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Use of Recombinant Cyprosin in the Manufacture of Ewe's Milk Cheese

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A recombinant cyprosin from the cardoon (*Cynara cardunculus* L.) was assayed and compared with calf rennet in batches of ewes' milk cheese by determining different chemical, biochemical, and microbiological parameters over 4 months of ripening. There were no differences between the two types of coagulants in most chemical parameters, a_w, and pH. Proteolysis was more marked and rapid in cheese containing recombinant cyprosin as coagulant, the soluble nitrogen content of which was significantly higher ($p < 0.01$) than that of the cheese obtained with animal rennet; at the end of ripening the nonprotein nitrogen of cheese produced with recombinant cyprosin was slightly higher ($p > 0.05$) as compared with that in cheeses obtained with animal rennet. Microbial counts in the milk used for making cheese were high in most of the groups analyzed. Despite slight differences in counts, the main microbial groups analyzed were similar in cheese produced with both types of coagulants throughout ripening.

KEYWORDS: Proteolysis; ovine cheese; vegetable coagulant; recombinant cyprosin

INTRODUCTION

Historically, rennet used to coagulate milk in cheesemaking has generally been extracted from the fourth stomach of calves, although kids and lambs have also been used. The primary enzyme obtained from this source is chymosin (EC 3.4.23.4). The younger the animal, the higher the chymosin content, ranging in calf rennet from 75 to 100%. The second milk-coagulating enzyme found in calf rennet is bovine pepsin (EC 3.4.23.1). Levels are affected by the animal's diet; thus, adult bovine rennet contains a much lower level of chymosin—<30%.

The increasing consumption of cheese and the decreasing number of calves slaughtered in the 1970s led to an increase in the price of calf rennet, to a shortage in chymosin-rich rennet, and to a search for alternative milk coagulants. More recently, the shortage was exacerbated by the outbreak of BSE in dairy cattle, which was diagnosed in 1986 in the United Kingdom and later spread to the rest of Europe and elsewhere. However, genetic engineering techniques have made it possible to isolate the gene responsible for chymosin production from calves' stomachs and incorporate it into suitable microorganisms (e.g., the bacteria *Escherichia coli*, the yeast *Kluyveromyces marxianus* var. *lactis*, and the fungus *Aspergillus niger* var. *awamori*), solving the problems posed by calf rennet shortages (*1*). The U.S. Food and Drug Administration affirmed the GRAS (Generally Regarded as Safe) status of the first microbial chymosin in 1989. At present, industry estimates are that more than half of the chymosin used is currently produced by this method.

The coagulation of milk can be achieved by a number of proteolytic enzymes from various sources, such as different animal species (e.g., pig, cow, and chicken pepsins), microbial proteinases (*Rhizomucor miehei*, *Rhizomucor pusillus*, and *Cryphonectria parasitica*), and proteinases extracted from fruits (e.g., pineapple, papaya, and sodom apple) and plants such as wild cardoons. A crude aqueous extract of the thistle flowers from *Cynara cardunculus* L. is used chiefly in the making of various Spanish and Portuguese cheeses (*2*−*4*). Some of these cheeses enjoying Appellation d'Origine Controllée status (*5*) can be produced from raw ewes' milk using only the proteinases extracted from *C. cardunculus* flowers as coagulant.

The proteinases of *C. cardunculus* L. have been isolated, purified, and partially characterized in terms of activity (*6*, *7*). This species contains three proteinases, the proteolytic activities of which are maximal at pH 5.1, 5.7, and 6.0, at variable temperatures under ionic strengths equivalent to 0.1−0.6 M in NaCl (*4*). They are thus acidic proteinases belonging to the aspartic proteinase group called "cynarases" (*7*) or "cyprosins" (*8*).

