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Hygienic quality of ewes' milk cheeses manufactured with artisan-produced lamb rennet pastes

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The use of artisan-produced lamb rennet pastes, but not any of the other commercial animal rennets, imparts a characteristic flavour to the cheese, so most Mediterranean ewes' milk cheeses are coagulated with this kind of rennet paste. In contrast to the advantages of using lamb or kid rennet pastes from the sensory point of view, questions are still raised as to their hygienic quality. The goal was to examine the microbiological and enzymic quality of lamb rennet pastes prepared by cheese manufacturers for their own use, and evaluate the hygienic quality of raw sheeps' milk cheeses made with them, using Idiazabal cheese as a model. Lamb rennet pastes prepared by artisan cheese makers from the Basque region of Spain (27), and Italy (8) were evaluated. For cheese making experiments 5 different lamb rennet pastes were selected among the 27 samples from the Basque Country region of Spain. Microbiological analyses were carried out on samples from rennet pastes, rennet extracts, milks and cheeses during ripening. Enzymic activities studied in rennet paste were: total coagulating strength and lipase. Analysis of variance and Student's *t*-tests was performed. The results show that the artisan-produced rennet pastes contain high levels of a variety of microorganisms. After 60 ripening days, which is the minimum ripening period required for Idiazabal cheese prior to its commercialization, no *Eschericia coli*, *Clostridium, Salmonella* spp. or *Listeria monocytogenes* were detected, and levels for the rest of the microorganisms were below the limits of the European legislative standards for cheese manufactured with raw milk. We can conclude that the use of artisan-produced lamb rennet pastes of questionable hygienic quality for the manufacture of raw milk hard cheeses yields products of good hygienic quality.

Keywords: Artisan lamb rennet pastes, ewes' milk cheeses, hygienic quality, enzymic activities.

Rennet is the enzymatic preparation extracted from the abomasa of young ruminants, consisting, mostly, of the aspartic acid proteinases, chymosin and pepsin. Bovine rennet is the most frequently used coagulant in the manufacture of most cheese varieties worldwide and in today's cheese manufacturing industry (Wigley, 1996). The exceptions to this are some Mediterranean cheeses coagulated with lamb rennet paste such as Italy's Provolone, Pecorino Romano, Pecorino Sardo (Battistotti & Corradini, 1993; Barzaghi et al. 1997) and traditionally produced Greece Kefalotyri and Feta cheeses (Anifantakis, 1976; Anifantakis & Green, 1980). In Spain, the use of animal rennet, irrespective of species, is accepted by most Denominations of Origin and, to the best of our knowledge, approximately 50% of the Idiazabal cheese manufacturers (unpublished data provided by the Idiazabal Denomination of Origin) and some artisan cheese makers of Majorero goat cheese in the Canary Islands (De la Fuente et al. 1993) use lamb or kid rennet pastes.

Artisan-produced rennet pastes, in addition to chymosin and pepsin, contain different amounts of lipolytic activities (Bustamante et al. 2000) which are responsible for the strong lipolysis and the sensory characteristics of Italian, Spanish and Greek cheeses made with them (Nelson et al. 1977; Bustamante et al. 2003; Piredda & Addis, 2003; Virto et al. 2003; Moatsou et al. 2004; Addis et al. 2005; Horne et al. 2005).

The time-consuming preparation procedure and the difficulty to obtain artisan-produced rennet pastes with

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the same levels of both milk-clotting and lipase activities resulted in a gradual decline in their use (Harboe, 1994). However, in recent years we have detected, at least in certain areas of Spain, a renewed interest in the use of lamb and kid rennet pastes to maintain the authenticity of traditional cheeses.

In contrast to the advantages of using lamb or kid rennet pastes from the sensory point of view, questions are still raised as to their hygienic quality. The few published reports indicate that the hygienic quality of these rennets is very low (Calandrelli et al. 1997; Irigoyen et al. 2001; Piredda & Addis, 2003; Çakmakçi & Boroglu, 2004; Calvo & Fontecha, 2004; Moatsou et al. 2004). To the best of our knowledge, there are even fewer studies about the hygienic quality of cheeses made with artisan-produced rennets (Calandrelli et al. 1997; Moatsou et al. 2004). Idiazabal cheese is a traditional semi-hard cheese from the Basque Country region of Northern Spain made with raw ovine milk which can be manufactured either with any commercial animal rennet or with artisan-produced lamb rennet paste, as approved by its Denomination of Origin (Boletín Oficial del Estado, 2002). Therefore, the goal of the present study was to examine the microbiological and enzymic quality of lamb rennet pastes prepared by cheese manufacturers for their own use, and evaluate the hygienic quality of raw sheeps' milk cheeses made with them, using Idiazabal cheese as a model.

