Chemical and Microbiological Characteristics of Ewes' Milk Cheese Manufactured with Extracts from Flowers of *Cynara cardunculus* and *Cynara humilis* as Coagulants

Montserrat Vioque, Rafael Gómez, Emilia Sánchez, Carmen Mata, Luis Tejada, and José Fernández-Salguero*

Departamento de Bromatología y Tecnología de los Alimentos, Universidad de Córdoba, Campus de Rabanales, Edificio C-1, E-14014 Córdoba, Spain

The chemical and microbial characteristics as well as the flavor and aroma of Los Pedroches cheese made using aqueous extracts of *Cynara cardunculus* L. flowers were compared with those of cheeses manufactured with extracts of *Cynara humilis* L. throughout ripening. The two thistle species assayed were found to have no appreciable effect on the moisture, fat, protein, and NaCl contents of the cheese or on its water activity, flavor, and aroma; however, the use of *C. humilis* resulted in reduced lactic acid content ($p < 0.001$) and higher pH values ($p < 0.05$) relative to those of cheese specimens produced with *C. cardunculus*. The protein breakdown of the cheeses was assessed in terms of soluble nitrogen (SN), nonprotein nitrogen (NPN), and amino acid nitrogen (AAN). Proteolysis was more marked and rapid in cheese containing *C. cardunculus* as coagulant, the SN and NPN contents of which were significantly higher ($p < 0.01$) than those of the cheese obtained with the species *C. humilis*; AAN contents were similar in both species of *Cynara* throughout ripening. Although total viable, coliform, and lactobacilli counts were similar in cheeses produced with both types of plant coagulant throughout ripening, enterobacteria and yeasts counts ($p < 0.01$) and molds counts ($p < 0.05$) were higher in cheese produced with *C. humilis* than in cheese obtained with *C. cardunculus*.

Keywords: *Proteolysis; ovine cheese; vegetable coagulants; Cynara cardunculus; Cynara humilis*

INTRODUCTION

Rennet produced from calf stomachs has by tradition been used since ancient times as a coagulant in most cheese manufacturing. However, the increasing consumption of cheese and the decreasing number of calves slaughtered have led to an increased price of calf rennet and a search for alternative milk coagulants. Those facts have been accompanied by the more recent BSE disease in dairy cattle. The industrial-scale production of recombinant calf chymosin from the pro-chymosin gene cloned into *Escherichia coli* and food grade yeasts and molds (Fox and McSweeney, 1996) has been solved in the past few years, but some countries forbid its use, for example, Germany and The Netherlands. Other rennet substitutes were also studied. Coagulation of milk can be achieved by a number of proteolytic enzymes from various sources, such as different animal species (e.g., pig, bovine, and chicken pepsins), microbial proteinases (*Rhizomucor miehei*, *Rhizomucor pusillus*, and *Cryphonectria parasitica*), and proteinases extracted from fruits (e.g., pineapple, papaya, sodom apple) and plants such as wild cardoons.

Some Spanish and Portuguese varieties of raw ewes' and goats' milk cheeses are made with aqueous extracts of dried wild thistle flowers from various species of the genus *Cynara* L. (Carr, 1981). Although *C. cardunculus* is the most used species, other *Cynara* species such as *C. humilis* L., which is more abundant, are also used in the making of cheeses as a replacement for or, allegedly, mixed with *C. cardunculus* when this is scanty (Tavaria et al., 1997; Fernández-Salguero and Sanjuán, 1999).

Three proteinases of *C. cardunculus* L. have been isolated, purified, and partially characterized in terms of activity (Heimgartner et al., 1990; Campos et al., 1990; Cordeiro et al., 1992). They are thus acidic proteinases belonging to the aspartic proteinase group called "cynarases" (Cordeiro et al., 1992) or "cyprosins" (Cordeiro et al., 1994). Also, two additional aspartic proteinases were isolated from fresh stigmas of a standard variety of *C. cardunculus* grown from selected seeds, namely, cardosins A and B, being in terms of specificity and kinetic parameters one of them similar to chymosin while the other is similar to pepsin (Veríssimo et al., 1995, 1996; Ramalho-Santos et al., 1996). On the other hand, the proteinases found in species *C. humilis* are different than the proteinases of *C. cardunculus*, the former containing only a component similar to chymosin (Pires et al., 1994), and also are different in terms of milk clotting activity (Fernández-Salguero and Gómez 1997).

Some cheeses enjoying *Appellation d'Origine Controllée* status, such as "Serra da Estrela" (Portugal) and "La Serena" (Spain) can be made only from raw ewes' milk using an extract of the cardoon flowers (*C. cardunculus*) as coagulant. However, flowers gathered by different pickers may well be a mixture of *C. cardunculus* and *C. humilis*, and these may even be contaminated by other species such as *Centaurea calcitrapa* and *Silybum marianum* (Sanjuán and Fernández-Salguero,

* Corresponding author (telephone +34 957 212010; fax +34 957 212000; e-mail ao1fecaj@lucano.uco.es).

