and plasmin on caseins, were progressively converted into small peptides and FAA by the microflora peptidases acting during cheese ripening (Beresford and Williams, 2004).

Rates of proteolysis in Piacentinu Ennese cheese were found to be significantly ($P < 0.05$) affected by farm technology. Levels of pH4.6SN and TCASN, expressed as percentages of total N, were significantly (P

< 0.05) higher in nontraditional cheeses than in traditional counterparts. These findings were in disagreement with most of the results from similar studies, where cheeses made from pasteurized milk are generally reported to have similar or lower levels of pH4.6SN and TCASN than raw milk cheeses (Gaya et al., 1990; Lau et al., 1991; Beuvier et al., 1997; Albenzio et al., 2001). However, it is known that contradictory results have been described for the effects of milk pasteurization on primary and secondary proteolysis, depending on the cheese variety (Grappin and Beuvier, 1997). Researchers working on Cheddar (Rosenberg et al., 1995), Idiazábal (Mendia et al., 2000), Serra da Estrela (Sousa and Malcata, 1996), and semi-hard goats' milk cheeses (Trujillo et al., 2002) obtained results consistent with our findings; i.e., cheeses made from pasteurized ewes' milk with native added starter had the highest levels of soluble nitrogenous fractions. Prieto et al. (2004) studied the effects of the type of coagulant on proteolysis of León cows' milk cheese and reported significantly higher contents of pH4.6SN and TCASN in cheeses made using commercial rennet than in those made using farmhouse rennet. Differences in the levels of proteolysis between nontraditional and traditional cheeses might have been caused also by changes in the levels of residual coagulant, as a greater retention of rennet was reported in cheeses made from pasteurized milk compared with those made from raw milk (Gaya et al., 1990). A large contribution of plasmin to primary proteolysis of nontraditional cheeses may also be suggested, as its activity is known to be increased by the heat processing of milk (Grappin and Beuvier, 1997). The higher levels of TCASN in nontraditional compared with traditional cheeses were probably due to the larger impact that starter bacteria exert on secondary proteolysis compared with NSLAB (Lane and Fox, 1996; Lynch et al., 1996, 1997). The highest levels of thermophilic

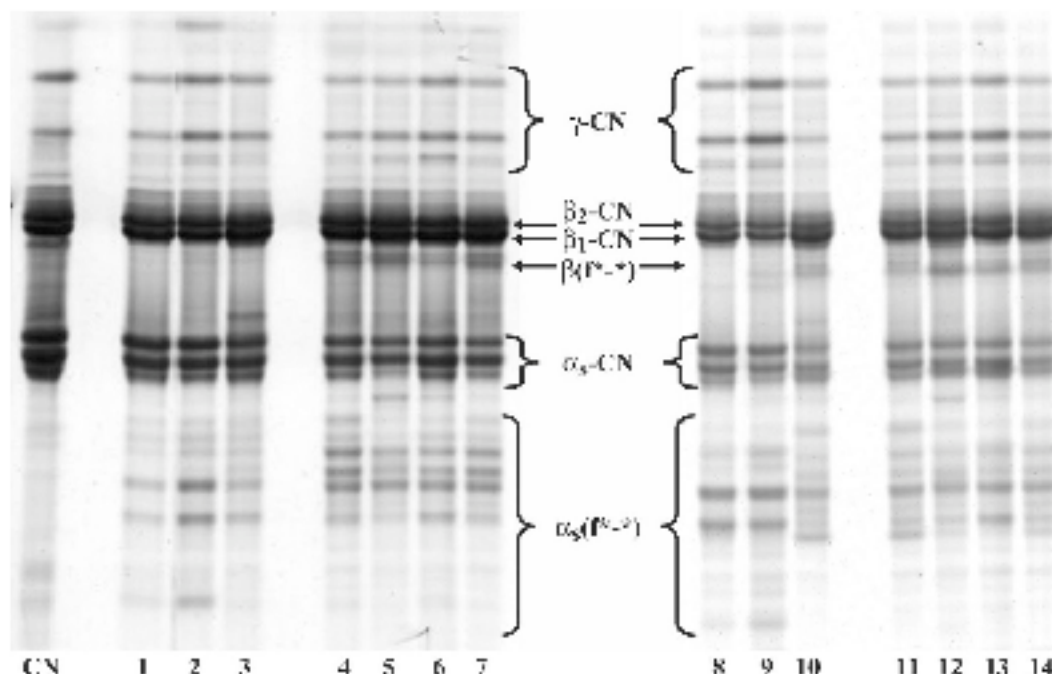


Figure 2. Urea-PAGE (pH 8.6) of pH 4.6-insoluble N fraction of Piacentinu Ennese cheese made using the traditional technology after 2 (Lanes 1 to 3) and 10 mo (Lanes 8 to 10) of age, in comparison with cheeses made using the nontraditional technology after 2 (Lanes 4 to 7) and 10 mo (Lanes 11 to 14) of age. Whole ovine casein was used as a reference standard.

