Comparison of Plant and Animal Rennets in Terms of Microbiological, Chemical, and Proteolysis Characteristics of Ovine Cheese

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Cheeses manufactured from ovine raw milk using crude aqueous extracts of flowers of *Cynara cardunculus* as rennet were compared with cheeses manufactured with a commercial animal rennet. Changes in a number of microbiological, chemical, and biochemical characteristics throughout ripening were followed in attempts to get scientific insight especially into the primary proteolysis brought about by this plant rennet in cheese. Using averages and corresponding 95% confidence intervals, it was concluded that the type of rennet had no significant effect on cheese composition (e.g., moisture, fat, protein, salt, and pH at the center and at the surface) over the ripening period but lower microbiological counts of *Enterobacteria*, *Lactococci*, and *Lactobacilli* were obtained for cheese manufactured with plant rennet until 28 days of the ripening. Conversely, several biochemical differences in cheese became apparent as ripening progressed. Electrophoretic analyses of the water insoluble fractions from cheeses manufactured with either rennet showed that β-caseins were less susceptible to proteolysis than α_s-caseins and that the animal rennet was more proteolytic on β- and α_s-caseins than the plant rennet; cheeses manufactured with the plant rennet exhibited higher levels of WSN/TN than cheeses manufactured with the animal rennet, although the former showed lower levels of TCA/TN and lower levels of PTA/TN. The peptide profiles of water-soluble extracts of the cheeses obtained by reversed-phase HPLC exhibited different patterns at all stages of ripening for the two rennets utilized, thus conveying important qualitative information for fundamental differentiation of proteolysis effected by either rennet.

Keywords: *Rennet substitute; Cynara cardunculus; ovine cheese; ripening; proteolysis*

INTRODUCTION

Proteolysis is usually regarded as the most important biochemical event during cheese ripening and one of the most important factors for development of typical cheese flavor and texture. Proteolytic agents in cheese originate from five sources: indigenous milk proteinases (plasmin and cathepsin D); rennet (chymosin) or rennet substitute (e.g., bovine, porcine, and chicken pepsins or acid proteinases from *Rhizomucor miehei*, *R. pusilus*, and *Cryphonectria parasitica*; proteinases and peptidases from starter microorganisms (e.g., *Lactococcus*, thermophilic *Streptococcus* and *Lactobacillus*); proteinases and peptidases from secondary microorganisms (e.g., *Propionibacterium* spp., *Brevibacterium linens*, yeasts, and molds); and enzymes from non-starter bacteria (Martley and Crow, 1993). Initial hydrolysis of caseins is caused chiefly by residual rennet, resulting in the formation of large and intermediate sized peptides that are subsequently degraded by rennet itself and by enzymes contributed by viable or lysed starter and non-starter microorganisms. The production of small peptides and free amino acids results from the catalytic action of bacterial proteinases and peptidases.

Although crude plant proteinases appear to have been used as rennets since prehistoric times, chymosin-rich crude mixtures of animal proteinases obtained from the stomachs of calves, kids, or lambs have been used almost exclusively in modern times (Fox, 1988; Fox and Law, 1991). However, increasingly higher prices and ethical concerns associated with the production of such crude enzyme preparations for general cheesemaking have led to a situation where systematic investigations on possible (and suitable) substitutes from microbial and plant origin are relevant. One of the most successful rennets of plant origin, which has been employed for ages in Portugal and bordering regions of Spain for the manufacture of traditional cheeses from raw ovine milk at the farm level, is obtained from *Cynara cardunculus* L. This prickly variety of thistle, which produces large heads and purple flowers throughout summer, grows wild and abundantly in dry, stony, uncultivated areas of the southern and northeastern parts of Portugal, although it has also been identified in several regions of Northern Africa, Canary Islands, and Madeira Island. Although the dried flowers of *C. cardunculus* have been used extensively in the manufacture of such traditional cheese varieties as Serra (Vieira de Sá and Barbosa, 1972; Barbosa, 1983; Macedo *et al.*, 1993), successful experiments have also been carried out on their use in the manufacture of such French cheeses as Camembert and Gruyère (Barbosa *et al.*, 1976) and such Italian cheeses as Bel Paese, Grana, and Provolone (Barbosa *et al.*, 1981). After collection from the mature plants, the flowers are dried in the shade in the open air, stored in a dry place, and sold at local markets. One of the most popular methods used to prepare crude, enzymatically active extracts from thistle flowers for cheesemaking involves soaking a handful of flowers in a bowl of tap water for several hours, smashing with a mortar and pestle, and filtering through a piece of cotton cloth. The brownish liquor thus obtained is added to raw ovine milk to induce coagulation.

In addition to clotting activity, the enzymes in aqueous extracts of flowers of *C. cardunculus* also possess general proteolytic activity: three proteinases have been

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isolated, purified, and partly characterized in terms of activity (Heimgartner *et al.*, 1990; Campos *et al.*, 1990; Faro, 1991; Cordeiro *et al.*, 1992) and specificity (Macedo, 1993) on pure bovine caseins; one such proteinase (tentatively termed cardosin A) is similar to chymosin in specificity and activity, whereas another (tentatively termed cardosin B) is similar to pepsin (Pires *et al.*, 1994). Vieira de Sá and Barbosa (1972) reported that the milk clotting activity of the flowers of *C. cardunculus* is more dependent on temperature, pH, and substrate concentration than calf animal rennets and showed that this plant rennet is a good technological substitute for animal rennets, especially for the manufacture of ovine cheese. However, fundamental work on the action of such plant rennet on ovine milk is scarce, and extrapolation from the conclusions obtained with pure bovine caseins must be done carefully. The objective of this work was to compare the microbiological, chemical and biochemical characteristics of ovine cheeses manufactured using *C. cardunculus* rennet or a commercial animal rennet.