10.1021/jf990326v CCC: $19.00
Published on Web 12/31/1999

Table 1. Average Values and Standard Deviations for Moisture, Fat, Protein, Lactic Acid, NaCl (Grams per 100 g of Cheese), pH, and a_w in the Cheese Batches Obtained with *C. cardunculus* (CC) or *C. humilis* (CH) throughout Ripening

		days of ripening					
	batch	2	8	15	30	60	90
moisture	CC	49.61 ± 2.30	48.70 ± 1.99	46.30 ± 2.28	44.55 ± 2.74	40.86 ± 2.48	38.08 ± 1.35
fat		24.75 ± 4.68	24.92 ± 5.34	26.83 ± 6.21	26.75 ± 5.85	29.00 ± 5.77	29.25 ± 5.06
protein		21.39 ± 2.58	21.26 ± 2.53	22.15 ± 2.42	22.88 ± 2.49	23.98 ± 2.96	25.32 ± 3.28
lactic acid		0.99 ± 0.16	0.98 ± 0.14	1.09 ± 0.15	1.27 ± 0.12	1.42 ± 0.10	1.66 ± 0.23
NaCl		0.95 ± 0.29	1.12 ± 0.11	1.32 ± 0.17	1.55 ± 0.05	1.76 ± 0.04	1.70 ± 0.14
pH		5.29 ± 0.23	5.11 ± 0.11	5.15 ± 0.12	5.14 ± 0.16	5.19 ± 0.21	5.17 ± 0.30
a_w		0.985 ± 0.004	0.980 ± 0.005	0.971 ± 0.003	0.964 ± 0.003	0.958 ± 0.003	0.948 ± 0.001
moisture	CH	51.41 ± 1.62	48.52 ± 1.43	46.42 ± 1.52	44.32 ± 1.62	40.73 ± 2.30	38.21 ± 2.35
fat		23.83 ± 2.10	24.17 ± 3.25	24.50 ± 2.75	25.83 ± 3.36	27.08 ± 2.63	28.92 ± 3.02
protein		20.16 ± 0.36	22.07 ± 1.32	23.53 ± 0.98	24.84 ± 1.43	25.79 ± 1.37	26.73 ± 1.02
lactic acid		0.87 ± 0.10	0.92 ± 0.11	0.90 ± 0.07	0.94 ± 0.04	1.24 ± 0.05	1.24 ± 0.09
NaCl		1.08 ± 0.08	1.27 ± 0.27	1.48 ± 0.16	1.49 ± 0.29	1.71 ± 0.27	1.91 ± 0.41
pH		5.39 ± 0.21	5.21 ± 0.17	5.29 ± 0.09	5.32 ± 0.13	5.39 ± 0.15	5.41 ± 0.18
a_w		0.980 ± 0.005	0.976 ± 0.004	0.970 ± 0.009	0.967 ± 0.012	0.960 ± 0.009	0.948 ± 0.012

1994), because wild thistle growth tends to be considerable in very rainy springs.

One of the most representative artisanal ewes' milk cheeses manufactured in Spain is Los Pedroches (Carr, 1981). It is a semihard cheese manufactured from raw Merino ewes' milk that is not inoculated with a lactic culture and is usually coagulated with plant coagulant. The physicochemical and biochemical characteristics during ripening of Los Pedroches cheese made with animal rennet have been studied (Fernández-Salguero, 1975). Also we have studied the influence of vegetable (*C. cardunculus*) and animal rennet on proteolysis (Fernández-Salguero and Sanjuán, 1999) and mineral content (Sanjuán et al., 1998) during ripening as well as the effect of addition of starter cultures on the physicochemical and biochemical features (Carmona et al., 1999) of Los Pedroches cheese. The effect of animal rennet and extracts of *C. cardunculus* have been also studied in the Spanish La Serena cheese (Núñez et al., 1991) and in the Portuguese Serra da Estrela cheese (Sousa and Malcata, 1997a).

The isolation and purification of the proteinases of *C. cardunculus* and *C. humilis* have shown some qualitative differences (Pires et al., 1994; Esteves, 1995). However, information on proteolysis changes in cheese made with extracts of flowers from *C. humilis* as coagulant is not available. The aim of this paper was to compare changes in the physicochemical, biochemical, and microbiological characteristics of Los Pedroches cheese during ripening manufactured using *C. cardunculus* or *C. humilis*.