C. cardunculus flowers are easy to handle for the extraction of proteinases. However, the picking, storage, handling, and marketing of these cardoon flowers are not subject to the legal regulations governing food additives. Flowers gathered by different pickers may well be a mixture of different cardoon species (*9*) and can be picked at different stages of flowering

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and in different geographical areas; they may also contain impurities such as bracts and the remains of leaves, making it very difficult to standardize their clotting activity. In addition, the poor microbiological quality of crude aqueous extracts from *C. cardunculus* cardoon flowers has recently been reported (*10*, *11*), with viable total and Enterobacteriaceae counts of >5.0 log colony-forming units (cfu)/mL and coliform counts of >3.5 log cfu/mL. Although most cheeses in which cardoon is used as a coagulant are made from raw milk, the adding of cardoon crude extracts produces additional contamination of the initial milk and, consequently, of the cheeses. As an alternative to the use of the crude aqueous extract (CAE) from *C. cardunculus*, we have developed a powdered vegetable coagulant from CAE obtained by freeze-drying, which has been patented (*5*). This powdered vegetable coagulant (PVC) is a product free of viable microorganisms, soluble in water or milk, shelf stable in an airtight container without the need for preservatives, and easy to typify and handle as a vegetable coagulant.

Another alternative to the use of the crude aqueous extract or the PVC from *C. cardunculus* is the use of recombinant cyprosin, which, like transgenic chymosin, has been produced in plant cells as well as from microbial sources (*8*, *12*). Like microbial rennets, the use of these vegetable proteinases as milk coagulants is of interest because they are natural enzymes and can also be used for producing cheeses and whey products intended for the vegetarian market. Vegetable proteinases can also be certified Kosher and Halal.

The aim of this paper was to study the physicochemical, biochemical, and microbiological characteristics during ripening of a ewe's milk cheese (Manchego type) manufactured with either recombinant cyprosin (RC) or animal rennet (AR).

MATERIALS AND METHODS

Recombinant Cyprosin. RC was obtained as described elsewhere (*6*, *8*, *12*). The expression for cyprosin in both microbial and plant cells has finally been optimized according to a patent described by Planta et al. (*13*).

Chees-Making Procedure and Sampling. Batches of Manchego type cheese were made on different days using raw ewes' milk and commercial starter culture. The amount obtained during milking day was split into two batches, one of which was coagulated with animal rennet by adding ~2.5 g/100 L of milk (commercial calf rennet powder with 80% chymosin and 20% pepsin, from Chr. Hansen) and recombinant cyprosin. The milk obtained on another day was split identically into two batches and coagulated with the same types of coagulant. Therefore, two experimental batches were coagulated with calf rennet and another two with RC. A commercial starter culture EZAL from Larbus S.A. (Madrid, Spain) containing a mixture of *Lactococcus lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris*, *L. lactis* subsp. *lactis* var. *diacetylactis* and *Streptococcus thermophilus* was added to the milk. Each of the four experimental batches consisted of six cheeses (using ~5.2 L of milk/kg of cheese). The clotting temperature for milk was 29 ± 1 °C, and the clotting took 45−55 min. After pressing, the cheeses were salting on brine, ripened in a controlled room at 11 °C and 85% relative humidity, and analyzed at 2, 15, 30, 60, 90, and 120 days of ripening.

Compositional Analyses. Determinations of the chemical composition of the samples included those of moisture, protein, fat, and sodium chloride, all of which were conducted in duplicate as described (*14*). The pH was measured by probing the cheese directly with a glass electrode of a Beckman 3500 digital pH-meter, and the water activity (a_w) was determined at 20 °C by means of a CX-1 dew-point hygrometer from Decagon Devices (Pullman, WA). All samples were analyzed in duplicate.

Nitrogen Fractions. Nitrogen fractions, namely, soluble nitrogen (SN) at pH 4.6, nonprotein nitrogen (NPN) in 12% trichloroacetic acid, and ammonia nitrogen (NH_3N), were determined as described elsewhere (*9*, *15*). Amino acid nitrogen (AAN) was determined as described by Folkertsma and Fox (*16*). All samples were analyzed in duplicate.

Microbiological Analyses. All microbial groups, except staphylococci, were analyzed according to the APHA (*17*) as follows: aerobic bacteria were determined on plate count agar (PCA, Oxoid Limited, Basingstoke, U.K.) and incubated at 30 °C for 72 h; total enterobacteria (Gram-negative and citocromo-oxidase negative) on violet-red bile glucose agar (VRBG) and incubated at 37 °C for 24−48 h; coliforms on violet-red bile agar (VRBA) and incubated at 37 °C for 24 h; micrococci on mannitol salt agar (MSA; Oxoid) incubated at 37 °C for 72 h; lactic acid bacteria on MRS agar in anaerobiosis and incubated a 37 °C for 24 h; and molds and yeasts on potato dextrose agar (PDA; Oxoid) and incubated at 26 °C for 96 h. Staphylococci on Baid Parker agar (BP; Oxoid) were incubated at 37 °C for 24−48 h as described by Buttiaux (*18*). All determinations were made in duplicate and expressed as log cfu per gram of sample.