lactic acid bacteria found in nontraditional cheeses by Horne et al. (2005) support this hypothesis. Finally, despite the generally higher mean values for nontraditional cheeses compared with traditional cheeses, farm technology had no significant ($P > 0.05$) influence on the remaining chemical parameters.

Electrophoretic Analysis

Primary proteolysis in Piacentinu Ennese cheese produced according to different farm technologies was compared by urea-PAGE analysis of the pH 4.6-insoluble N fraction (Figure 2). Native caseins in ewes' milk cheeses are distributed within 2 groups of bands showing different electrophoretic mobility. The group with lower mobility consists of 2 genetic variants (β_1 and β_2) of β -CN, which differ in the level of phosphorylation (6 and 5 phosphate groups, respectively). The group with higher mobility is formed instead with 3 fractions of α_s -CN, whose microheterogeneity is due to different degrees of glycosylation or phosphorylation coupled with genetic polymorphism (Sousa and Malcata, 1998). Moderate hydrolysis of α_s -CN and β -CN fractions were common features of both traditional and nontraditional cheese profiles after 2 mo of age. A slight increase in proteolysis occurred in both cheese-types during aging, although a considerable amount of native caseins remained intact after 10 mo of age. This finding was in

agreement with the very low level of primary proteolysis, measured as pH4.6SN, that was found in Piacentinu Ennese (Table 1). Regardless of cheese-type and age, the α_s -CN fractions were degraded to a larger extent than β -CN, which was similar to the trends reported for Canestrato Pugliese (Albenzio et al., 2001), Fiore Sardo, Pecorino Romano (Di Cagno et al., 2003), and Roncal (Irigoyen et al., 2000). Chymosin is thought to be largely inactivated by the high cooking temperatures (50 to 55°C) used during the manufacture of most hard cheese varieties (e.g., Parmigiano Reggiano, Swiss-type cheeses; Sousa et al., 2001). However, the lower temperatures (45°C) used for cooking of curd in Pecorino cheeses, compared with the hard varieties, probably result in larger residual rennet activity producing extensive hydrolysis of α_s -CN. Minor degradation of β -CN at the beginning of cheese ripening was associated with a small production of γ -CN, indicating limited activity of plasmin. Although increasing levels of γ -CN were found as the cheese aged, plasmin seemed to play a minor role in the proteolytic maturation of Piacentinu Ennese.

Farm technology affected the electrophoretic profiles of primary proteolysis in cheese, varying the patterns of peptides produced. A larger hydrolysis of α_s -CN fractions occurred in nontraditional cheeses compared with traditional cheeses, leading to the production of a greater number of peptides moving in the high mobility

side of the gel. Similar electrophoretic profiles were reported for Canestrato Pugliese (Albenzio et al., 2001) and Idiazábal (Mendia et al., 2000) ewes' milk cheeses. Nontraditional cheese also had a higher level of pH4.6SN (Table 1), made up of nitrogenous compounds that are principally due to the action of residual coagulant. Both findings suggested an increased activity of coagulant in nontraditional cheeses, which was probably related to the use of commercial rennet that was reported to have higher enzymatic activity than farmhouse rennet (Prieto et al., 2004) and was also related to the greater retention of coagulant caused by milk pasteurization (Gaya et al., 1990). Plasmin is known to contribute to pH4.6SN and to determine the highest levels of proteolysis in cheeses from pasteurized milk also (Grappin et al., 1985). Unexpectedly, plasmin activity seemed to be unaffected by thermal treatment of milk, as similar levels of γ -CN were measured between traditional and nontraditional cheeses (results not shown). This finding was consistent with the general trend that showed great resistance of β -CN fractions to hydrolysis during aging of Piacentinu Ennese. McSweeney et al. (1993) found no difference in the level of β -CN hydrolysis between pasteurized and raw milk Cheddar cheese, and similar results were obtained by others working on different cheese varieties, such as Arzua (Centeno et al., 1994) and Canestrato Pugliese (Albenzio et al., 2001). These results have been attributed to a lower level of plasmin activity compared with other hard cheese varieties (e.g., Swiss-type and Manchego) or, more likely, to the elimination of raw milk microflora caused by the pasteurization process (Grappin and Beuquier, 1997). Finally, the largest extent of primary proteolysis in nontraditional cheeses distinctively produced 2 peptides migrating faster than β -CN in the correspondent electrophoretic profiles.