MATERIALS AND METHODS

Cheese-Making Procedure and Sampling. Cheese samples were made at a traditional factory in the production area by using raw milk from Merino ewes to which no starter culture was added. The amount obtained during a milking day was split into two batches, one of which was coagulated with an extract of *C. cardunculus* and the other with an extract of *C. humilis* (using ~6.1 kg of milk/kg of cheese). The milk obtained on another two days was split identically into two batches and coagulated with the same types of vegetable coagulant. Therefore, three experimental batches were coagulated with *C. cardunculus* and another three with *C. humilis*. Each of the six experimental batches consisted of six cheeses. The aqueous crude extracts from *C. cardunculus* or *C. humilis* were obtained as has been previously mentioned (Fernández-Salguero and Sanjuán, 1999). The clotting temperature for milk was 29 ± 1 °C. After pressing, the cheese was salted by rubbing its surface with dry salt and transported, under refrigeration, to the laboratory and ripened in a controlled room at 9 °C and 85% relative humidity. The cheeses were turned upside down daily the first 20 days of ripening and then every 2 days. The cheeses were analyzed at 2, 8, 15, 30, 60, and 90 days of ripening.

Compositional Analyses. Determinations of the chemical composition of the samples included those of moisture, protein, fat, lactic acid, and sodium chloride, all of which were conducted in duplicate as described (Fernández-Salguero et al., 1991). The pH was measured by probing the cheese directly with the 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, viz., soluble nitrogen (SN) at pH 4.6 and nonprotein nitrogen [NPN; soluble in 12% trichloroacetic acid (TCA)] and amino acid nitrogen (AAN) were determined as described elsewhere (Fernández-Salguero et al., 1989; Carmona et al., 1999). All samples were analyzed in duplicate.

Microbiological Analyses. All microbial groups were analyzed according to APHA (1984) methods as follow: 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; lactobacilli 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. All determinations were made in duplicate and expressed as log colony-forming units (cfu) per gram of sample.

Sensory Evaluation. Cheese flavor and aroma were determined by 12 trained panelists on a 10−0 point scale with anchor points (from extremely strong to extremely mild). The sensory evaluation of the cheeses was carried out at 60 and 90 days of ripening.

Statistical Treatment. The results obtained at the different ripening stages were subjected to an analysis of variance (ANOVA) using the SAS 6.09 sofware package (SAS, 1989).

RESULTS AND DISCUSSION

Compositional Characteristics. Overall mean values and standard deviations for moisture, fat, protein (TN × 6.38), lactic acid, NaCl (in grams per 100 g of cheese), pH, and a_w throughout ripening (at 2, 8, 15, 30, 60, and 90 days) of cheeses manufactured with *C. cardunculus* and *C. humilis* extracts are shown in Table 1. The analysis of variance (ANOVA) of all data obtained in this study are shown in Table 5. Table 5 shows that no significant differences were observed in moisture, fat, protein, and NaCl contents or in a_w of cheeses produced

Table 2. Average Values and Standard Deviations in TN and the Different Soluble Nitrogen Components (SN, NPN, and AAN; as Grams per 100 g of TN) in the Cheese Batches Produced with *C. cardunculus* (CC) or *C. humilis* (CH) throughout Ripening

		days of ripening					
	batch	2	8	15	30	60	90
TN	CC	3.36 ± 0.40	3.33 ± 0.39	3.47 ± 0.38	3.59 ± 0.39	3.76 ± 0.46	3.97 ± 0.52
SN		22.11 ± 5.41	24.54 ± 5.75	27.32 ± 3.56	31.15 ± 7.98	34.52 ± 6.33	37.31 ± 5.09
NPN		6.87 ± 0.80	9.7 ± 1.90	9.77 ± 1.91	11.10 ± 1.28	12.39 ± 1.66	14.67 ± 1.20
AAN		0.88 ± 0.05	1.24 ± 0.19	1.23 ± 0.15	2.49 ± 0.76	3.71 ± 0.21	5.57 ± 0.69
TN	CH	3.19 ± 0.06	3.45 ± 0.19	3.69 ± 0.16	3.89 ± 0.22	4.05 ± 0.23	4.19 ± 0.16
SN		17.45 ± 2.34	21.07 ± 2.24	20.82 ± 1.84	23.27 ± 3.47	27.95 ± 5.67	31.97 ± 2.99
NPN		6.46 ± 0.37	8.61 ± 2.71	8.17 ± 1.08	9.22 ± 0.90	10.20 ± 0.96	11.82 ± 1.04
AAN		0.77 ± 0.11	1.10 ± 0.48	1.51 ± 0.45	2.19 ± 0.46	3.58 ± 0.26	5.73 ± 1.01