Statistical Treatment. The results obtained at the different ripening stages were subjected to an analysis of variance (ANOVA) using the SAS 6.09 software package (*19*).

RESULTS AND DISCUSSION

Compositional Characteristics and Nitrogen Fractions. The mean values and standard deviations for moisture, fat, protein, and NaCl (as grams per 100 g of cheese) and different nitrogen fractions [SN, NPN, AAN, and NH_3N; as grams per 100 g of total nitrogen (TN)] as well as pH and a_w throughout ripening (at 2, 15, 30, 60, 90, and 120 days) of cheeses obtained with animal rennet (AR) or recombinant cyprosin (RC) are shown in **Table 1**. All chemical parameters, pH, and a_w changed significantly during ripening ($p < 0.01$). The ANOVA showed significant differences in moisture and fat of cheeses ($p < 0.01$) produced with both coagulants assayed. Also was found (*3*) a similar difference between cheeses produced with AR and a crude extract of *C. cardunculus*. The lower moisture found in cheeses obtained with RC, as well as the higher proteolysis of this enzyme, led to lower water activity throughout ripening ($p < 0.001$) in comparison to cheeses produced with AR. In ripened cheese, the decrease in a_w is mainly influenced by the presence of salt, moisture loss, and the gradual hydrolysis of proteins to soluble low molecular weight components. Recorded pH values also displayed significant differences throughout ripening; the pH of cheese produced with AR was lower ($p < 0.001$) than those obtained with RC, which is consistent with a slightly higher level of lactic acid bacteria throughout ripening of cheeses coagulated with AR (**Table 2**).

The nitrogen fraction values differed markedly between the cheeses produced using the two types of coagulants. After 2 days of ripening, the SN values for the cheeses made using RC were almost double those of samples produced with AR (**Table 1**). These high levels of SN during the early stages of ripening, also reported by other authors for different varieties of ewes' milk cheese made with crude extract of *C. cardunclus* (*3*, *20*, *21*), are the result of the intense proteolytic action of the cyprosin in the plant coagulant, which exhibited virtually maximum activity at the pH studied (*6*). At the end of ripening, mean SN values approached 30% of TN in cheeses made with recombinant cyprosin, whereas those made with animal rennet approached only 20.0% TN ($p < 0.001$). In cheese made from ewes' milk from the Serra de la Estrela region (Portugal) using AR and crude extract of *C. cardunculus*, water-soluble N (WSN) levels were similar (*22*) to those recorded here. Nevertheless, the variations in soluble nitrogen between these different experiments are explained by the different factors that influence proteolysis of casein and their first breakdown products, the most important factors being temperature and relative moisture

Table 1. Averages Values and Standard Deviations for Moisture, Fat, Protein, Lactic Acid, NaCl (Grams per 100 g of Cheese), pH, a_w, and Different Soluble Nitrogen Components (SN, NPN, AAN, and NH_3N; as Grams per 100 g of TN) in the Cheese Batches Obtained with Animal Rennet (AR) and Recombinant Cyprosin (RC) throughout Ripening[a]