RP-HPLC Analysis of EIN and ESN

Chromatograms of the EIN and ESN fractions of traditional and nontraditional cheeses at different ages (2, 4, 6, 8, and 10 mo) are shown in Figures 3 and 4, respectively. Farm technology was found to affect the qualitative HPLC profiles of Piacentinu Ennese cheese. A larger proportion of peptides eluted at high acetonitrile concentrations in the chromatograms of the EIN fraction of traditional cheeses compared with NT counterparts, mainly at the beginning of cheese ripening (Figure 3). Differences in peptide profiles between cheese-types became less evident as the cheeses aged; hydrophobic peak heights progressively decreased, and eluting peaks appeared early. Qualitative differences were also found between the ESN fractions of traditional and nontraditional cheeses by RP-HPLC analysis

(Figure 4). Larger amounts of FAA and hydrophilic peptides eluted in the first 35 min of chromatograms of nontraditional cheeses compared with those of traditional cheeses; these latter cheeses showed a higher proportion of hydrophobic peptides. Larger amounts of soluble compounds in the ESN cheese fraction were in agreement with the higher levels of TCASN (Table 1) that were found in nontraditional compared with traditional cheeses. These findings suggested that peptidase activities from commercial starters made a greater contribution to secondary proteolysis in Piacentinu Ennese cheese than raw milk microbiota. The highest levels of thermophilic lactic acid bacteria found in nontraditional cheeses by Horne et al. (2005) supported this hypothesis. However, it was likely that the enzymatic activities of adventitious microflora surviving the pasteurization process or deriving from the manufacturing environment (Beresford and Williams, 2004) also provided a contribution to the larger production of soluble peptides and FAA in nontraditional cheeses. Farm-dependent differences were found among the HPLC profiles of traditional and nontraditional cheeses, showing that there was also a large amount of variability within farm technology.

Literature data concerning the influence of milk pasteurization on cheese peptide profiles were again almost contradictory. In agreement with our findings, Albenzio et al. (2001) observed large differences between the HPLC profiles of raw and pasteurized milk Canestrato Pugliese cheese; the latter lacked some of the peptides in the hydrophobic zone. Gomez et al. (1997) and Trujillo et al. (2002), instead, found similar levels of hydrophobic and hydrophilic peptides in the water-soluble fraction of Hispanico and of a semi-hard goats' milk cheese, respectively, made from pasteurized and raw milk. The reports of Lau et al. (1991) and McSweeney et al. (1993) for Cheddar cheese were in opposition to our findings. Cheddar made from pasteurized milk had a generally higher concentration of hydrophobic peptides, resulting in a higher hydrophobic to hydrophilic peptide ratio than raw milk cheese. Both groups of researchers attributed these differences to the aminopeptidase activities of NSLAB.

Cheese Microstructure

The structural characteristics of traditional and nontraditional Piacentinu Ennese cheese after 2, 4, and 6 mo of age are shown in Figure 5. The protein matrix of traditional cheese (Figure 5a, b, c) has a sponge-like structure permeated by a well-distributed system of empty spaces that were originally occupied by fat globules and whey. Casein micelles join each other to form clusters and strands with no defined orientation; this