with *C. cardunculus* as compared with those obtained with *C. humilis* (type of coagulant); however, significant differences were observed in the lactic acid contents ($p < 0.001$) and pH values ($p < 0.05$) of cheeses made using both types of coagulants. A comparison of the changes in these physicochemical components throughout ripening (ripening time; Table 5) revealed significant differences in moisture, lactic acid, NaCl, and a_w ($p < 0.001$) contents and in protein ($p < 0.01$) contents. These moisture contents were very similar throughout ripening to those obtained previously for Los Pedroches cheese (Fernández-Salguero and Sanjuán, 1999) prepared using *C. cardunculus*. Fat contents throughout ripening were slightly higher in cheese obtained using *C. cardunculus* than in cheese made using *C. humilis* ($p > 0.05$). Changes in protein and NaCl contents of both cheeses (Table 1) were similar. Lactic acid contents throughout ripening were significantly higher in cheeses produced with *C. cardunculus* ($p < 0.001$) than in those obtained with *C. humilis*. The pH values in cheeses dropped during the first days of ripening and then increased slightly, reaching values of almost 5.2 and 5.4 after 90 days ripening in the case of cheeses produced using *C. cardunculus* and *C. humilis*, respectively. With regard to a_w, almost identical values were obtained with both types of coagulants throughout ripening. Similar changes in the a_w of cheeses coagulated with *C. cardunculus* were reported by Carmona et al. (1999) in Los Pedroches cheese. 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 (Marcos et al., 1981).

Nitrogen Fractions. Table 2 shows average values and standard deviations in total nitrogen (TN) and different soluble nitrogen fractions (SN, NPN, and AAN, in grams per 100 g of TN) during the ripening of cheeses manufactured with *C. cardunculus* and *C. humilis*. SN values in cheeses manufactured with both coagulants increased throughout the whole of the ripening process ($p < 0.001$; Table 5). SN values also differed markedly between the cheeses coagulated with the two types of coagulant. Although SN is produced by the action of rennet, bacteria in the curd, and milk plasmin (Visser, 1977), the high SN contents observed at the beginning of ripening resulted from the intense proteolytic action of enzymes in the plant coagulants, which exhibited virtually maximum enzyme activity at the pH studied (Heimgartner et al., 1990; Veríssimo et al., 1996). Slightly lower SN values at the start of ripening have been reported in Los Pedroches cheese manufactured with *C. cardunculus* with 14−17% TN (Fernández-Salguero and Sanjuán, 1999; Carmona et al., 1999), although Fernández del Pozo et al. (1988) and Núñez et al. (1991) obtained values of 25.6 and 34.3%, respectively, after 2 days of ripening in La Serena cheese prepared with *C. cardunculus*. These variations in SN 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 during ripening, amount of coagulant added (especially in vegetable coagulant that is not properly standardized), salt-in-moisture concentration, and the pH of the cheese. With regard to the evolution of SN throughout ripening, much higher values (Table 2) were observed ($p < 0.01$; Table 5) in cheeses coagulated with *C. cardunculus* than in those coagulated with *C. humilis*. After 90 days of ripening, SN values in cheeses produced with *C. cardunculus* (37.3% on average) were >14% higher than those found in cheeses manufactured with *C. humilis* (≈32% on average). SN values >30% of TN at the end of ripening were observed by different authors who have studied the evolution of proteolysis in cheeses produced with vegetable coagulant. The higher amount of soluble nitrogen obtained in cheeses produced with *C. cardunculus* suggests stronger proteolytic activity of *C. cardunculus* enzymes than those of *C. humilis*. This observation is in line with the findings of Pires et al. (1994), who found that the proteinase system of *C. cardunculus* contains two components, one of which is similar to chymosin and the other to pepsin, whereas *C. humilis* contains only one component, which is similar to chymosin. Nevertheless, SN levels in cheese produced with *C. humilis*, which rose from 17.5% of TN at 2 days of ripening to 32% at the end of ripening, were much higher than those found in Los Pedroches cheese made with chymosin (Fernández-Salguero and Sanjuán, 1999), which after 100 days of ripening reached only 22.6% of TN.

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 ($p < 0.001$; ripening time, Table 5) in cheese manufactured with both coagulants throughout ripening. From the start, the increase in NPN was more marked in cheeses produced with *C. cardunculus* ($p < 0.01$). NPN values of 13−17% of TN at the end of ripening were obtained in Los Pedroches cheese (Fernández-Salguero and Sanjuán, 1999; Carmona et al., 1999) and La Serena cheese (Fernández del Pozo et al., 1988; Núñez et al., 1991) manufactured with *C. cardunculus*. However, Sousa and Malcata (1997a), in experiments with Serra da Estrela cheese also coagulated with *C. cardunculus* and kept in a ripening chamber at 6 °C, obtained NPN values (12% TCA-N) that were lower than 7% of TN after 68 days of ripening. Although lactic bacteria and other enzymes (O'Keeffe et al., 1978) are the principal agents for the production