Material and Methods

Artisan lamb rennet pastes

Rennet pastes from the Basque region of Spain were prepared by 27, randomly selected, artisan cheese makers, belonging to the Idiazabal Denomination of Origin. Italian rennet pastes were prepared by 8 different artisan cheese makers, and given to us by a rennet manufacturer. Each individual cheese maker used his or her particular procedure to prepare the rennet paste which was intended for his or her own use, and not as part of a research project. Pastes were kept in the refrigerator until analysed. A generalized recipe for preparing rennet pastes in the Basque Country is as follows. Pastes are usually prepared from the abomasa of lambs, sacrificed between three and four weeks of age, air dried in a ventilated room with no heating for about 40–45 d and ground with salt to a final concentration of between 30 and 50% (by weight). Cheese makers select light to medium ground abomasa, indicating that were fed milk before sacrifice.

Cheese manufacture

Cheeses were made by artisan cheese makers in Spain. For cheese making experiments 5 different lamb rennet pastes having high bacterial counts were selected among the 27 samples from the Basque Country region. Rennet extracts were prepared by suspending a given amount of paste in 100–150 ml water (0·025–0·25 g/ml) at room temperature for 45–60 min and filtered through cheese cloth. The final concentration of rennet added was between 0·1–0·26 g/l milk.

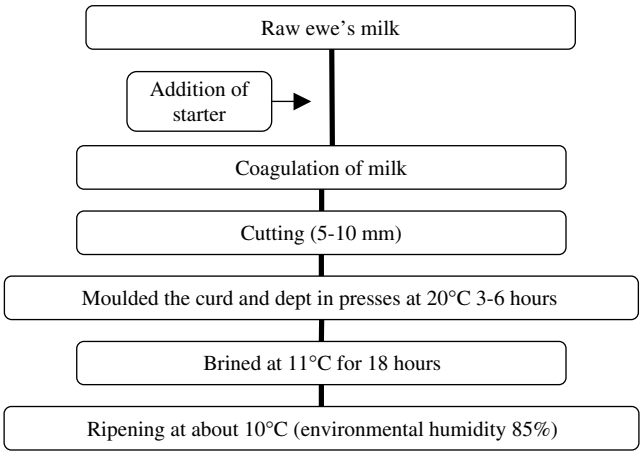

Fig. 1. Manufacturing protocol for Idiazabal cheese.

Each cheese maker made cheese in her habitual manner, in 300–500 l vats, with ewes' raw milk according to the traditional method for the artisan production of Idiazabal cheese, as approved by its Denomination of Origin (Boletín Oficial del Estado, 2002; Fig. 1). In order to minimize the influence of the microbiological characteristics of the milk, one manufacturing replicate was carried out within 1 week in the same farmhouse and with the same rennet paste.

Microbiological analyses

Microbiological analyses were carried out on samples from rennet pastes, rennet extracts, milks prior to addition of the starter cultures, and cheeses after 2, 60 and 150 ripening days. All samples were maintained at 3–4 °C during transport to the laboratory and duplicate analyses were performed within a maximum of 4 h.

Microbiological analyses in samples of rennet pastes and rennet extracts were carried out as required by the Spanish legislation (Boletín Oficial del Estado, 1988). The following microorganisms were determined: aerobic mesophilic bacteria, *Enterobacteriaceae*, *Eschericia coli*, coagulase-positive staphylococci, sulphur-reducing *Clostridium*, moulds and yeasts and *Salmonella* spp. In addition to these species, the presence of *Listeria monocytogenes* was studied in rennet extracts, whereas in milk and cheese samples coliforms, *List. monocytogenes*, and the above-mentioned microorganisms except *Enterobacteriaceae*, were determined.