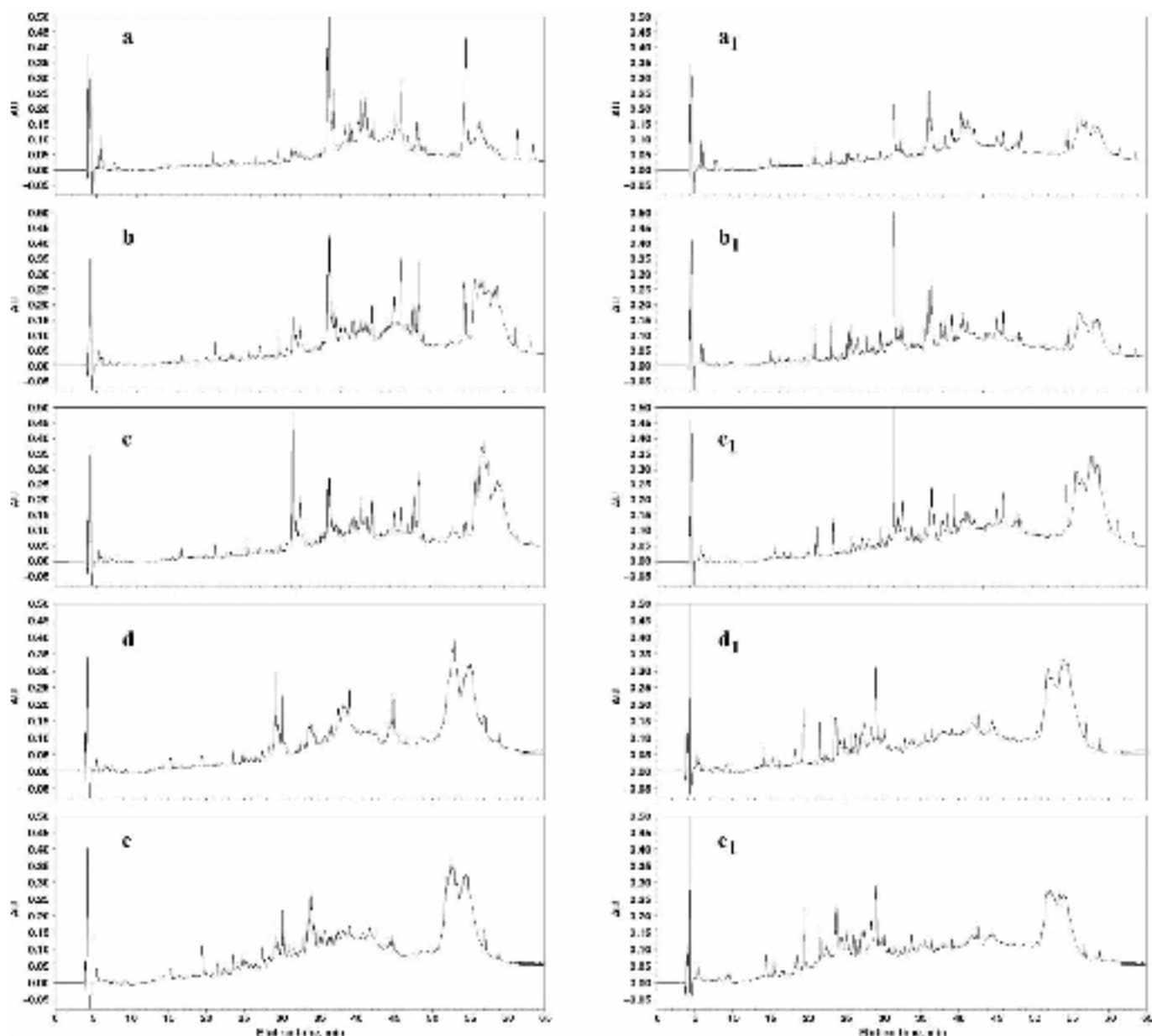


Figure 3. Reversed phase-HPLC chromatograms of the 70% ethanol-insoluble N (EIN) fraction of Piacentinu Ennese cheese made using traditional (left) or nontraditional (right) technology after 2 (a, a₁), 4 (b, b₁), 6 (c, c₁), 8 (d, d₁), and 10 mo (e, e₁) of age.

imparts to Piacentinu Ennese an amorphous texture as observed in Edam, Gouda (Kalab, 1977), and Prato cheeses (Spadoti et al., 2004). Traditional cheese aged for 2 mo had large empty gaps in the protein matrix, which conceivably held moisture. Over the maturation period (4 to 6 mo), the microstructure changed to become more compact, with cavities of smaller size, as a result of the gradual loss of moisture caused by the evaporation at the cheese surface. The protein matrix of nontraditional vs. traditional Piacentinu Ennese cheese (Figure 5d, e, f) had a coarser and less continuous

appearance; this reflected a poorer curd fusion and a more porous matrix. Similar differences were reported by Buffa et al. (2001) for the microstructure of a goats' milk cheese produced from raw and pasteurized milk. The more open structure observed in nontraditional cheeses might have been determined by increased hydration of the protein matrix resulting from milk pasteurization. This effect is known to be a consequence of the decreased fusion of casein micelles caused by the formation of a complex between denatured β -LG and κ -CN and to the lower level of soluble Ca in cheese