MATERIAL AND METHODS

Cheesemaking and Sampling. Raw ovine milk from the Serra da Estrela region was collected from the sheep flock on the morning of cheesemaking and transported to the pilot plant. The milk was divided in two equal portions, and cheeses were manufactured simultaneously at the pilot plant following a slight modification of the traditional technology. The milk was heated to 28 °C and salted (3 g/L); 16 cheeses were manufactured using extracts of *C. cardunculus* as rennet at a level of 0.16 g of dry flowers per L of milk (stylets of the dry flowers were ground for 1 min and soaked in tap water for 10 min with stirring), and another 16 cheeses were manufactured using animal rennet (1:10 000 Stabo, Chris. Hansen's, Denmark). Coagulation time was ca. 50 and 20 min for extracts of *C. cardunculus* and animal rennet, respectively. In both vats, the coagulum was cut, stirred for 30 min, allowed to stand to promote whey draining, placed into cylindrical molds, and lightly pressed by hand. The cheeses were salted with dry salt on both surfaces and 24 h later placed in a ripening room maintained at 6 °C and 92% relative humidity. After two weeks, the cheeses were washed with warm and slightly salted water. The cheeses (500 ± 100 g, 10 ± 1 cm in diameter, and 5 ± 1 cm high) were inverted daily for 68 d.

Two cheeses manufactured with each type of rennet were selected randomly for microbiological, chemical, and biochemical sampling and analysis after 0, 7, 14, 28, 42, 56, and 68 days, and the average for the two cheeses of every analytical determination was considered as a datum point. The overall experimental variability of each data set generated was assessed via computation of the standard error of the mean (which is obtained from all replications of all data).

Microbiological Analyses. Microbiological analyses were conducted according to the methods described in detail by Freitas *et al.* (1995): total viable counts were determined on plate count agar (PCA), *Enterobacteria* on violet red bile glucose agar (VRBGA), yeasts and molds on potato dextrose agar (PDA), *Lactococcus* on M17 agar (M17A), *Lactobacillus* on Rogosa agar (RA), and *Pseudomonas* on agar base (AB). All determinations were made in duplicate and expressed as cfu/g_{cheese}. (As discussed below, the microflora were not quantitatively affected by the type of rennet used because both rennets were not contaminated; however, this conclusion was to be experimentallly double-checked before changes observed could be attributed to the rennet only.)

Compositional Analyses. The total solids content (TS) was determined by oven drying at 100 °C an aliquot of the sample, as described by Kosikowski (1982). Salt (NaCl) was assayed in another aliquot of the sample by the modified Volhard method using silver nitrate and potassium thiocyanate (Merck), as described by Kosikowski (1982). The pH was measured by probing directly the cheese with a glass electrode connected to a potentiometer Microph 2001 (Crison, Spain). The total nitrogen (TN) content of cheese was determined by the micro-Kjeldahl procedure (IDF, Standard 20B, 1993) using a Kjeltec system 1002 Distilling unit (Tecator, Sweden). The fat content was determined by the Van Gulik method (Anonymous, 1975). All determinations were made in duplicate.

Proteolysis Analyses. Water-soluble extract (WSE) of an aliquot of the cheese sample was determined by the procedure of Kuchroo and Fox (1982). Samples of water-insoluble extract (WISE) and WSE were freeze-dried before further analysis. The trichloroacetic acid (TCA) soluble extract was prepared by adding *y* mL of 48% TCA to 4*y* mL of WSE. The mixture was allowed to stand for 30 min at 20 °C and filtered through Whatman No. 42 filter paper (Maidstone, U.K.). The nitrogen content was determined on an aliquot of the filtrate. Phosphotungstic acid (PTA)-soluble extract was prepared by adding 14.0 mL of 3.95 M sulfuric acid and 6 mL of PTA (33.3%, w/v) to 20 mL of WSE. The mixture was allowed to stand overnight at 4 °C and subsequently filtered through Whatman No. 542 filter paper. The nitrogen content in aliquots of water-soluble, TCA-soluble, and PTA-soluble extracts were determined by the micro-Kjeldahl procedure. The ripening extension index, represented by the ratio of WSE/TN (which is proportional to proteolytic activity), the ripening depth index, represented by the ratio TCA/TN (Furtado and Partridge, 1988), and the free amino acid index, represented by the ratio PTA/TN (which reflects the aminopeptidase activities of the starter bacteria in cheese) (Aston *et al.*, 1983), were then calculated. All determinations were made in duplicate.

Urea−polyacrylamide gel electrophoresis (urea−PAGE) (12.5% C, 4% T, pH 8.9) was performed on cheese samples, as well as on their WSE and WISE, using a Protean II XI vertical slab-gel unit (Bio-Rad Laboratories, Watford, U.K.) and the stacking gel system of Andrews (1983) with modifications (Shalabi and Fox, 1987); the gels were stained with Coomassie Blue G-250 (Bio-Rad) using the method of Blakesley and Boezi (1977). Quantitation of intact β- and α_s-caseins was by densitometry using a model CD60 densitometer (Desaga, Germany).

Peptide profiles of the WSE were obtained by reversed-phase HPLC using the method by Singh *et al.* (1995) in a Beckman system (U.S.A.) composed by an autosampler with temperature control for the column (Autosampler 502), a solvent delivery system with two pumps (Programmable Solvent Module 126), a programmable multiwavelength spectrophotometer (Diode Array Detector 168), a personal computer with a software package for system control and data acquisition (GOLD v6.01), a Lichrosorb 250 × 4 mm RP-8 (5 μm) column, and a Lichrocart 4-4 guard column (Merck, Germany). Elution was effected at 30 °C using a mobile phase of two solvents, A [0.1% trifluoroacetic acid (TFA) in H_2O] and B [0.1% TFA in acetonitrile (Romil, U.K.)], starting with pure A for 5 min, and continuing with a linear gradient to 50% B over 55 min, 50% B for 6 min, a linear gradient to 60% B over 4 min, and 60% B for 3 min; absorbance of the eluate was read at 214 nm. Samples (10 mg/mL) of freeze-dried WSE were dissolved in a mixture of solvents A and B (1: 0.01, v/v) and filtered through 0.22 μm cellulose acetate filter, and an aliquot (75 μL) of the filtrate was injected using an eluant flow rate of 1.0 mL/min.

Statistical Analyses. The Statview v.4.0 statistical package (Haycock *et al.*, 1992) was used for statistical treatment of the results by ANOVA. This test was used to determine overall statistical differences between microbiological, chemical, and biochemical data brought about by the type of coagulant, the ripening time, and the interaction thereof.