Samples (10 g or 10 ml) were homogenized in 90 ml sterile 2% (w/v) sodium citrate solution (Merck, Darmstadt, Germany) in a Colworth Stomacher 400 (A.J. Seward Ltd., London, UK). Decimal dilutions of the homogenate were prepared by mixing 10 ml with 90 ml 0·1% (w/v) sterile

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Table 1. Microbiological counts (log_{10} cfu/g) and enzymatic activities (means ± standard deviation and ranges) of lamb rennet pastes artisanally produced in the Basque Country (B) and in Italy (I)

	Mean ± SD		Range ± SD	
	B (n=27)	I (n=8)	B	I
Aerobic mesophilic bacteria	3·99 ± 0·61	4·19 ± 0·62	2·61–5·33	2·81–4·58
Enterobacteriaceae	ND	ND	ND	ND
Esch. coli	ND	ND	ND	ND
Enterotoxigenic staphylococci	0·99 ± 1·02	0·19 ± 0·52**	ND–2·43	ND–1·48
Clostridium sulphur–reducing	1·94 ± 0·96	2·76 ± 0·39**	ND–3·77	2·06–3·34
Moulds – yeasts	1·16 ± 1·15	1·68 ± 1·00	ND–3·12	ND–3·47
Salmonella spp.	ND	ND	ND	ND
Coagulating strength (RU/g)	103·6 ± 83·0	56·0 ± 6·0	28·8–383·3	48·7–64·6
Lipase activity (LU/g)	10·08 ± 10·20	0·63 ± 0·59**	0·61–30·26	0·00–1·25

ND, below detection limit. Significance of effects: ** P<0·01

peptone water solution (Oxoid, Unipath Ltd., Basingstoke, UK) as described by International Dairy Federation (IDF, 1992).

Specific media were used to enumerate the different microbial groups: aerobic mesophilic bacteria on standard Plate Count Agar (PCA) (Oxoid) after incubation at 30 °C for 72 h; *Enterobacteriaceae* on Violet Red Bile Dextrose Agar (VRBGA) (Oxoid) after incubation at 37 °C for 24 h, and coliforms on Violet Red Bile Agar (VRBA) (Oxoid) after incubation at 30 °C for 24 h (Pascual & Calderón, 1999); *Esch. coli* on Selective Chromogenic Medium Coli ID (BioMérieux, Marci, L'Etoile, France) incubated at 44 °C for 24 h (AFNOR, 1999); enterotoxigenic staphylococci on Baird-Parker RPF-Agar (BioMérieux) incubated at 37 °C for 48 h (UNE, 2000); sulphur-reducing *Clostridium* on Sulphite Polymyxin Sulfadiazine Agar (SPS) (Merck) incubated at 46 °C for 48 h; and moulds and yeasts on Oxytetracycline Glucose Yeast Extract Agar (OGYE) (Oxoid) incubated at 20 °C for 5 d (Pascual & Calderón, 1999).

One ml of each dilution was depth-inoculated in standard PCA, VRBG, VRBA, Coli ID, SPS and OGYE agar, in duplicate, and homogenized before solidification. Plates of VRBG and VRBA were covered with a layer of the same medium before incubation. In Baird-Parker RPF-agar, 0·1 ml of each dilution was surface plated in duplicate.

For the investigation of *Salmonella* spp. 25 g or 25 ml sample was homogenized in 225 ml 1% (w/v) sterile peptone water solution (BioMérieux) using a Colworth Stomacher 400 and incubated at 37 °C for 20 h. An aliquot of homogenate (0·1 ml) was incubated in 10 ml of Rappaport Vassiliadis medium (Oxoid) at 42 °C for 48 h and a second aliquot of 1 ml was incubated in 10 ml of Mueller Kauffman Tetrathionate Broth Medium (Oxoid) at 37 °C for 48 h. Xilose Lysine Decarboxylase agar (XLD) (BioMérieux) and Chromogenic SMID (BioMérieux) were surface-plated with each of the cultures obtained from the previous media and incubated at 37 °C for 24 h. Representative numbers of suspicious colonies were verified by standard biochemical and serological procedures (Pascual & Calderón, 1999).

List. monocytogenes was determined as described in ISO (1998).

