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Volatile compounds in cheeses made from raw ewes' milk ripened with a lactic culture

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In some European regions ewes' milk is transformed into cheese without a previous heat treatment. The microbiota and the native enzymes present in raw milk are considered to be responsible for the characteristic strong flavour of raw ewes' milk cheeses (Nuñez et al. 1989). Although pasteurization ensures a higher uniformity of the product, heat treatment of ewes' milk may impair the sensory characteristics of cheeses (Gaya et al. 1990).

Storage of raw milk at low temperatures promotes the selection of psychrotrophic gram-negative bacteria producing extracellular proteases and lipases which may influence cheese flavour (Nuñez et al. 1984; Banks et al. 1988; Cromie, 1992). Attempts have been made to minimize the growth of psychrotrophic gram-negative bacteria in refrigerated raw milk by the use of temperatures close to 0 °C, inoculation (ripening) with lactic cultures, activation of milk lactoperoxidase system or addition of carbon dioxide (Juffs & Babel, 1975; Uceda et al. 1994).

No information is available on the influence of milk storage conditions on the formation of volatile compounds in ewes' milk cheese. The aim of the present work was to study the effect of ripening raw ewes' milk with a commercial lactic culture at low temperatures (5 and 10 °C) on the formation of volatile compounds of cheese, in comparison with volatile compounds of cheeses made from milk stored without lactic culture at the same temperatures.

Materials and Methods

Cheese manufacture

Cheeses were manufactured in three experiments, carried out on consecutive weeks, from refrigerated bulk raw ewes' milk collected on its arrival at a dairy in Central Spain. Cheese making procedures were those described for raw milk Manchego cheese by Fernández-García et al. (2002). Each experiment consisted of four vats containing 20 l milk. Vats 1 and 2 were inoculated with 0·05 g commercial frozen concentrated (FC) lactic culture/kg milk and ripened for 18 h at 5 °C (R5) or 10 °C (R10), respectively. The FC culture used was DVS R-603 (Chr. Hansen, E-28760 Tres Cantos, Spain), consisting of *Lactococcus lactis* subsp. *cremoris* and *Lc. lactis* subsp. *lactis* strains. Vats 3 and 4 were made in the same experiment from milk not inoculated with FC culture, stored for 18 h at 5 °C (S5) or 10 °C (S10). Milk in vats 1 and 2 was heated to 30 °C, and 20 min later rennet (2·7 ml Maxiren, 1 : 15 000 strength; Gist Brocades, NL-02611 Delft XT, The Netherlands) was added. A fresh lactic culture for vats 3 and 4 was prepared from raw ewes' milk thermized at 67 °C for 15 s, which was inoculated with 0·05 g FC culture/kg and incubated for 16 h at 25 °C. Milk in vats 3 and 4 was heated to 30 °C and inoculated with 10 g fresh lactic culture/kg; rennet was added 20 min later. Curds were cut 40 min after rennet addition into 6–8 mm cubes, scalded at 37 °C for 15 min, and distributed into cylindrical moulds. Two cheeses of ~2 kg were obtained from each vat. Cheeses were pressed at 20 °C for 18 h, salted in brine (150 g NaCl/l) at 12 °C for 24 h, and ripened at 12 °C. One cheese per vat was sampled on days 1, 15 and 30, and the other on day 60.

Microbiological and chemical analyses

Samples were homogenized in a sterile sodium citrate solution (20 g/l) using a Stomacher (Seward Laboratory, London SE1 9UG, UK) and decimal dilutions of milk and cheese homogenate prepared in sterile peptone water (1 g/l). Total viable counts were determined on plate count agar (Difco, Becton Dickinson France, F-38800 Le Pont de Claix, France) incubated at 30 °C for 72 h, gram-negative bacteria on PMK agar (Biolife, I-20128 Milano, Italy) incubated at 30 °C for 48 h, and coliforms on violet red bile agar (Oxoid, Basingstoke RG24 0PW, UK) incubated at 30 °C for 24 h, all in duplicate plates.