	days of ripening					
	2	15	30	60	90	120
			Batch AR			
moisture	39.16 ± 0.46a	36.93 ± 0.13b	38.15 ± 0.98ab	33.58 ± 0.16c	31.95 ± 0.21c	29.15 ± 1.63d
fat	32.75 ± 0.35a	33.50 ± 0.00ab	34.25 ± 0.35b	35.75 ± 0.35c	39.25 ± 1.06e	37.50 ± 0.00d
protein	21.24 ± 0.81a	22.26 ± 0.93ab	22.30 ± 1.78ab	22.62 ± 3.68ab	23.25 ± 1.35ab	26.08 ± 0.01b
NaCl	0.82 ± 0.11a	1.03 ± 0.13a	1.41 ± 0.01b	1.54 ± 0.08b	1.62 ± 0.12b	1.59 ± 0.14b
ash	3.30 ± 0.88a	4.25 ± 0.18ab	4.50 ± 0.24b	4.06 ± 0.21b	4.75 ± 0.35b	4.84 ± 0.23b
a_w	0.978 ± 0.001a	0.964 ± 0.002b	0.962 ± 0.002bc	0.957 ± 0.002c	0.948 ± 0.003d	0.933 ± 0.00e
pH	5.02 ± 0.05b	4.90 ± 0.00a	5.00 ± 0.04b	5.17 ± 0.04cd	5.14 ± 0.05c	5.2 ± 0.01d
SN	5.82 ± 0.86a	9.48 ± 0.70ab	15.79 ± 5.50bc	16.35 ± 3.17c	19.07 ± 1.34c	19.32 ± 1.63c
NPN	2.19 ± 0.49a	4.54 ± 0.41ab	7.20 ± 0.58bc	10.39 ± 1.45cd	12.85 ± 2.57de	14.44 ± 1.03e
AAN	0.27 ± 0.00a	0.60 ± 0.13ab	0.96 ± 0.20b	2.40 ± 0.33c	3.65 ± 0.18d	3.76 ± 0.23d
NH_3N	0.42 ± 0.05a	0.69 ± 0.05ab	0.72 ± 0.25ab	1.51 ± 0.08abc	1.79 ± 0.85bc	2.11 ± 0.86c
			Batch RC			
moisture	36.57 ± 0.32a	34.92 ± 0.93ab	34.40 ± 1.56b	29.32 ± 0.48c	28.66 ± 0.76c	27.25 ± 0.35c
fat	35.00 ± 0.00a	35.00 ± 0.00a	36.00 ± 0.00b	39.25 ± 0.35c	40.50 ± 0.71d	39.25 ± 0.35c
protein	20.64 ± 0.98b	22.59 ± 1.33c	18.47 ± 0.21a	24.69 ± 0.09d	24.97 ± 0.63de	26.64 ± 0.28e
NaCl	0.82 ± 0.25a	1.31 ± 0.26ab	1.64 ± 0.21b	1.49 ± 0.28b	1.58 ± 0.02b	1.51 ± 0.03b
ash	3.78 ± 0.54a	3.83 ± 0.47a	4.51 ± 0.23ab	3.96 ± 0.12a	4.92 ± 0.35b	4.59 ± 0.12ab
a_w	0.981 ± 0.002a	0.966 ± 0.006b	0.957 ± 0.003c	0.946 ± 0.003d	0.941 ± 0.001d	0.919 ± 0.001e
pH	5.23 ± 0.04b	5.10 ± 0.01a	5.19 ± 0.01b	5.36 ± 0.02c	5.36 ± 0.01c	5.43 ± 0.04d
SN	8.02 ± 0.80a	18.15 ± 2.24b	24.57 ± 0.65c	27.00 ± 0.32d	28.66 ± 0.52d	29.25 ± 0.10d
NPN	2.66 ± 0.42a	4.57 ± 0.95a	8.05 ± 0.20b	10.99 ± 0.21b	14.68 ± 0.39c	15.41 ± 2.15c
AAN	0.26 ± 0.01a	0.66 ± 0.03b	1.45 ± 0.02c	2.18 ± 0.04d	3.38 ± 0.04e	3.46 ± 0.01f
NH_3N	0.50 ± 0.02a	0.59 ± 0.02a	0.97 ± 0.19ab	1.37 ± 0.42ab	1.94 ± 0.64bc	2.41 ± 0.64c

[a] Means of the same parameter in the same row without a common letter differ significantly ($p < 0.05$).

Table 2. Log Counts of Different Microbial Groups in Raw Initial Milk, Curd, and Cheeses Manufactured with Animal Rennet (AR) and Recombinant Cyprosin (RC) during Ripening