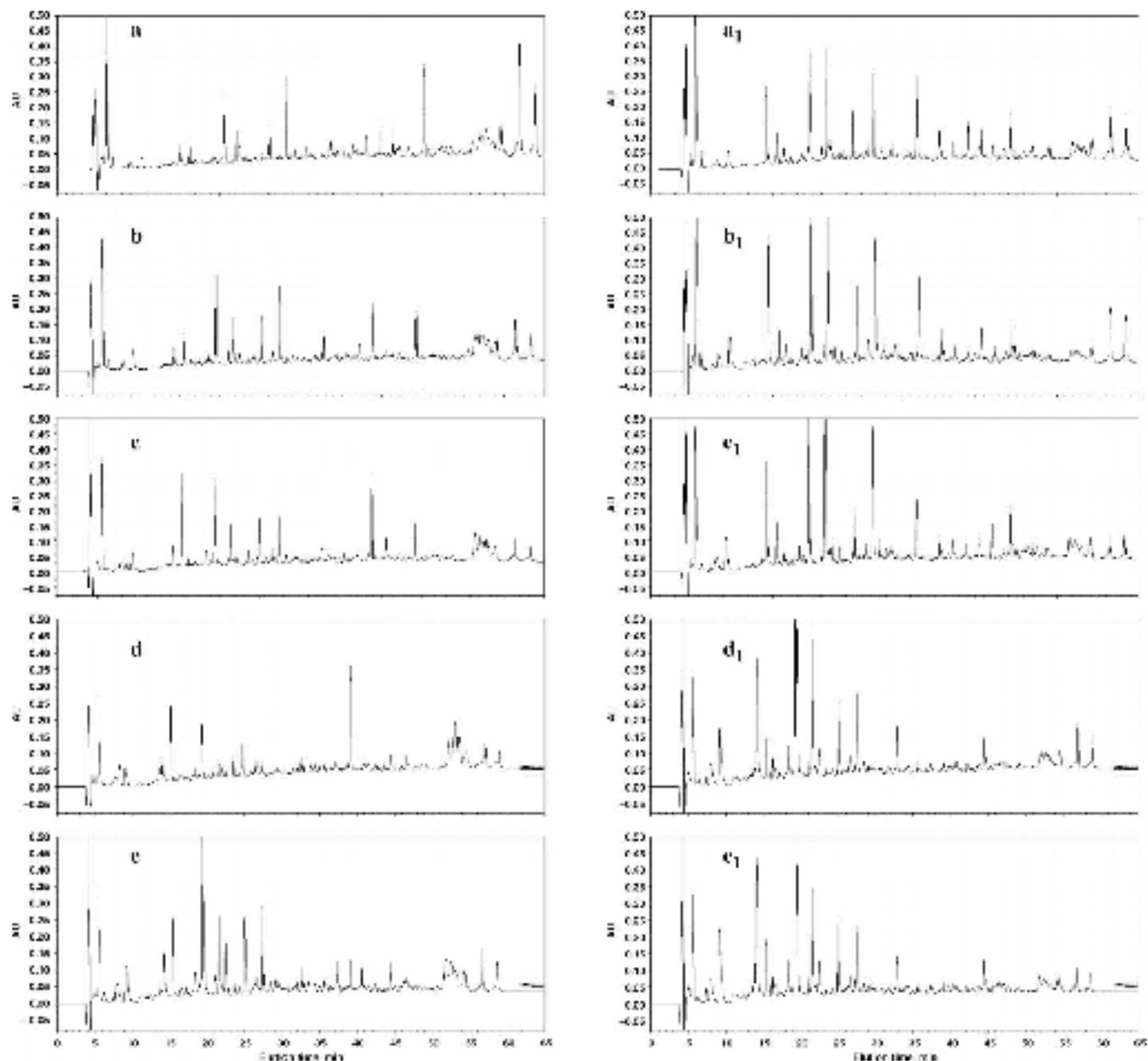


Figure 4. Reversed phase-HPLC chromatograms of the 70% ethanol-soluble N (ESN) fraction of Piacentinu Ennese cheese made using traditional (left) or nontraditional (right) technology after 2 (a, a₁), 4 (b, b₁), 6 (c, c₁), 8 (d, d₁), and 10 mo (e, e₁) of age.