Table 3. Log Counts of Different Microbial Groups in Raw Initial Milk and Cheeses Manufactured with Extracts from Flowers of *C. cardunculus* (CC) or *C. humilis* (CH) during Ripening

		days of ripening					
	milk	2	8	15	30	60	90
total viable							
CC	5.85 ± 0.02	10.53 ± 0.27	10.54 ± 0.02	9.57 ± 0.11	9.38 ± 0.39	9.39 ± 0.15	8.84 ± 0.54
CH	5.85 ± 0.02	10.76 ± 0.02	10.42 ± 0.12	9.80 ± 0.06	9.37 ± 0.14	9.29 ± 0.11	8.81 ± 0.17
enterobacteria							
CC	4.69 ± 0.08	8.30 ± 0.07	8.04 ± 0.16	8.03 ± 0.29	7.47 ± 0.16	5.39 ± 0.45	4.73 ± 0.49
CH	4.69 ± 0.08	8.22 ± 0.09	7.95 ± 0.11	8.21 ± 0.11	7.47 ± 0.22	6.43 ± 0.19	5.38 ± 0.07
coliforms							
CC	4.31 ± 0.05	7.52 ± 0.21	7.76 ± 0.12	6.66 ± 0.15	6.37 ± 0.06	5.48 ± 0.15	3.39 ± 0.11
CH	4.31 ± 0.05	7.51 ± 0.03	7.40 ± 0.02	6.39 ± 0.21	6.73 ± 0.26	5.42 ± 0.09	4.53 ± 0.78
lactobacilli							
CC	5.10 ± 0.26	8.49 ± 0.13	9.09 ± 0.58	8.87 ± 0.77	9.24 ± 0.14	8.30 ± 0.19	8.23 ± 0.14
CH	5.10 ± 0.26	8.73 ± 0.11	9.05 ± 0.32	8.60 ± 0.18	8.41 ± 0.32	8.40 ± 0.29	8.36 ± 0.47
molds							
CC	1.28 ± 0.19	1.66 ± 0.49	2.39 ± 0.70	1.33 ± 0.43	0.33 ± 0.58	0.00 ± 0.00	0.00 ± 0.00
CH	1.28 ± 0.19	2.31 ± 0.05	3.23 ± 0.21	2.22 ± 0.12	0.42 ± 0.73	0.00 ± 0.00	0.00 ± 0.00
yeasts							
CC	4.56 ± 0.40	3.50 ± 0.13	3.72 ± 0.31	2.35 ± 0.31	1.77 ± 0.27	0.87 ± 0.90	0.00 ± 0.00
CH	4.56 ± 0.40	4.13 ± 0.15	3.45 ± 0.13	3.22 ± 0.21	2.98 ± 0.19	2.10 ± 0.09	0.67 ± 1.16

of NPN, the higher proteolytic activity in the breakdown of caseins and their first breakdown products shown by *C. cardunculus* enzymes than by *C. humilis* enzymes (SN values in Table 2) suggests that cheeses obtained with *C. cardunculus* contain more substrates (casein polypeptides) to produce higher amounts of low molecular weight nitrogen.

It is well-known that the acidification process influences the proteolysis of cheese. The NS and NPN contents (Table 2) of cheeses were correlated positively with the amount of lactic acid (Table 1), correlations being higher in cheeses obtained with *C. cardunculus* ($r = 0.9728$, $p < 0.01$; and $r = 0.9317$, $p < 0.01$, respectively) than in those obtained in cheese produced with *C. humilis* ($r = 0.9058$, $p < 0.05$; and $r = 0.8764$, $p < 0.05$, respectively). However, no significant correlation ($p > 0.05$) was observed between the amount of NS and NPN and the pH of cheeses using either type of coagulant.

AAN values (Table 2) increased steadily and significantly ($p < 0.001$; ripening time, Table 5) in cheeses manufactured with both coagulants throughout ripening, rising from 0.9 to 5.6% of TN in cheeses produced with *C. cardu*nculus and from 0.8 to 5.7% in cheeses manufactured with *C. humilis*. No significant differences ($p > 0.05$) were observed in the amount of AAN with either coagulant. Similar amounts of AAN were obtained (Carmona et al., 1999) in Los Pedroches cheese produced with *C. cardunculus* after 90 days of ripening. Fernández-Salguero and Sanjuán (1999) and Núñez et al. (1991), in Los Pedroches and La Serena cheeses, respectively, found no significant difference in AAN values in cheese produced with animal rennet compared to those obtained with vegetable coagulant (*C. cardunculus*). Finally, Sousa and Malcata (1997a) and Macedo and Malcata (1997), in experiments with Serra cheese, obtained much lower phosphotungstic acid-soluble nitrogen values (<1.5%) than those obtained in the present study. The similar AAN values obtained (Table 2) using both types of coagulant, *C. cardunculus* and *C. humilis*, suggest that both vegetable coagulants had little peptidase activity to produce free amino acids; therefore, the peptidases of microorganisms are the main source of its production (McSweeney and Fox, 1993).