Enzymic activities

Total coagulating strength. Lamb rennet pastes were homogenized (0·1 g/l deionized water) on ice for 10 min in a Potter-Elvejhem type homogenizer and solids were removed by filtering through a fine plastic mesh. The coagulating activity was determined as described by IDF (1987). Chymosin and pepsin were separated by ion exchange chromatography on Fractogel EMD DEAE.650 (Merck), and quantified as described by IDF (1987), with the modifications described by Bustamante et al. (2000). The content of chymosin and pepsin was expressed as percentage of the total coagulating activity.

Lipase activity. Lipase activity assay was adapted from the pH-stat method described by Barton et al. (1996) with the modifications described by Svensson et al. (2006) using tributyrin as subtract.

Statistical analysis

Analysis of variance was performed using SPSS statistics package version 12.0 for Windows (SPSS Inc., Chicago, IL, USA). Student's *t*-tests were used to compare the differences in microbiological and enzymatic parameters in Basque Country and Italian rennets and to compare the differences in microbiological parameters in rennet paste and rennet extract used for cheese manufacture.

Results and Discussion

Artisan lamb rennet pastes

In Spain current legislation establishes the maximum levels of the microbial groups listed in Table 1 that are allowed in rennets (Boletín Oficial del Estado, 1988). None of the

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27 Basque rennet pastes analysed complied with it. As can be seen, the range of values found for each microorganism was large, most likely due to the different preparation procedures used by each artisan cheese maker. Taking into account each rennet paste sample, the level of aerobic mesophilic bacteria (upper legal limit 10^5 cfu/g), enterotoxigenic staphylococci (absence/g required) and moulds and yeasts (upper legal limit 10 cfu/g) detected in our study were below the legal limits in 92, 44·4 and 51·8% (data not shown), respectively. Only 3·7% of the samples were in compliance with the Spanish legislation as far as sulphur-reducing *Clostridium* was concerned (upper legal limit 1 cfu/g). Considering that rennet is a type of product that is not directly consumed by humans, the maximum amount required of this last microorganism by the Spanish legislation appears to be excessively rigorous, particularly when compared with 10^2 cfu/g, which is the maximum amount authorised in meat products to be consume without a prior thermal treatment, such as ham (Boletín Oficial del Estado, 1984).

Published reports (Calandrelli et al. 1997; Piredda & Addis, 2003) indicate that the hygienic quality of Italian artisan rennets is also very low. We had the opportunity to obtain small samples of Italian rennet pastes and decided to investigate them. The results are included in Table 1 for comparison. The hygienic quality of both Basque and Italian rennets was similar. As can be seen, *Enterobacteriaceae*, *Esch. coli* and *Salmonella* were not present in any of the samples studied.

A review of the literature indicated that artisan rennets from Greece, Egypt and Turkey countries in which these rennets are extensively used in the manufacture of traditional cheeses exhibited high levels of these microorganisms (Moatsou et al. 2004).

The enzymatic characteristics of Basque and Italian rennets were quite different. The mean value of total coagulating activity of Basque rennets was approximately 2-fold higher than that found in Italian rennets. Likewise, lipase activity of Basque rennets was approximately one order of magnitude higher than that found in Italian rennets.

Cheesemaking

In all the above mentioned countries traditional cheeses made with rennet pastes are highly appreciated by consumers because of their distinct sensory characteristics and have been extensively consumed by humans without any public health problems. Therefore, we decided to study the hygienic quality of cheeses made with artisan-produced rennet pastes. The 5 Basque pastes having highest microbiological counts were selected as the worst-case scenario.

Neither *Salmonella* spp. nor *List. monocytogenes* were detected in any of the rennet extracts studied. The levels of microorganisms from fecal origin, such as *Enterobacteriaceae*, and those from environmental contamination, such as mould and yeasts in the rennet extracts were significantly ($P<0·01$) higher than those found in the rennet pastes. This is most likely due to careless handling by two cheese makers for *Enterobacteriaceae* during the preparation of the rennet extract. For moulds and yeasts, three cheese makers contributed to the increase of 2–3 log, independently of the dilution that was achieved. By contrast, during cheese making with commercial rennets no measurable levels of microorganisms are detected.

Poor microbiological quality was also reported in a study of 12 samples of lamb rennet extracts by Irigoyen et al. (2001). Plant rennets, such as extracts of cardoon flowers (*Cynara cardunculus* and *Cynara humilis*), were also found to contain very high levels of aerobic mesophiles, *Enterobacteriaceae* and moulds and yeasts (Fernandez-Salguero et al. 1999).