RESULTS AND DISCUSSION

Microbiological Characteristics. The numbers of total viable counts on PCA, *Enterobacteria* on VRBGA, yeasts and molds on PDA, *Lactococcus* on M17A, *Lactobacillus* on RA, and *Pseudomonas* on AB for milk and cheeses are tabulated in Table 1. Using averages and corresponding 95% confidence intervals (see Table 2), no significant differences were found between counts on

Table 1. Total Viable Counts on PCA, *Enterobacteria* on VRBGA, Yeasts and Molds on PDA, *Lactococcus* on M17A, *Lactobacillus* on RA, and *Pseudomonas* on AB for Raw Ovine Milk and Cheeses Renneted with Extracts of Flowers of *C. cardunculus* (a) or Animal Rennet (b)

viable counts[a] (cfu/g_{cheese})	milk	cheese		
		0 days	28 days	68 days
PCA				
a	55×10^2	47×10^5	25×10^8	34×10^7
b	55×10^2	70×10^5	14×10^8	11×10^6
VRBGA				
a	46×10^2	62×10^4	17×10^5	17×10^6
b	46×10^2	21×10^4	15×10^6	42×10^4
PDA				
a	$<10^3$	$<10^3$	60×10^3	10×10^4
b	$<10^3$	$<10^3$	67×10^3	18×10^4
M17A				
a	52×10^2	47×10^5	65×10^7	32×10^7
b	52×10^2	50×10^5	12×10^8	57×10^5
RA				
a	37×10^4	23×10^5	87×10^7	97×10^7
b	37×10^4	22×10^5	30×10^8	95×10^6
AB				
a	$<10^3$	$<10^3$	$<10^3$	$<10^3$
b	$<10^3$	$<10^3$	$<10^3$	$<10^3$

[a] Standard errors of the means: PCA, 2.70×10^8; VRBGA, 7.37×10^6; M17A, 1.20×10^8; RA, 1.20×10^8; PDA, 2.98×10^4.

Table 2. Results of the ANOVA of Microbiological Data Obtained for Total Viable Counts on PCA, *Enterobacteria* on VRBGA, Yeasts and Molds on PDA, *Lactococcus* on M17A, and *Lactobacillus* on RA

factor	*p* value				
	PCA	VRBGA	PDA	M17A	RA
type of rennet, T	0.3714	0.7468	0.1614	0.2248	0.0008
ripening time, t	0.0346	0.2760	0.0017	<0.0001	<0.0001
interaction, $T \times t$	0.6633	0.0758	0.2270	0.0045	<0.0001

PCA, VRBGA, M17A, and PDA throughout the ripening time. The viable counts on VRBGA were not significantly different between fresh cheeses and 68-day-old cheeses; these results disagree with Nuñez *et al.* (1991), who reported significantly higher counts of *Enterobacteria* in fresh cheeses manufactured with plant rennet than with animal rennet. The higher numbers of *Enterobacteria* in the interior of the 68-day-old cheeses manufactured with plant rennet may be due to the higher pH in this cheese by the end of ripening which favors survival of these bacteria (Llano *et al.*, 1992); high numbers have also been reported for La Serena and Majorero cheeses (Fernández del Pozo *et al.*, 1988a; Fontecha *et al.*, 1990). Numbers of *Pseudomonas* were negligible in both cheeses, which agrees with observations by Macedo *et al.* (1995) on Serra cheese.

Compositional Characteristics. The mean overall values for moisture, fat, protein, NaCl, and pH at 0, 7, 14, 28, 42, 56 and 68 days of ripening are shown in Figure 1. The moisture contents (%, w/w) (see Figure 1a) of cheeses manufactured with either extracts of *C. cardunculus* or animal rennet were found to decrease throughout the 68-day ripening period and were higher than those reported by Nuñez *et al.* (1991) for La Serena cheese manufactured with either plant or animal rennet at the same stage of ripening. The fat content (see Figure 1b) of both types of cheese decreased slightly during ripening, as well as total protein content (see Figure 1c). Although data on fat and protein contents seem erratic throughout the ripening period, it should be emphasized that samples from two independent cheese replicates were assayed (in duplicate) at each sampling time, and so a given cheese was only sampled once throughout the entire experimental program; the percent contents of protein, fat, moisture, and salt did not sometimes add up to 100%, but the confidence interval always overlapped the 100% threshold. The NaCl content (see Figure 1d) of the cheeses increased during the first 7 days of ripening, probably due to diffusion of NaCl into, and loss of water by, the cheese and remained relatively constant. The salt concentration is of great importance in cheese ripening due to its influence on the proteolytic activity of enzymes (Fernandez del Pozo *et al.*, 1988b), on the specificity of the rennet (Mulvihill and Fox, 1980), and on the growth and activity of lactic acid bacteria (Fontecha *et al.*, 1994). The pH (see Figure 1e) at the surface of fresh cheeses manufactured with extracts of *C. cardunculus* or animal rennet decreased only slightly relatively to that of raw milk (results not shown), probably as a consequence of the absence of lactic starters. The decrease in pH during whey drainage at both the center and the surface of the cheeses manufactured with either rennet may be due to the metabolic activity of such groups of microorganisms that show high growth rates in the interior of the cheese as *Enterobacteria*, *Lactococcus*, and *Lactobacillus*. The pH increased slightly after 28 days of ripening at the surface of the cheese, particularly in cheeses manufactured with extracts of *C. cardunculus*. This observation is probably due to metabolism by lactic acid-utilizing yeasts and/or to ammonia production by yeasts following proteolysis (Fernández del Pozo *et al.*, 1988a). A similar trend in pH was reported for La Serena cheese (Fernandez del Pozo *et al.*, 1988b) and for Casar de Cáceres cheese (Poulet *et al.*, 1991) during ripening. Using averages and corresponding 95% confidence intervals, it is concluded that the type of rennet had no significant effect on moisture, fat, protein, and NaCl contents and pH at the center (see Table 3), but pH at the surface by 28 days was significantly higher in cheese manufactured with plant than with animal rennet.

Proteolysis Characteristics. Figure 2a shows the WSE/TN ratio plotted as a function of ripening time; this ratio has been used by a number of researchers to follow aging of cheese. The values of WSE/TN in cheeses manufactured with either rennet increased throughout the ripening period, but the levels were significantly higher for the plant rennet (see Table 4). A similar trend was reported by Nuñez *et al.* (1991), who found higher values for pH 4.6-soluble nitrogen throughout the ripening period in cheeses manufactured with extracts of *C. cardunculus* than those with animal rennet; this difference was attributed to the type of rennet used. Soluble nitrogen components in cheese are produced mainly by the action of rennet but can also be produced by starter bacteria or plasmin (Visser, 1977); however, as the cheeses in this study contained no starter bacteria and the levels of indigenous plasmin were virtually the same, it appears that the higher levels of WSN/TN are due only to the action of *C. cardunculus*.