Cheese Microbiology. As mentioned above, the same initial milk that was split into two vats was used for the batches of cheese manufactured with *C. cardunculus* and the those produced with *C. humilis*. Table 3 shows average values (log cfu/g of sample) and standard deviations for microbial groups: total viable, total enterobacteriacae, coliforms, lactobacilli, molds, and yeasts obtained for initial milk and cheeses during ripening for the two trials manufactured with *C. cardunculus* (CC) or *C. humilis* (CH). The microbiological quality of the extracts from flowers of *C. cardunculus* and *C. humilis* have been obtained (Fernández-Salguero et al., 1999) and have high counts for total viable, enterobacteria, and coliforms in the flowers of both species of cardoon. Microbial counts in the milk used for making cheese 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 (Fernández del Pozo et al., 1988; Núñez et al., 1991; Poullet et al., 1991; Sousa and Malcata, 1997b). After 2 days of ripening, total viable counts in cheeses were almost 5 log units higher that in milk and 4 log units higher in terms of the enterobacteria, coliforms, and lactobacilli. This is partly explained by microbial growth during the milk clotting stage at ~30 °C, the physical entrapment of bacteria in the curd (Tatini et al., 1971), and the fact that the use of cardoon aqueous extracts entails additional microbial contamination of initial milk (Fernández-Salguero et al., 1999). Núñez et al. (1991) and Sánchez (1999) also obtained significantly higher microbial counts in La Serena and Los Pedroches cheese, respectively, produced with *C. cardunculus* compared to those obtained with chymosin.

Total viable microorganisms reached a maximum in the initial stages of ripening (Table 3), presenting values of 8.8 log cfu/g after 90 days in both types of coagulants. Other microbial groups, enterobacteria, coliforms, lac-

Table 4. Sensory Characteristics[a] of the Cheese Batches Obtained with *C. cardunculus* (CC) or *C. humilis* (CH) at 60 and 90 Days of Ripening

characteristics	days after manufacture	type of coagulant	
		CC	CH
flavor	60	5.37 ± 0.23	5.74 ± 1.53
	90	6.54 ± 0.29	6.80 ± 0.00
aroma	60	5.37 ± 0.34	5.04 ± 0.74
	90	6.31 ± 0.17	5.70 ± 0.66

[a] Mean values from 12 panelists.

Table 5. Results of the ANOVA for Physicochemical Parameters, Soluble Nitrogen Components, and Microbial Data of the Cheeses

Physicochemical Parameters, *p* Value

factor	moisture	fat	protein	lactic acid	NaCl	pH	a_w
type of coagulant, *T*	0.7169	0.4230	0.1539	<0.0001	0.2190	0.0205	0.8653
ripening time, *t*	<0.0001	0.3683	0.0019	<0.0001	<0.0001	0.5311	<0.0001
interaction, *T* × *t*	0.9538	0.9986	0.7918	0.1408	0.8290	0.9924	0.9815

Soluble Nitrogen Components, *p* Value

factor	SN/TN	NPN/TN	AAN/TN
type of coagulant, *T*	0.0042	0.0019	0.8038
ripening time, *t*	<0.0001	<0.0001	<0.0001
interaction, *T* × *t*	0.9725	0.7689	0.9131

Microbial Data, *p* Value

factor	viable	enterobacteria	coliforms	lactobacilli	molds	yeasts
type of coagulant, *T*	0.7097	0.0025	0.1112	0.3318	0.0103	0.0002
ripening time, *t*	<0.0001	<0.0001	< 0.0001	0.0081	<0.0001	< 0.0001
interaction, *T* × *t*	0.6291	0.0021	0.0002	0.1542	0.2652	0.1622

Sensory Characteristics, *p* Value

factor	flavor	aroma
type of coagulant, *T*	0.5831	0.2089
ripening time, *t*	0.0489	0.0356
interaction, *T* × *t*	0.9221	0.6134

tobacilli, molds, and yeasts, reached maximum counts between 2 and 8 days of ripening and then decreased at different rates until the end of ripening. Enterobacteria counts throughout ripening were significantly higher ($p < 0.01$, Table 5) in cheese produced with *C. humilis* than in cheese obtained with *C. cardunculus*. The higher enterobacteria counts after 60 and 90 days in cheeses manufactured with *C. humilis* could be due to the higher pH in these cheeses (Table 1), which favors the survival of these bacteria (González et al., 1992); enterobacteria counts of $>10^4$ and $>10^6$ g^{-1} have been reported for La Serena cheese after 60 days of ripening (Medina et al., 1991) and for Serra da Estrela cheese after 68 days (Sousa and Malcata, 1997b), respectively, and produced with vegetable coagulant. Coliform counts presented a similar evolution throughout ripening in cheese manufactured with both types of coagulants ($p > 0.05$). Only at 90 days of ripening were the counts in cheese made with *C. humilis* >1 log unit; mean log counts of 3.81 for coliforms in La Serena cheese have been reported by Medina et al. (1991), but Núñez et al. (1991) obtained counts of $>10^5$ g^{-1} at 60 days of ripening in this variety of cheese.