			cheese, days of ripening					
	milk	curd	2	15	30	60	90	120
				Batch AR				
total viable	5.96 ± 0.00a	7.23 ± 0.00b	9.15 ± 0.15g	9.18 ± 0.13g	8.43 ± 0.46ef	8.72 ± 0.06f	8.24 ± 0.06de	7.97 ± 0.17cd
Enterobactericeae	4.43 ± 0.00c	6.78 ± 0.00a	5.59 ± 0.81b	4.51 ± 0.05c	2.98 ± 0.03d	2.72 ± 0.45d	1.63 ± 0.39e	0.00 ± 0.00f
coliforms	4.81 ± 0.00c	6.92 ± 0.00ab	6.46 ± 0.57b	4.52 ± 0.00c	2.92 ± 0.00d	2.35 ± 0.00e	1.55 ± 0.00e	0.00 ± 0.00f
staphylococci	3.42 ± 0.00cd	4.70 ± 0.00a	4.27 ± 0.06b	3.74 ± 0.00c	3.73 ± 0.40c	3.74 ± 0.04c	3.25 ± 0.01d	3.25 ± 0.01d
micrococci	4.29 ± 0.00 d	6.20 ± 0.00ab	6.32 ± 0.45a	5.66 ± 0.01abc	5.37 ± 0.53abcd	4.75 ± 1.47cd	5.55 ± 0.01abc	5.03 ± 0.01bcd
lactic acid bacteria	6.10 ± 0.00 a	7.30 ± 0.00b	9.13 ± 0.03de	9.17 ± 0.04e	8.45 ± 0.45cd	8.65 ± 0.14cde	8.72 ± 0.78cde	8.06 ± 0.14c
molds, yeasts	5.30 ± 0.00ab	5.47 ± 0.00 a	4.27 ± 0.08cd	3.80 ± 0.13d	4.79 ± 0.08bc	4.15 ± 0.98abc	2.78 ± 0.19e	2.50 ± 0.18e
				Batch RC				
total viable	5.96 ± 0.00f	7.70 ± 0.00e	8.44 ± 0.18b	8.74 ± 0.13a	8.66 ± 0.11a	8.38 ± 0.11bc	8.21 ± 0.00c	7.92 ± 0.01d
Enterobacteriaceae	4.43 ± 0.00e	6.70 ± 0.00b	6.30 ± 0.23c	5.66 ± 0.13d	3.83 ± 0.04f	2.40 ± 0.14g	1.59 ± 0.15g	0.00 ± 0.00h
coliforms	4.81 ± 0.00d	6.48 ± 0.00a	6.54 ± 0.00b	5.77 ± 0.00c	3.79 ± 0.00e	2.41 ± 0.00f	1.48 ± 0.00g	0.00 ± 0.00g
staphylococci	3.42 ± 0.00f	4.41 ± 0.00b	4.12 ± 0.06c	3.71 ± 0.05e	4.49 ± 0.08b	4.22 ± 0.04c	3.92 ± 0.11d	3.86 ± 0.01d
micrococci	4.29 ± 0.00a	6.98 ± 0.00d	6.43 ± 0.40c	6.07 ± 0.01bc	6.16 ± 0.01c	6.36 ± 0.13c	6.15 ± 0.07c	5.66 ± 0.36b
lactic acid bacteria	6.10 ± 0.00a	8.14 ± 0.00d	8.34 ± 0.16e	8.74 ± 0.14f	8.76 ± 0.06f	8.63 ± 0.01f	8.28 ± 0.01de	7.93 ± 0.01c
molds, yeasts	5.30 ± 0.00a	5.47 ± 0.00a	4.09 ± 0.28cd	3.57 ± 0.24de	4.49 ± 0.27bc	3.36 ± 0.02ef	2.83 ± 0.54fg	2.53 ± 0.64g

[a]Means of the same microbial group in the same row without a common letter differ significantly ($p < 0.05$).

during ripening, amount of coagulant added, salt-in-moisture concentration, and the pH of the cheese.

The proportion of total soluble nitrogen (SN) has traditionally been regarded as a "ripening index" for cheese because it reflects the extent of proteolysis. The strong proteolytic activity of RC as well as the natural enzymes extracted, as crude extract, from *C. cardunculus* L. flowers compared to chymosin in the formation of soluble nitrogen makes it suitable for use as a proteinase system for accelerated ripening in some ewes' milk cheese varieties involving the addition of enzymes (*23*).