To establish the conditions of the starting materials for cheese making, both rennet extracts and milk were analysed. In our study mean counts for aerobic mesophilic bacteria, *Esch. coli* and moulds and yeasts in rennet extracts and in milk were similar. However, levels of staphylococci were lower in rennet extract than in milk, whereas counts for sulphur-reducing *Clostridium* were higher in rennet extract than in milk (Table 2). The low amount of sulphur-reducing *Clostridium* added with the extract rennet to the milk could be important, as this microorganism is sporulated and could resist cheese making and ripening conditions, such as low pH, a_w and high salt concentrations. Taking into account the individual cheese makers (Fig. 2) this microorganism was present in all of the rennet extracts, whereas it was detected only in milk samples of one cheese maker (D).

The trends in the variation of counts throughout cheese making (from milk to 150 d ripening) were similar for all microorganisms except for staphylococci (Fig. 2). Considering that the trends described in Fig. 2 were similar, mean values for each microorganism and d ripening are listed in Table 2.

The counts in cheese milk were quite low for all microorganisms studied (Table 2). In fact, all milks fulfilled the European legislation for raw milk for cheese manufacture (DOCE, 2005). Some authors have reported substantially greater values for raw ewes' milk levels of aerobic mesophiles, coliforms, and moulds and yeasts (Nuñez et al. 1991; Sousa & Malcata, 1997; Fernandez-Salguero et al. 1999; Pérez-Elortondo et al. 1999a; Salmeron et al. 2002).

Counts of aerobic mesophilic bacteria, coliforms, *Esch. coli* and moulds and yeasts were statistically higher in 2 d-old cheeses than in milk (Table 2). This increase could be due to the physical entrapment of microorganisms in the curd and above all to the microbial multiplication during coagulation and draining period (approximately 12 h; Arenas et al. 2004). Welthagen & Viljoen (1998) suggested that a significant source of yeast contamination is probably the brine solution. However, no statistically significant differences were observed for the mean values

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Table 2. Microbiological counts (log_{10} cfu/g or mL) in Basque lamb rennet extract, milk and cheese during ripening. (Means, standard deviations and ANOVA during ripening time)

			Cheese (days of ripening)		
	Rennet extract	Milk	2 d	60 d	150 d
Aerobic mesophilic bacteria	4·02 ± 0·80	4·03 ± 0·62^{a}	9·68 ± 0·16^{d}	8·43 ± 0·47^{c}	7·50 ± 0·14^{b}
Enterobacteriaceae	1·32 ± 1·46	—	—	—	—
Coliforms	—	1·44 ± 1·03^{a}	4·07 ± 1·03^{b}	1·07 ± 1·18^{a}	ND
Esch. coli	0·16 ± 0·50	0·83 ± 1·23^{a}	2·65 ± 1·56^{b}	ND	ND
Enterotoxigenic staphylococci	0·50 ± 0·80	1·74 ± 0·69^{b}	1·88 ± 1·58^{b}	1·64 ± 1·20^{b}	0·19 ± 0·38^{a}
Clostridium sulphur-reducing	1·49 ± 0·63	0·27 ± 0·59^{a}	0·24 ± 0·58^{a}	ND	ND
Moulds and yeasts	2·50 ± 0·82	1·90 ± 0·77^{b}	3·58 ± 1·03^{c}	1·42 ± 0·83^{a,b}	1·10 ± 0·73^{a}
Salmonella spp.	ND	ND	ND	ND	ND
Listeria monocytogenes	ND	ND	ND	ND	ND

Note: The values presented are means of duplicate analyses from 5 independent experiments
^{a,b,c} Means in the same row with the same superscript are not significantly different ($P<0·05$). ND: below detection limit. —: not mentioned in Norms