Lactobacilli presented a similar evolution in cheeses produced with both types of coagulants ($p > 0.05$), reaching maximum counts after 8 days of ripening (values $>10^9$ g^{-1}; Table 3), and, together with other lactic acid bacteria, were probably responsible for the fall in pH for cheeses produced with both types of coagulant (minimum values at 8 days; Table 1). From this point onward, lactobacilli decreased slightly until the end of ripening, with values ≈8 log cfu g^{-1} after 90 days. Similar lactobacilli counts have been reported by other authors in different types of cheeses. Mold counts reached a maximum after 8 days of ripening in cheeses manufactured using both types of coagulant ($p > 0.05$), decreasing thereafter; they were no longer detected after 60 days of ripening. Yeast counts were significantly higher ($p < 0.001$) in cheeses produced with *C. humilis* than in those obtained with *C. cardunculus*. Mold and yeast counts at 2 days of ripening were almost similar to those found in initial milk. Higher mold and yeast counts than those obtained here (Table 3) have been reported by other authors who have studied the microbiology of ovine cheeses (Fernández del Pozo et al., 1988; Poullet et al., 1991; Sousa and Malcata, 1997b) produced with vegetable coagulant; these authors reported mean log counts of ≥3.0 in different cheeses at the end of ripening.

Sensory Characteristics. Table 4 shows average values and standard deviations for flavor and aroma scores at 60 and 90 days of ripening of cheeses manufactured with *C. cardunculus* and *C. humilis*. The flavor and aroma scores increased ($p < 0.05$; ripening time) with the age of cheeses, but no significant differences were observed in both sensory characteristics of cheeses produced with *C. cardunculus* as compared with those obtained with *C. humilis* (type of coagulant).

Conclusions. The use of the two thistle species *C. cardunculus* and *C. humilis* had no significant effect on most of the chemical components analyzed and sensory characteristics (flavor and aroma) or on the water activity of cheeses throughout ripening, although higher lactic acid contents were observed in the former. The type of coagulant also had no significant influence on total viable, coliform, and lactobacilli counts, although higher enterobacteria, mold, and yeast counts were obtained in cheese produced with *C. humilis*. The higher microbial counts obtained at 2 days of ripening in cheese manufactured with both types of coagulants than in initial milk suggests that the use of aqueous flower extracts led to further microbial contamination. Although cheeses made with *C. cardunculus* displayed greater proteolytic activity in terms of soluble nitrogen and nonprotein nitrogen than cheeses manufactured with *C. humilis*, the levels of free amino acids were similar for cheeses obtained with both types of cardoons. This all therefore suggests that aqueous extracts of *C. humilis* could be used as a commercial alternative to *C. cardunculus* in the manufacture of ewes' milk cheese.

LITERATURE CITED

APHA. *Standard Methods for the Examination of Dairy Products*, 15th ed.; New York, American Public Health Association: Washington, DC, 1984.

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.

Carmona, M. A.; Sanjuán, E.; Gómez, R.; Fernández-Salguero, J. Effect of starter cultures on the physicochemical and biochemical features in ewe cheese made with extracts from flowers of *Cynara cardunculus* L. *J. Sci. Food Agric.* **1999**, *79*, 737−744.

Carr, S. *Pocket Guide to Cheese*; Mitchell Beazley Publishing: London, U.K., 1981.

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

Cordeiro, M.; Pais, M. S.; Brodelius, P. Tissue-specific expression of multiple forms of cyprosin (aspartic proteinase) in flowers of *Cynara cardunculus*. *Physiol. Plant.* **1994**, *92*, 645−653.

Esteves, C. Comparative study of biochemical characteristics of rennets *Cynara cardunculus* L., *Cynara scolymus* L., and *Cynara humilis* L. M.Sc. Thesis, University of Coimbra, Portugal, 1995.

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

Fernández-Salguero, J. Chemical composition and changes in nitrogen components during ripening of Los Pedroches cheese. Ph.D. Thesis, University of Córdoba, Spain, 1975.

Fernández-Salguero, J.; Gómez, R. *Study of the Traditional Cheeses of Andalucía*; Universidad de Córdoba-Cajasur: Córdoba, Spain, 1997.

Fernández-Salguero, J.; Sanjuán, E. Influence of vegetable and animal rennet on proteolysis during ripening in ewes' milk cheese. *Food Chem*. **1999**, *64*, 177−183.

Fernández-Salguero, J.; Marcos, A.; Alcalá, M.; Esteban, M. A. Proteolysis of Cabrales cheese and other European blue vein cheese varieties. *J. Dairy Res.* **1989**, *56*, 141−146.

Fernández-Salguero, J.; Sanjuán, E.; Montero, E. A preliminary study of the chemical composition of Guía cheese. *J. Food Compos. Anal.* **1991**, *4*, 262−269.