NPN (containing mainly small peptides of 2 and 20 residues and free amino acids), which has traditionally been regarded as an index of "ripening depth", increased steadily and significantly ($p < 0.001$) in cheese manufactured with both coagulants throughout ripening. At the end of ripening the NPN of cheese produced with RC was slightly higher than that of cheese produced using AR. Although lactic bacteria and other enzymes (*24*) are the principal agents for the production of NPN, higher proteolytic activity in the breakdown of caseins and their first breakdown products in cheeses obtained with RC, which has been detected in cheeses made with *C. cardunculus* enzymes as opposed to AR (*21*), suggests that cheeses obtained with the RC contain more substrates (casein polypeptides) for producing higher amounts of low molecular weight nitrogen than those obtained with AR. NPN values of 13−17% of TN at the end of ripening were obtained in Los Pedroches cheese (*3*, *25*) and La Serena cheese (*20*, *26*) manufactured with *C. cardunculus*. However, in experiments with Serra da Estrela cheese, also coagulated with *C. cardunculus* and kept in a ripening chamber

at 6 °C, were obtained (*27*) NPN values (12% TCA-N) lower than 7% of TN after 68 days of ripening.

The other nitrogen fractions (AAN and NH_3N) also increased steadily and significantly during cheese ripening ($p < 0.001$) in cheeses manufactured with both coagulants throughout ripening. However, no significant differences ($p > 0.05$) were observed in the amount of AAN and NH_3N with either coagulant. The similar AAN and NH_3N values obtained (**Table 1**) using both types of coagulant, recombinant cyprosin and animal rennet, suggest that both coagulants had little peptidase activity; peptidases of microorganisms thus being the main source of production (*24*).

Cheese Microbiology. Table 2 shows average values (log cfu/gram of sample) and standard deviations (SD) for microbial groups, total viable, total Enterobacteriaceae, coliforms, staphylococci, micrococci, lactic acid bacteria, and molds and yeasts, obtained for initial milk, curd, and cheeses during ripening for the two trials manufactured with AR and RC. Microbial counts in the milk used for cheesemaking were high in most of the groups analyzed. These findings are in line with those of other authors who have studied the microbiology of raw ewes' milk (*20*, *27*, *28*). In curd and in the cheeses after 2 days of ripening, all microbial groups analyzed were higher than in initial milk, except for molds and yeasts. This is due to microbial growth during the milk clotting stage at ~30 °C, the physical entrapment of bacteria in the curd (*29*), and the starter culture added.

Total viable and lactic acid bacteria reached maximum values in the first 15 days of ripening in cheeses obtained with AR and between 15 and 30 days in cheeses produced with RC. Afterward, these two microbial groups decreased slightly until the end of ripening, with values ≈8 log cfu/g after 120 days. Enterobacteriaceae and coliforms reached maximum counts at 2 days of ripening and then decreased at similar rates until 90 days of ripening; they were not detected at 120 days of ripening. However, in cheeses obtained with crude aqueous extracts of cardoon and with no starter culture added, the counts of enterobacteria and coliforms after 60 and 90 days were still $>10^4$ g^{-1} (*9*). Enterobacteriaceae counts of over 10^4 and 10^6 g^{-1} have been reported for La Serena cheese after 60 days of ripening (*30*) and for Serra da Estrela cheese after 68 days (*27*), respectively, both varieties of cheeses using vegetable coagulant. Also, mean log counts of 3.81 for coliforms in La Serena cheese have been reported by Medina et al. (*30*) at 60 days of ripening.

In general, staphylococci and micrococci reached maximum counts in the first month of ripening and subsequently decreased at similar rates until the end of ripening. Mold and yeast counts reached a maximum in the first month of ripening in cheeses manufactured using both types of coagulant, decreasing thereafter. Higher mold and yeast counts than those obtained here (**Table 2**) have been reported by other authors who have studied the microbiology of ovine cheeses (*27*, *28*) produced with crude extract of vegetable coagulant; these authors reported mean log counts of ≥3.0 in different cheeses at the end of ripening.

Conclusions. The use of recombinant cyprosin had a significant effect on some chemical components analyzed, such as moisture and fat, as well as on pH and water activity of cheeses throughout ripening. In general, the type of coagulant had no significant influence on the microbial groups analyzed. Cheeses made with recombinant cyprosin displayed greater proteolytic activity in terms of soluble nitrogen and, to a lesser extent, in terms of nonprotein nitrogen than cheeses manufactured with animal rennet. The level of free amino acids and ammonia nitrogen were similar for cheese obtained with both types of coagulants. This therefore suggests that recombinant cyprosin produces a proteolysis similar to that obtained with natural enzymes present in the crude aqueous extract from *C. cardunculus* and thus quite different from that obtained using calf rennet.

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