The TCA/TN ratio has been used to evaluate the action of lactic acid bacteria in the formation of soluble nitrogen compounds in cheese (Furtado and Partridge, 1988); this fraction contains small peptides of between 2 and 20 amino acid residues. The TCA/TN ratio in cheeses manufactured with *C. cardunculus* and animal rennet increased with ripening time, but the values were significantly higher in the latter by the end of the ripening period (see Table 4 and Figure 2b). These

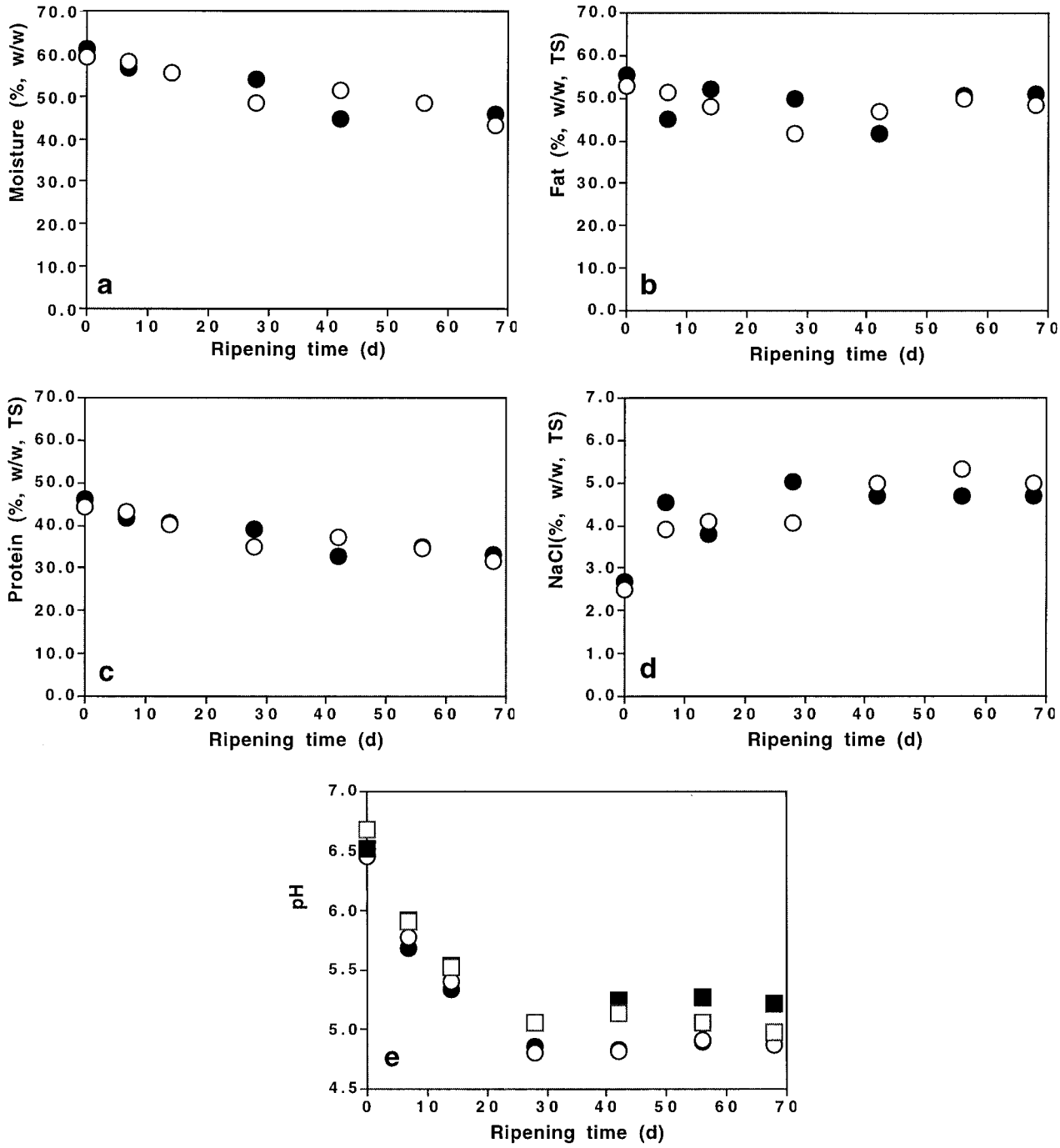

Figure 1. Average values for (a) moisture, (b) fat, (c) protein, (d) NaCl, and (e) pH at the center (circles) and at the surface (squares) of the cheese throughout ripening, for raw ovine milk cheeses manufactured with extracts of *C. cardunculus* (closed symbols) or animal rennet (open symbols). Standard errors of the means: moisture, 1.03; fat, 1.32; protein, 0.720; NaCl, 0.320; pH (center), 0.045; pH (surface), 0.055.

Table 3. Results of the ANOVA of Compositional Data Obtained for Moisture, Fat, Protein, and Salt Contents and pH at the Center and at the Surface of the Cheeses

	p-value					
					pH	
factor	moisture	fat (%TS)	protein (%TS)	NaCl (%TS)	center	surface
type of rennet, *T*	0.4588	0.0791	0.3233	0.8024	0.8310	0.0003
ripening time, *t*	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
interaction, $T \times t$	<0.0001	<0.0001	<0.0001	0.0328	0.1188	0.0174

results are not in agreement with those obtained by Nuñez *et al.* (1991), who reported that TCA-soluble nitrogen in La Serena cheese manufactured with plant rennet was slightly higher than its animal rennet counterpart. Rennet is generally recognized as possessing the ability to produce large pH 4.6-soluble peptides from casein but a limited capacity to breakdown casein further than polypeptides (Desmazeaud and Gripon, 1977); the action of starter bacteria or other enzymes is less important at this level of proteolysis but is