produced by heat treatment of milk (Singh and Waungana, 2001; Pastorino et al., 2003). The more open curd structure found in nontraditional cheese was also associated with a higher level of primary proteolysis, in agreement with findings on a semi-hard cheese. Guinee et al. (1995) reported that the lower degree of curd fusion and the greater surface area of the protein matrix, at the protein-void interface, increased the accessibility of the paracasein to proteinases and thereby contributed to higher level of primary proteolysis in cheese.

Scanning electron microscopy examination of traditional and nontraditional Piacentinu Ennese cheese revealed the presence of circular, oval, and kidney-shaped crystalline inclusions associated, singularly or in small aggregates, with the casein matrix (Figure 6a, b). Their morphology was similar to those of the Ca phosphate crystals observed in Mahón (Frau et al., 1997), Grana (Bottazzi et al., 1982), Edam, and Gouda cheeses (Kalab, 1977). These have been reported to originate during cheese ripening by crystallization of Ca phosphate of

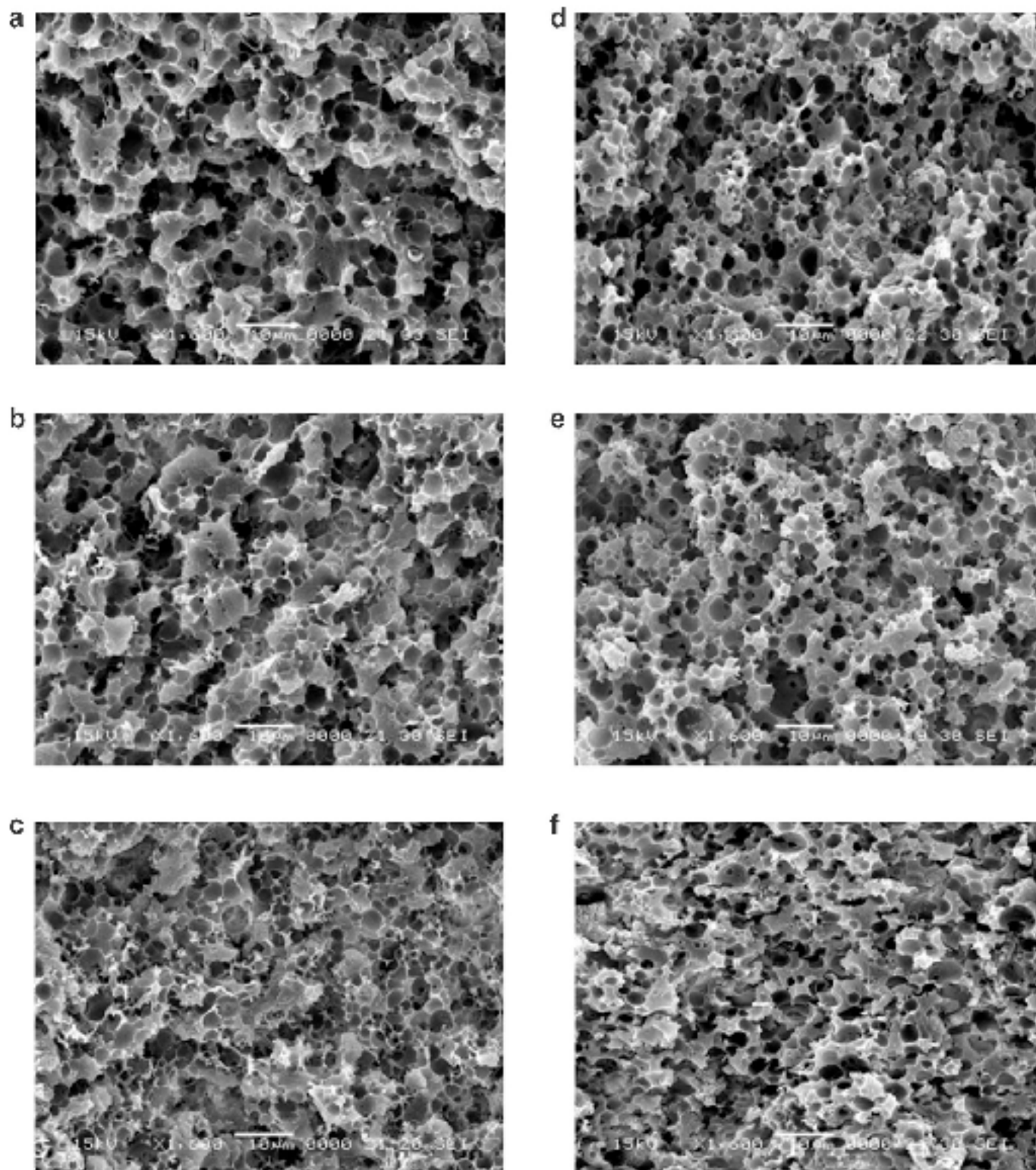


Figure 5. Scanning electron micrographs showing the structure of Piacentini Emnese cheese made using traditional (left) or non-traditional (right) technology after 2 (a, d), 4 (b, e), and 6 mo (c, f) of age.

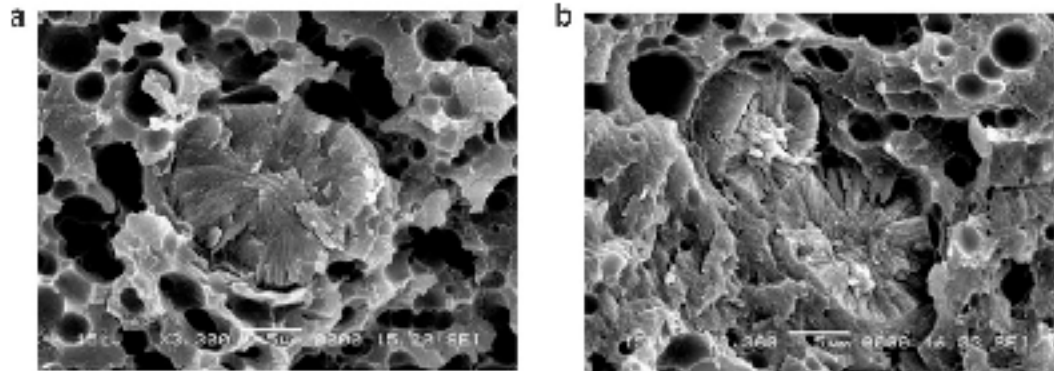


Figure 6. Scanning electron micrographs showing the microstructure of calcium phosphate crystals, singularly associated with the casein matrix (a) or aggregated inside the casein matrix (b).

the residual whey entrapped in the casein network (Brooker, 1987).

CONCLUSIONS