Fernández-Salguero, J.; Sánchez, E.; Gómez, R.; Mata, C.; Vioque, M.; Tejada, L. A preliminary study of microbiological quality of cardoons of the genus *Cynara* L. used in manufacture of traditional cheeses. *Michwissenschaft* **1999**, *54*, in press.

Fox, P.; McSweeney, P. L. H. Proteolysis in cheese during ripening. *Food Rev. Int.* **1996**, *12*, 3.1−3.56.

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

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

Macedo, A. C.; Malcata, F. X. Secondary proteolysis in Serra cheese during ripening and throughout the cheese-making season. *Z. Lebensm. Unters. Forsch. A* **1997**, *204*, 173−179.

Marcos, A.; Alcalá, M.; León, F.; Fernández-Salguero, J.; Esteban, M. A. Water activity and chemical composition of cheese. *J. Dairy Sci.* **1981**, *64*, 622−626.

McSweeney, P. L. H.; Fox, P. F. Cheese: methods of chemical analysis. In *Cheese: Chemistry, Physics, and Microbiology*, 2nd ed.; Fox, P. F., Ed.; Chapman and Hall: London, U.K., 1993; Vol. I.

Medina, M.; Fernández del Pozo, B.; Rodríguez Marín, M. A.; Gaya, P.; Núñez, M. Effect of lactic starter inoculation on chemical, microbiological, rheological and sensory characteristics of La Serena cheese. *J. Dairy Res.* **1991**, *58*, 355−361.

Núñez, M.; Fernández del Pozo, B.; Rodríguez Marín, 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.

O'Keeffe, A. M.; Fox, P. F.; Daly, C. Proteolysis in Cheddar cheese: role of coagulant and starter bacteria. *J. Dairy Res.* **1978**, *45*, 465−477.

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

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

Ramalho-Santos, M.; Veríssimo, P.; Faro, C.; Pires, E. Action on bovine α_{s1}-casein of cardosins A and B, aspartic proteases from the flowers of the cardoon *Cynara cardunculus* L. *Biochim. Biophys. Acta* **1996**, *1297*, 83−89.

Sánchez, E. Changes in the microflora of artisanal ewes' milk cheese made in different conditions during ripening. Ph.D. Thesis, University of Córdoba, Spain, 1999.

Sanjuán, E.; Fernández-Salguero, J. Influencia de algunos factores sobre el tiempo de coagulación por cuajo vegetal (*Cynara* sp.). *Aliment. Equip. Tecnol.* **1994**, *13*, 69−73.

Sanjuán, E.; Saavedra, P.; Millán, R.; Castelo, M.; Fernández-Salguero, J. Effect of ripening and type of rennet on the mineral content of Los Pedroches cheese. *J. Food Qual.* **1998**, *21*, 187−200.

SAS. General Lineal Model (GLM) procedures. In *SAS/STAT User's Guide: Statistics*, 4th ed.; SAS institute Inc.: Cary, NC, 1989.

Sousa, M. J.; Malcata, F. X. Comparison of plant and animal rennets in terms of microbiological, chemical and proteolysis characteristics of ovine cheese. *J. Agric. Food Chem*. **1997a**, *45*, 74−81.

Sousa, M. J.; Malcata, F. X. Influence of pasteurization of milk and addition of starter cultures on protein breakdown in ovine cheeses manufactured with extracts from flowers of *Cynara cardunculus*. *Food Chem*. **1997b**, *57*, 549−556.

Tatini, S. R.; Jezeski, J. J.; Morris, H. A.; Olson, J. C.; Casman, E. P. Production of staphylococcal enterotoxin A in Cheddar and Colby cheese. *J. Dairy Sci.* **1971**, *54*, 815−825.

Tavaria, F. K.; Sousa, M. J.; Domingos, A.; Malcata, F. X.; Brodelius, P.; Clemente, A.; Pais, M. S. Degradation of caseins from milk of different species by extracts of *Centaurea calcitrapa*. *J. Agric. Food Chem*. **1997**, *45*, 3760−3765.

Veríssimo, P.; Esteves, C.; Faro, C.; Pires, E. The vegetable rennet of *Cynara cardunculus* L. contains two proteinases with chymosin and pepsin-like specificities. *Biotechnol. Lett.* **1995**, *17*, 621−625.

Veríssimo, P.; Faro, C.; Moir, A. J. G.; Lin, Y.; Tang, J.; Pires, E. Purification, characterization and partial amino acids sequencing of two new aspartic proteinases from fresh flowers of *Cynara cardunculus* L. *Eur. J. Biochem*. **1996**, *235*, 762−768.

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.

Received for review April 6, 1999. Revised manuscript received September 27, 1999. Accepted October 29, 1999. We acknowledge financial funding of Spain's CICyT to Project ALI98-1135 and of the Plan Andaluz de Investigación to Group AGR 0120.

JF990326V