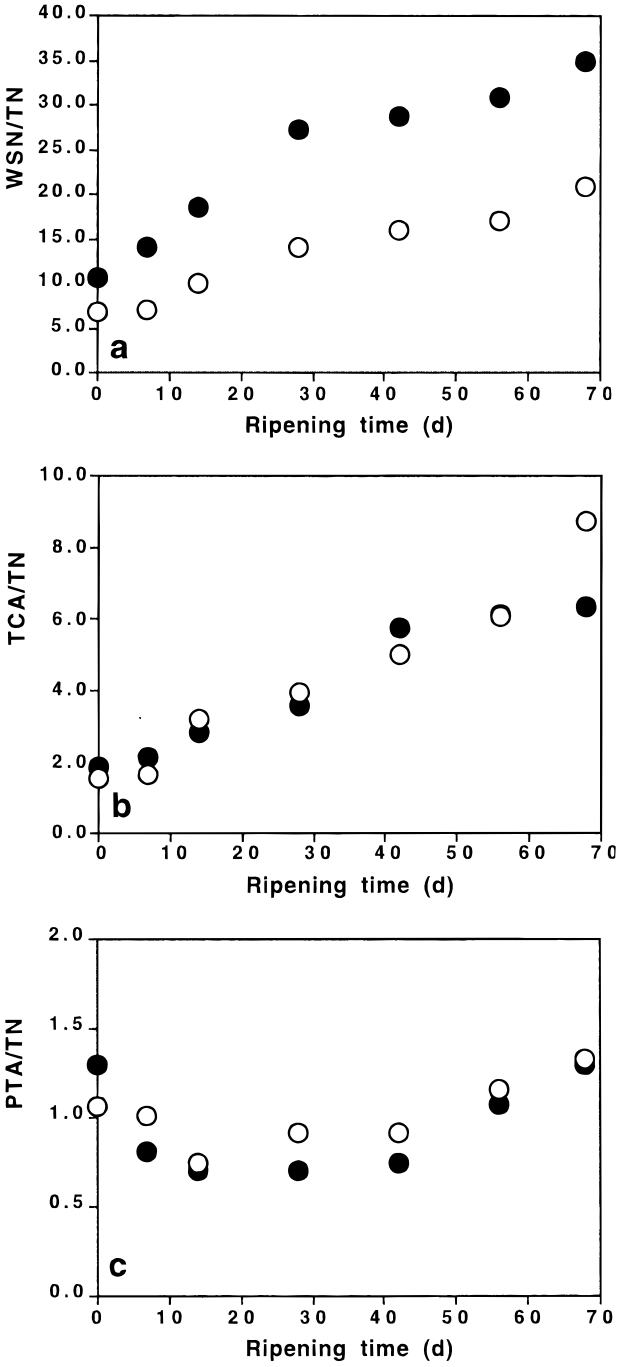

Figure 2. Average values for the ripening indices (a) WSN/TN (ripening extension index), (b) TCA/TN (ripening depth index), and (c) PTA/TN (free amino acid index) throughout ripening for raw ovine milk cheeses manufactured with extracts of *C. cardunculus* (●) or animal rennet (○). Standard errors of the means: WSN/TN, 0.501; TCA/TN, 0.0904; PTA/TN, 0.0707.

Table 4. Results of the ANOVA of Proteolysis Data Obtained for Water-Soluble Nitrogen, Trichloroacetic Acid-Soluble Nitrogen, and Phosphotungstic Acid-Soluble Nitrogen

	p-value		
factor	WSN/TN	TCA/TN	PTA/TN
type of rennet, *T*	<0.0001	<0.0001	0.0145
ripening time, *t*	<0.0001	<0.0001	<0.0001
interaction, $T \times t$	<0.0001	<0.0001	0.0055

primarily responsible for the production of small peptides and free amino acids that are 12% TCA-soluble.

The free amino acid index, represented by PTA/TN, decreased up to 14 days and increased toward the end of ripening. These results reflect microbial consumption of free amino acids, which is confirmed by the increasing numbers of microorganisms from 0 to 28 days of ripening (see Table 1). The lower values obtained for the ratio PTA/TN for cheeses manufactured with plant rennet than with animal rennet suggest that casein was cleaved by the proteinases of flowers of *C. cardunculus* into high-molecular weight peptides but that these were not extensively broken down to low-molecular weight peptides and free amino acids. The type of rennet had a significant effect on the ratio PTA/TN of both cheeses (see Table 4 and Figure 2c).

Urea−PAGE electrophoregrams of the WISE from ovine cheese manufactured with the two rennets (see Figure 3a and 3b) showed a group with lower mobility containing two β-casein variants, β_1- and β_2-casein (Richardson and Creamer, 1979) and also showed that they were less susceptible to proteolysis than α_s-caseins; the percentage of degradation of the combined β_1- and β_2-caseins was 33.1% or 50.3% in cheese manufactured with extracts of *C. cardunculus* or animal rennet, respectively. Ovine casein hydrolyzed by calf chymosin at various pH values and 5% NaCl leads to a pair of bands with higher electrophoretic mobility than β-casein and comparable to bovine β-I-casein (Whyte, 1995); the bonds in bovine β-casein most susceptible to the action of chymosin and of proteinases contributed by the flowers of *C. cardunculus* are Leu192-Tyr193 and Ala189-Phe190 (Sousa, 1993; Macedo, 1993), and β-I-casein results from breakage of either of these bonds (Sousa, 1993); the corresponding cleavage sites in ovine β-casein are Leu190-Tyr191 and Ala187-Phe188 (Whyte, 1995). The group with higher mobility (see Figure 3a and 3b) consisting of three bands with different mobilities was designated as α_s-casein region despite the various α_s variants claimed in the literature (Richardson and Creamer, 1976; Boisnard *et al.*, 1985; Mercier *et al.*, 1985; Whyte, 1995); quantification of this region indicated degradation of α_s-caseins by 47.0% or 87.6% in cheese manufactured with extracts of *C. cardunculus* or animal rennet, respectively, with concomitant appearance of bands with higher electrophoretic mobility tentatively designated as a whole by α_s-I-casein. Two (or three) such bands were already apparent by 28 days of ripening (see Figure 3a) and increased with ripening time for cheese manufactured with *C. cardunculus* but remained unchanged for its animal rennet counterpart (see Figure 3b). Ovine α_s-casein hydrolyzed by calf chymosin at various pH values and 5% NaCl leads to a set of bands of higher electrophoretic mobility than α_s-casein, and comparable to bovine α_{s1}-I-casein (Whyte, 1995); the bond in bovine α_{s1}-I-casein most susceptible to chymosin is Phe23-Phe24, hydrolysis of which yields peptides f1-23 and f24-199 (McSweeney, 1993); the corresponding bond in ovine α_s-casein is Phe23-Val24 (Whyte, 1995), and so the electrophoretic band designated as α_s-I-casein in ovine cheese is probably the peptide f24-199. Two bands with higher electrophoretic mobility than the α_s-casein region (see A in Figure 3a) were produced from the very beginning of ripening in cheeses manufactured with *C. cardunculus* but not with animal rennet (see Figure 3b) and became more intense as ripening time elapsed; similar bands were found by Fernandez del Pozo *et al.* (1988b) in cheese manufac-