Cheese pH was measured in duplicate using a penetration electrode (Xerolyt 52-32, Crison, E-08328 Barcelona, Spain). Cheese proteolysis was determined in

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Table 1. Microbial counts, pH and proteolysis in cheeses made from milk ripened with a lactic culture at 5 °C (R5) or 10 °C (R10) or from milk cold stored at 5 °C (S5) or 10 °C (S10)

Mean ± SD of duplicate determinations from three experiments

	Days	R5	R10	S5	S10
Gram-negative bacteria, log cfu/g	1	7·40 ± 0·36^{a}	7·47 ± 0·30^{a}	6·84 ± 0·05^{a}	7·52 ± 0·25^{a}
	15	7·17 ± 0·18^{b}	7·30 ± 0·31^{b}	6·21 ± 0·29^{a}	6·91 ± 0·44^{ab}
	30	6·21 ± 0·75^{a}	6·56 ± 0·35^{a}	5·08 ± 0·51^{a}	5·88 ± 0·63^{a}
	60	4·38 ± 0·87^{ab}	5·13 ± 0·22^{b}	3·24 ± 0·11^{a}	3·72 ± 0·58^{a}
Coliforms, log cfu/g	1	6·75 ± 0·55^{ab}	7·00 ± 0·56^{b}	5·41 ± 0·21^{a}	6·65 ± 0·66^{ab}
	15	5·41 ± 0·53^{a}	5·81 ± 0·51^{a}	4·70 ± 0·48^{a}	5·29 ± 0·67^{a}
	30	3·71 ± 0·42^{ab}	4·62 ± 0·62^{b}	2·68 ± 0·25^{a}	3·21 ± 0·31^{a}
	60	2·06 ± 0·10^{a}	3·22 ± 0·42^{b}	1·57 ± 0·15^{a}	2·00 ± 0·05^{a}
Cheese pH	1	5·05 ± 0·05^{b}	5·04 ± 0·06^{b}	4·94 ± 0·05^{a}	4·95 ± 0·04^{a}
	15	5·17 ± 0·09^{a}	5·26 ± 0·09^{a}	5·19 ± 0·12^{a}	5·25 ± 0·11^{a}
	30	5·16 ± 0·09^{a}	5·29 ± 0·10^{a}	5·18 ± 0·08^{a}	5·24 ± 0·10^{a}
	60	5·18 ± 0·05^{ab}	5·25 ± 0·02^{b}	5·16 ± 0·02^{a}	5·26 ± 0·02^{b}
Proteolysis, $A_{340\ nm}$	1	0·16 ± 0·02^{a}	0·16 ± 0·03^{a}	0·14 ± 0·02^{a}	0·15 ± 0·01^{a}
	15	0·23 ± 0·01^{a}	0·30 ± 0·03^{b}	0·22 ± 0·02^{a}	0·28 ± 0·02^{b}
	30	0·34 ± 0·02^{a}	0·45 ± 0·03^{b}	0·36 ± 0·01^{a}	0·44 ± 0·02^{b}
	60	0·47 ± 0·03^{a}	0·55 ± 0·02^{b}	0·49 ± 0·01^{a}	0·58 ± 0·01^{b}

Means in a row with the same superscript do not differ significantly ($P<0·05$)

duplicate by the *o*-phthaldialdehyde method, with 3 ml *o*-phthaldialdehyde reagent and 50 µl of filtered cheese extract in the assay mixture which was incubated at 20 °C for 2 h (Oumer et al. 2001).

Pieces (~100 g) of 60 d-old cheeses were wrapped in aluminium foil, vacuum packed and frozen at –40 °C until analysis of volatile compounds. Preparation of samples, extraction using an automatic dynamic headspace apparatus and analysis of volatile compounds by GC-MS were carried out in duplicate as previously described (Fernández-García et al. 2002). Quantification was carried out by sum of all ions abundance (TIC). The exceptions (ions used for quantification in brackets) were 2-butanol (ion 59), 1-propanol (ion 59), isoamyl butyrate (ions 71, 70, 89, 55), ethyl butyrate (ion 88) co-eluting with 1-propanol, and propyl butyrate (ions 71, 89, 101) co-eluting with isoamyl acetate (ions 70, 87). Data are presented as relative abundance, with reference to the cyclohexanone peak.