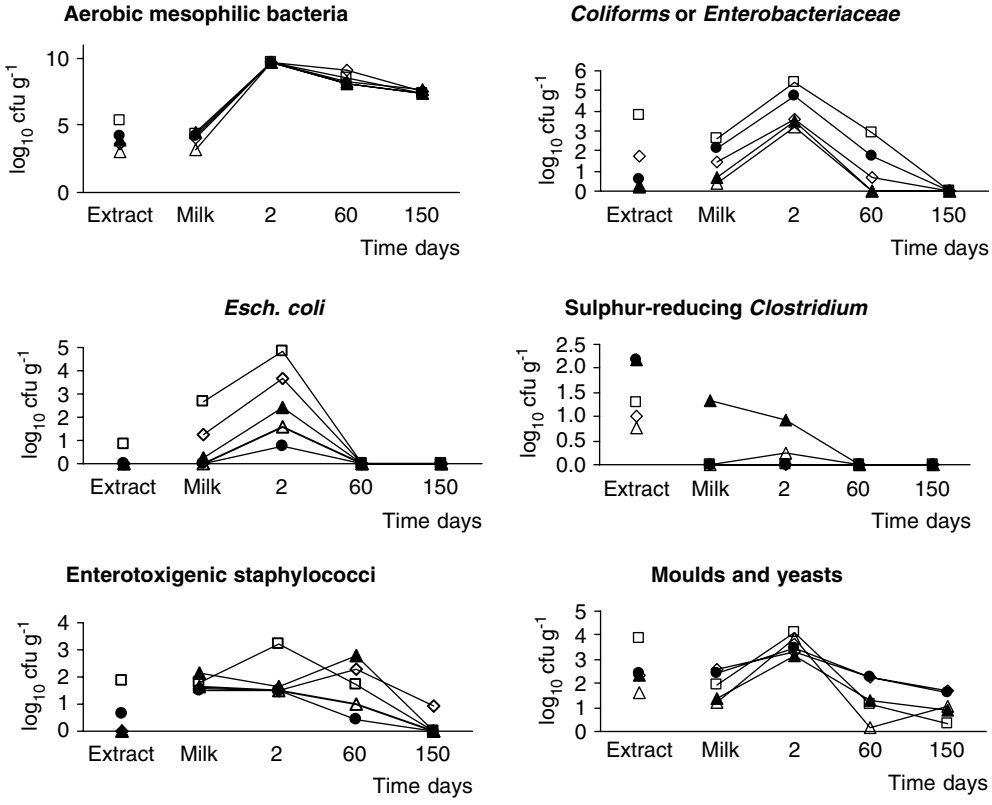

Fig. 2. Trends for microbiological counts during cheese ripening for individual cheese makers. Cheese makers: A, B, C, D and E. □, A; ◇, B; △, C; ▲, D; ●, E.

of the levels of staphylococci and sulphur-reducing *Clostridium* between milk and 2 day-old cheeses. In spite of the high concentration of *Clostridium* in rennet extracts, the concentration of this microorganism in 2 day-old cheeses was similar to that in milk. After 60 d ripening, which is the minimum ripening period required for Idiazabal cheese prior to its commercialization (Boletín Oficial del Estado, 2002), no *Esch. coli* or *Clostridium* were detected, and levels for the rest of the microbial groups were below the limits required by the European

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legislative standards for cheese manufacturing with raw milk (DOCE, 2005).

Special attention should be given on the trends of *Clostridium* for cheese maker D: in spite of having with the highest levels in rennet extract (2·19 log) and in milk (1·34 log) (Fig. 2), after 60 d ripening *Clostridium* was not detected like in the other cases. These results indicated that ripening conditions were not favourable for this microorganism.

No coliforms were detected after 150 d ripening and the levels of all the microbial groups studied were significantly reduced. The trends for the levels of microbial populations during ripening reported in this work are habitual for ewes' cheeses manufactured with raw milk and commercial rennets (Pérez-Elortondo et al. 1999b; Salmeron et al. 2002; Caridi et al. 2003; Tejada & Fernandez-Salguero, 2003; Moatsou et al. 2004). Calandrelli et al. (1997) and Moatsou et al. (2004) reported similar trends in ewes' and goats' milk cheeses manufactured with artisan-produced rennets containing high microbial counts.

In conclusion, the use of artisan-produced lamb rennet pastes of questionable hygienic quality for the manufacture of raw milk cheeses yields products of good hygienic quality. This is most likely due to the very low absolute amounts of microorganisms added to a large volume of milk, as well as to the unfavorable conditions for their multiplication during cheese ripening. Considering our results, the use of artisan-produced lamb rennet pastes does not appear to pose an added risk of contamination to raw milk hard cheeses.

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