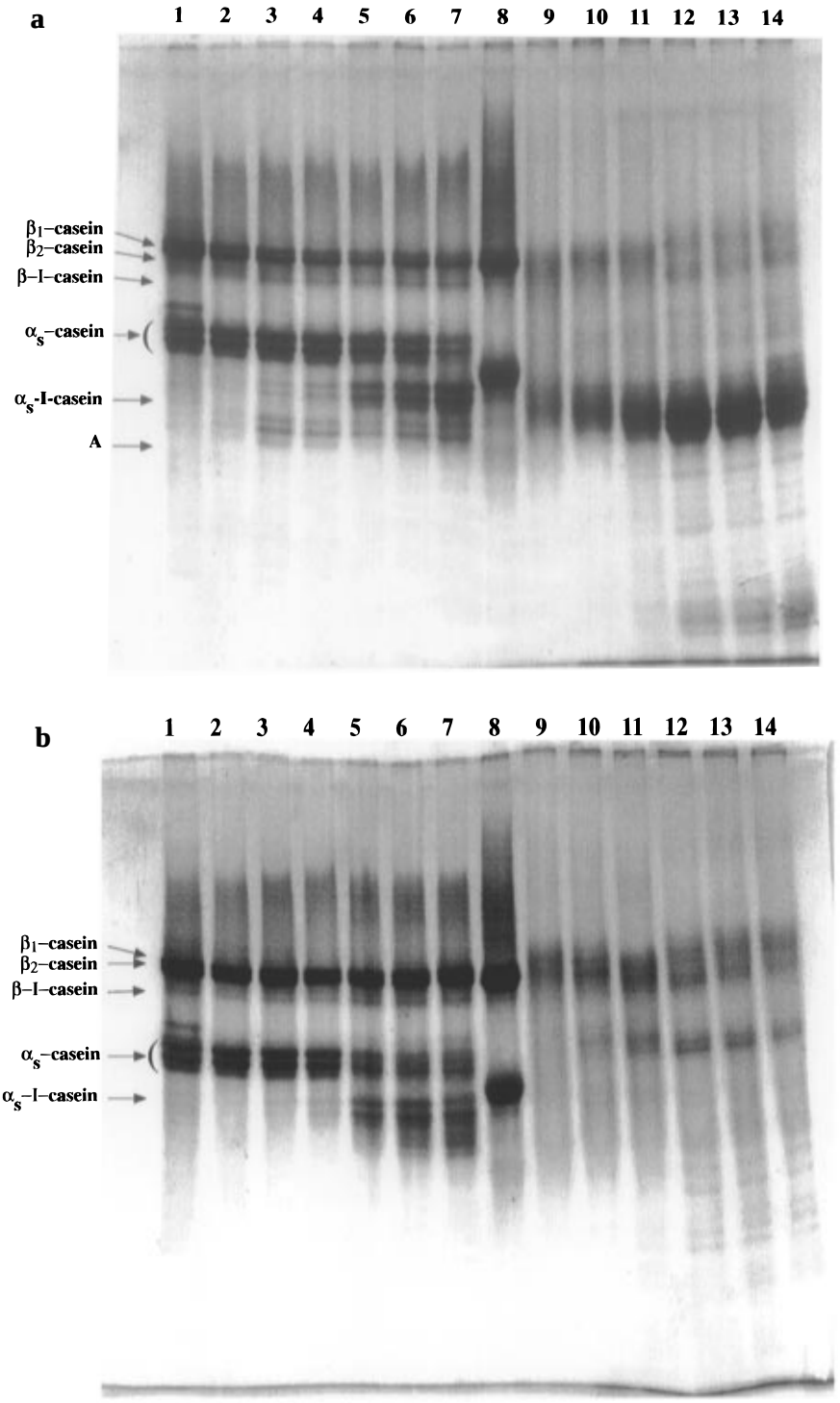

Figure 3. Urea−PAGE electrophoregrams (12.5% T, 4% C; pH 8.9) of water-insoluble (lanes 2−7) and water-soluble nitrogen fractions (lanes 9−14) for 0, 7, 14, 28, 42, or 68 days of ripening, respectively, for ovine milk cheeses manufactured with extracts of *C. cardunculus* (a) or animal rennet (b). Bovine sodium caseinate (lane 8) and ovine sodium caseinate (lane 1) were included as references.

tured, and by Sousa (1993) in solutions of bovine α_{s1}-casein incubated, with extracts from flowers of *Cynara* spp.

The RP-HPLC peptide profiles of the WSE of cheeses manufactured with plant and animal rennets at 0 d were similar (see Figure 4); two small peaks were eluted at ca. 22 and 50 min and ca. 22 and 38 min, respectively, but in both cases most peptides were eluted in the hydrophobic region (50−60 min). The number, and corresponding concentration, of peptides was very low during early stages of ripening (data not shown), but increased quickly thereafter: at 14, 28, and 68 days, peptide profiles of cheeses manufactered with plant and animal rennets showed considerable differences, both qualitatively and quantitatively (see Figure 4ii and 4iv). These differences are likely due to the different specificities of the plant and animal rennets because the feedstock milk, cheesemaking protocol, ripening conditions, and native microflora were similar for both types of cheese.