Sensory evaluation and statistical analyses

Four cheeses (R5, R10, S5 and S10) corresponding to each experiment were presented per session as representative slices in closed individual Petri dishes to 15 trained panellists for sensory analysis. Flavour intensity and flavour quality of cheeses were evaluated on a ten point scale, as previously described (Fernández del Pozo et al. 1988).

Analyses of variance with ripening (R) or storage (S) of milk, refrigeration temperature (5 or 10 °C) and experiment as main effects were performed using the SPSS program Win version 9.0 (SPSS, Chicago, IL 60611, USA). Comparisons of means ($P<0·05$) by Tukey's test at different cheese ages were carried out using the same program. Pearson correlation coefficients were also calculated (Steel & Torrie, 1980).

Results and Discussion

Microbial groups

Microbial counts in bulk raw ewes' milk before ripening or storage were high, with mean values (log cfu/ml) of 6·44 for total viable counts, 6·05 for gram-negative bacteria and 3·70 for coliforms. Milk inoculation with a lactic culture did not prevent growth of gram-negative bacteria during overnight storage. After 18 h, total viable counts (log cfu/ml) ranged from 7·23 to 7·62, and gram-negative bacteria from 7·16 to 7·35, with no significant differences between milks (data not shown). Refrigeration temperature had a significant ($P<0·001$) effect on coliforms, with counts (log cfu/ml) of 4·37, 5·25, 4·41 and 5·33 in R5, R10, S5 and S10 milks, respectively, after 18 h. Milk pH ranged from 6·50 to 6·56 after 18 h, with no significant differences between milks.

All microbial groups decreased in cheese during ripening. Total viable counts in cheese were not influenced by milk ripening or storage conditions (data not shown). However, milk ripening had a significant ($P<0·001$) effect on gram-negative bacteria, with higher levels from day 15 in R5 than in S5 cheeses, and in R10 than in S10 cheeses (Table 1). Coliforms were significantly influenced by ripening of milk ($P<0·001$) and by refrigeration temperature ($P<0·001$), the highest levels being those of R10 cheeses (Table 1).

Cheese pH and proteolysis

On day 1 minimum pH values corresponded to S5 and S10 cheeses (Table 1). The fresh lactic culture added to the vat of S5 and S10 cheeses was more active lowering cheese pH during manufacture than the FC culture inoculated before overnight storage in the milk of R5 and

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Table 2. Relative abundance of volatile compounds significantly influenced by milk treatment in 60-d-old cheeses made from milk ripened with a lactic culture at 5 °C (R5) or 10 °C (R10) or from milk cold stored at 5 °C (S5) or 10 °C (S10)