Piacentinu Ennese cheese undergoes a very low extent of proteolysis during aging, and chymosin plays a major role in regulating the primary hydrolysis of caseins and peptidases from microflora, contributing to the slow formation of small peptides and FAA. These proteolytic rearrangements give rise to a sponge-like microstructure, permeated by a well-distributed system of empty spaces. The cheese structure becomes more compact during ripening, with cavities of smaller size, as a result of the gradual loss of moisture caused by evaporation at the cheese surface.

Results from the comparative study showed that farm technology has a large impact on cheese quality, significantly affecting the development of proteolysis and microstructure in Piacentinu Ennese. The high levels of pH4.6SN and the large hydrolysis of α_s -CN fractions suggested high residual activity of the commercial rennet paste used for the manufacture of nontraditional cheeses. Similarly, patterns of secondary proteolysis in cheese were largely altered by replacing the native milk microflora by the starter cultures. Nontraditional cheeses had higher levels of TCASN and showed larger proportions of small soluble compounds in the HPLC profiles of the corresponding ESN fraction than traditional cheeses. Changes in rates and extent of proteolysis in nontraditional cheeses were also associated with negative modifications of the structure; nontraditional cheeses had a coarser and less continuous appearance, resulting in a more open structure than traditional cheeses. Furthermore, a large amount of variability was found in cheese proteolysis and structure within non-traditional treatment, reflecting farm-dependent changes in manufacturing conditions related to the use

of various types of rennet and starter. Considering that producers are hoping to obtain European recognition as a Protected Denomination of Origin cheese, technology and relative parameters need to be strictly defined to provide a quality range for Piacentinu Ennese.

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