CONCLUSIONS

No significant microbiological differences were found between ovine raw fresh cheeses manufactured with

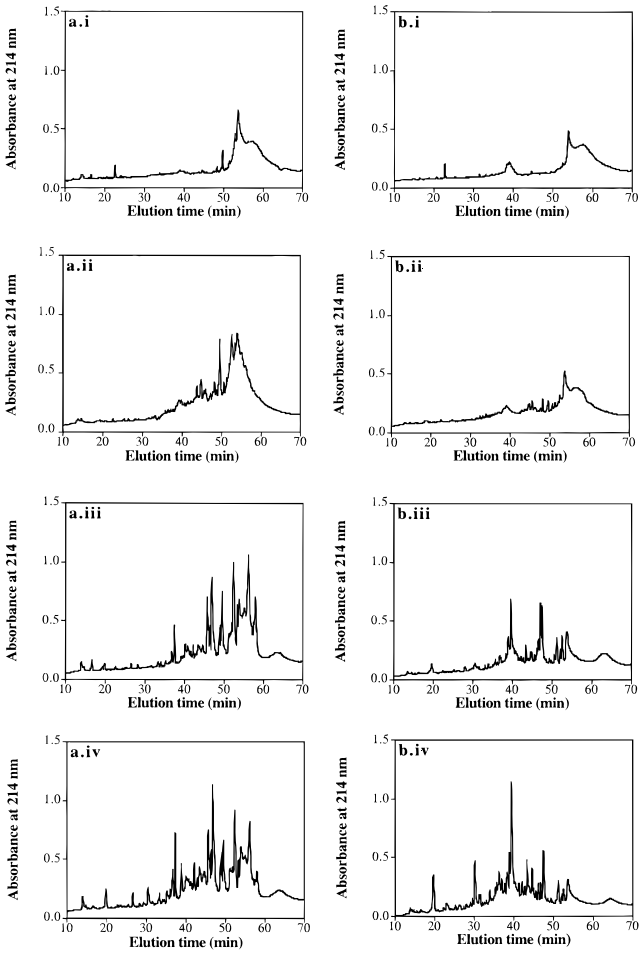

Figure 4. Reversed-phase HPLC peptide profiles of WSE obtained from ovine milk cheeses manufactured with *C. cardunculus* (a) or animal rennet (b) at (i) 0, (ii) 14, (iii) 28, and (iv) 68 days of ripening.

either *C. cardunculus* rennet or animal rennet, but higher counts of *Enterobacteria*, *Lactococcus*, and *Lactobacillus* were observed in the former by the end of ripening. The type of rennet had no significant effect on cheese chemical composition during the first 28 days of ripening, but several biochemical differences in cheese have become apparent as ripening time elapsed. In general, β-caseins were less susceptible to proteolysis than α_s-caseins by either rennet, and the animal rennet was more proteolytic on β- and α_s-caseins than the plant rennet. Cheeses manufactured with *C. cardunculus* exhibited higher levels of WSE/TN (which is a measure of proteolytic activity) than cheeses manufactured with animal rennet, although the former showed lower levels of TCA/TN and PTA/TN. Different peptide profiles were observed at all stages of ripening arising from the different specificities of the plant and animal rennets tested. Primary proteolysis (especially if measured by the amount of soluble nitrogen compounds) in cheese manufactured with extracts of *C. cardunculus* is due to the action of this rennet rather than to the prevailing microflora or native enzymes.

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LITERATURE CITED

Andrews, A. T. J. Proteinases in normal bovine milk and their action on caseins. *J. Dairy Res.* **1983**, 50, 45−55.

Anonymous. ISO 3433, Fromages. Determination de la teneur en matière grasse. Method Van Gulik. 1975.

Aston, J. W.; Durward, I. G; Dulley, J. R. Proteolysis and flavour development in Cheddar cheese. *Aust. J. Dairy Technol.* **1983**, 38, 55−59.

Barbosa, M. *O cardo (Cynara cardunculus L.) como coagulante vegetal*. LNETI-DTIA, Lisboa, Portugal, 1983.

Barbosa, M.; Vassal, E.; Mocquot, G. L'utilization d'extrait de *Cynara cardunculus* L. comme agent coagulant en fabrication de fromages à pâte mole et à pâte cuite. *Lait* **1976**, 54, 1−7.

Barbosa, M.; Corradini, C.; Battistoni, B. Cheesemaking experiments carried out on some Italian cheeses with vegetable rennet from cardo (*Cynara cardunculus* L.). *Sci. Tec. Latt.-Casearis* **1981**, 32, 203−221.

Blakesley, R. W.; Boezi, J. A. A new staining technique for proteins in polyacrylamide gels using Coomassie Brillant Blue G-250. *Anal. Biochem*. **1977**, 82, 55−581.

Boisnard, M.; Petrissant, G. Complete sequence of ovine-casein messenger RNA. *Biochimie* **1985**, 67, 1043−1051.

Campos, R.; Guerra, R.; Aguiar, M.; Ventura, O.; Camacho, L. Chemical characterization of proteases extracted from wild thistle (*Cynara cardunculus*). *Food Chem*. **1990**, 35, 89−97.

Chianese, L.; Garro, G.; Ferranti, P.; Malorni, A.; Addeo, F.; Rabasco, A.; Pons, P. M. Discrete phosphorylation generates the electrophoretic heterogeneity of ovine β-casein. *J. Dairy Res*. **1995**, 62, 89−100.

Cordeiro, M. C.; Jakob, E.; Puhan, Z.; Pais, M. S.; Brodelius, P. E. Milk clotting and proteolytic activities of purified cynarases from *Cynara cardunculus* - a comparison to chymosin. *Milchwissenschaft* **1992**, 47, 683−700.

Desmazeaud, M. J.; Gripon, J. C. General mechanism of protein breakdown during cheese ripening. *Milchwissenschaft* **1977**, 32, 731−734.

Faro, C. Purificação e caracterização físico-química da protease de *Cynara cardunculus* L. Ph.D. Thesis, Universidade de Coimbra, Portugal, 1991.

Fernández del Pozo, B.; Gaya, P.; Medina, M.; Rodriguez-Martín, A.; Nuñez, M. Changes in the microflora of La Serena ewe's milk during ripening. *J. Dairy Res*. **1988a**, 55, 449−455.

Fernández del Pozo, B.; Gaya, P.; Medina, M.; Rodriguez-Martín, A.; Nuñez, M. Changes in chemical and rheological characteristics of La Serena ewe's milk during ripening. *J. Dairy Res*. **1988b**, 55, 457−464.

Fontecha, J.; Peláez, C.; Juarez, M.; Requena, M. T.; Gómez, C.; Ramos, M. Biochemical and microbiological characteristics of artisanal hard cheese varieties. *J. Dairy Sci*. **1990**, 73, 1150−1157.

Fontecha, J.; Peláez, C.; Juarez, M. Biochemical characteristics of a semi-hard ewe's-milk cheese. *Z. Lebensm.-Unter. -Forsch*. **1994**, 198, 24−28.

Fox, P. F. Rennets and their action in cheese manufacture and ripening. *Biotechnol. Appl. Biochem*. **1988**, 10, 522−535.

Fox, P. F.; Law, J. Enzymology of cheese ripening. *Food Biotechnol*. **1991**, 5, 239−262.

Freitas, A. C.; Sousa, M. J.; Malcata, F. X. Effect of ripening time and the combination of ewe and goat milk on the microflora of Picante cheese. *Ital. J. Food Sci*. **1995**, 4, 361-377.

Furtado, M. M.; Partridge, J. A. Characterization of nitrogen fractions during ripening of a soft cheese made from ultrafiltration retentates. *J. Dairy Sci*. **1988**, 71, 2877−2885.

Haycock, K.; Roth, J.; Gagnon, J.; Finzer, W. F.; Soper, C. *Statview v 4.0: The ultimate integrated data analysis and presentation system*. Abacus Concepts, Berkeley, 1992.

Heimgartener, U.; Pietrzak, M.; Geertsen, R.; Brodelius, P.; Silva Figueiredo, A. C.; Pais, M. S. S. Purification and partial characterization of milk clotting proteases from flowers of *Cynara cardunculus*. *Phytochem*. **1990**, 29, 1405−1410.

Kosikowski, F. V. *Cheese and Fermented Milk Foods*, Kosikowski and Associates, Brooktondale, 1982.

Llano, D. G.; Ramos, M.; Rodriguez, A.; Montilla, A.; Juarez, M. Microbiological and physicochemical characteristics of Gamonedo blue cheese during ripening. *Int. Dairy J*. **1992**, 2, 121−135.

Kuchroo, C. N.; Fox, P. F. Soluble nitrogen in Cheddar cheese: Comparison of extraction procedures. *Milchwissenschaft* **1982**, 37, 331−335.

Macedo, A.; Malcata., F. X.; Oliveira, J. C. The technology, chemistry and microbiology of Serra cheese: a review. *J. Dairy Sci.* **1993**, 76, 1725−1739.

Macedo, A.; Malcata., F. X.; Hogg, T. A. Microbiological profile in Serra ewe's cheese during ripening. *J. Appl. Bacteriol.* **1995**, 79, 1−11.

Macedo, I. Especificidade caseinolítica da protease de *Cynara cardunculus* L. Ph.D. thesis, Universidade de Coimbra, Portugal, 1993.

Martley, F. G.; Crow, V. L. Interactions between non-starter microorganisms during cheese manufacture and ripening. *Int. Dairy J*. **1993**, 3, 461−483.

McSweeney, P. L. H.; Olson, N. F.; Fox, P. F.; Heally, A.; Hojrup, P. Proteolytic specificity of chymosin on bovine α_{s1}-casein. *J. Dairy Res*. **1993**, 60, 401−412.

Mercier, J. C.; Gaye, P.; Soulier, S.; Hue-Delahaie, D.; Vilotte, J. L. Construction and identification of recombinant plasmids carrying cDNAs coding for ovine α_{s1}-, α_{s2}-, β-, κ-casein and β-lactoglobulin. Nucleotide sequence of α_{s1}-casein cDNA. *Biochimie* **1985**, 67, 959−971.

Mulvihill, D. M.; Fox, P. F. Proteolysis of α_{s1}-casein by chymosin in dilute NaCl solutions and in Cheddar cheese. *Ir. J. Food. Sci. Technol.* **1980**, 4, 13−23.

Nuñez, M.; Fernandez del Pozo, B.; Rodriguez-Martin, M. A.; Gaya, P.; Medina, M. Effect of vegetable and animal rennet on chemical, microbiological, rheological and sensory characteristics of La Serena cheese. *J. Dairy Res*. **1991**, 58, 511−519.

Pires, E.; Faro, C.; Macedo, I.; Esteves, C.; Morgado, J.; Veríssimo, P.; Pereira, D.; Gomes, D. Flor de cardo versus quimosina no fabrico de queijos artesanais. *Química* **1994**, 54, 66−68.

Poulet, B.; Huertas, M.; Sánchez, A.; Cáceres, P.; Larriba, G. Microbial study of Casar de Cáceres cheese throughout ripening. *J. Dairy Res*. **1991**, 58, 231−238.

Richardson, B. C.; Creamer, L. K. Comparative micelle structure. V. The isolation and characterization of the major ovine caseins. *N. Z. J. Dairy Sci. Technol.* **1976**, 11, 46−53.

Richardson, B. C.; Mercier, J. C. The primary structure of the ovine β-caseins. *Eur. J. Biochem*. **1979**, 99, 285−297.

Shalabi, S. I.; Fox, P. F. Electrophoretic analysis of cheese: comparison of methods. *Ir. J. Food Sci. Technol.* **1987**, 11, 135−151.

Singh, T. K.; Fox, P. F.; Healy, A. Water-soluble peptides in Cheddar cheese: isolation and identification of peptides in the diafiltration retentate of the water-soluble fraction. *J. Dairy Res.* **1995**, 62, 629−640.

Sousa, M. J. C. F. Plant rennet substitute from flowers of *Cynara cardunculus*. M.Sc. Thesis, National University of Ireland, Cork, 1993.

Vieira de Sá, F.; Barbosa, M. Cheesemaking with a vegetable rennet from cardo *Cynara cardunculus* L. *J. Dairy Res.* **1972**, 39, 335−343.

Visser, F. M. W. Contribution of enzymes from rennet, starter bacteria and milk to proteolysis and flavour development in Gouda cheese. 3. Protein breakdown: analysis of the soluble nitrogen and amino acid nitrogen fraction. *Neth. Milk Dairy J*. **1977**, 31, 210−239.

Whyte, N. H. Proteolysis of bovine and ovine caseins by lamb and calf chymosin. M.Sc. Thesis, National University of Ireland, Cork, 1995.

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