Mean ± SD of duplicate determinations from three experiments

	R5	R10	S5	S10
Aldehydes				
2-Methyl-1-butanal	0·15 ± 0·12^{a}	0·35 ± 0·09^{b}	0·22 ± 0·05^{ab}	0·32 ± 0·06^{b}
3-Methyl-1-butanal	2·31 ± 0·79^{a}	4·85 ± 2·10^{b}	1·95 ± 0·44^{a}	2·19 ± 0·44^{a}
Phenyl acetaldehyde	0·09 ± 0·05^{ab}	0·03 ± 0·04^{a}	0·11 ± 0·03^{b}	0·11 ± 0·03^{b}
Ketones				
Acetone	1·67 ± 0·51^{a}	4·64 ± 2·03^{b}	2·17 ± 0·80^{a}	2·30 ± 0·73^{a}
2-Butanone	183·9 ± 46·8^{a}	532·7 ± 278·6^{b}	216·8 ± 40·8^{a}	352·9 ± 65·8^{ab}
2,3-Heptanedione	0·18 ± 0·02^{b}	0·22 ± 0·08^{b}	ND^{a}	ND^{a}
Alcohols				
Ethanol	245·4 ± 66·9^{a}	1035 ± 234^{b}	172·8 ± 92·9^{a}	223·9 ± 112·1^{a}
2-Propanol	20·02 ± 1·83^{a}	18·18 ± 4·24^{a}	26·86 ± 6·55^{b}	22·37 ± 1·19^{b}
2-Methyl-1-propanol	15·68 ± 2·47^{ab}	29·89 ± 17·65^{b}	3·66 ± 1·46^{a}	8·33 ± 1·65^{a}
3-Methyl-1-butanol	74·05 ± 7·84^{b}	136·0 ± 56·9^{c}	18·48 ± 7·38^{a}	43·11 ± 5·22^{ab}
2-Heptanol	0·53 ± 0·10^{a}	0·55 ± 0·33^{a}	1·74 ± 0·97^{b}	1·24 ± 0·99^{ab}
1-Pentanol+2-methyl-3-buten-1-ol	0·90 ± 0·21^{a}	1·22 ± 0·23^{ab}	1·08 ± 0·08^{ab}	1·40 ± 0·23^{b}
Esters				
Methyl acetate	0·07 ± 0·06^{a}	0·44 ± 0·37^{b}	0·04 ± 0·05^{a}	0·13 ± 0·10^{ab}
Ethyl acetate	31·87 ± 21·69^{a}	219·7 ± 117·5^{b}	8·28 ± 4·09^{a}	31·20 ± 19·50^{a}
Ethyl propionate	0·22 ± 0·08^{a}	2·72 ± 1·41^{b}	0·05 ± 0·01^{a}	0·19 ± 0·09^{a}
Ethyl butyrate	4·57 ± 1·60^{ab}	7·16 ± 3·03^{b}	2·87 ± 1·27^{a}	4·93 ± 2·40^{ab}
Ethyl caproate	10·36 ± 6·06^{ab}	15·81 ± 9·52^{b}	4·90 ± 3·40^{a}	9·89 ± 6·52^{ab}
Ethyl caprylate	0·66 ± 0·37^{b}	0·36 ± 0·18^{ab}	0·21 ± 0·08^{a}	0·44 ± 0·25^{ab}
Propyl acetate	0·78 ± 0·23^{a}	5·52 ± 4·49^{b}	0·37 ± 0·09^{a}	0·63 ± 0·32^{a}
Propyl butyrate	0·08 ± 0·06^{ab}	0·25 ± 0·21^{b}	ND^{a}	ND^{a}
Isoamyl acetate	0·33 ± 0·14^{ab}	2·44 ± 1·45^{b}	ND^{a}	0·18 ± 0·04^{ab}
Isoamyl butyrate	0·07 ± 0·04^{a}	0·19 ± 0·13^{b}	0·01 ± 0·01^{a}	0·04 ± 0·02^{a}
2-Butyl acetate	2·62 ± 1·73^{a}	55·89 ± 35·15^{b}	1·24 ± 0·32^{a}	3·26 ± 2·67^{a}

Means in a row with the same superscript do not differ significantly ($P<0·05$)
ND, below detection limit

R10 cheeses. There were no more significant differences in cheese pH until day 60, when higher values were recorded for R10 and S10 cheeses than for R5 and S5 cheeses.

Cheese proteolysis was significantly ($P<0·001$) influenced by milk storage temperature, with higher levels from day 15 in R10 and S10 cheeses than in R5 and S5 cheeses (Table 1). The higher storage temperature of milk probably favoured growth of bacterial strains with a strong proteolytic activity, which enhanced cheese proteolysis during ripening.

Volatile compounds

The 55 volatile compounds identified in the present work included aldehydes, alcohols, ketones, esters, hydrocarbons, sulphur compounds, benzenic compounds and acids. Three chromatographic peaks corresponded each to two compounds which could not be separated, 1-pentanol+2-methyl-3-buten-1-ol, 2-hexanone+hexanal and methyl caproate+heptanal. Relative abundances of 22 volatile compounds and of one peak corresponding to 1-pentanol+2-methyl-3-buten-1-ol in 60 d-old cheeses were influenced by milk treatment (Table 2).

Aldehydes with no significant differences due to milk treatment (mean values for their relative abundance in 60 d-old cheeses in brackets) were acetaldehyde (0·82), propanal (0·12), 2-propenal (1·00), butanal (0·08) and nonanal (0·12). Those influenced by milk treatment were 2-methyl-1-butanal, 3-methyl-1-butanal and phenyl acetaldehyde. Phenyl acetaldehyde was more abundant in S5 and S10 cheeses whereas 2-methyl-1-butanal and, particularly, 3-methyl-1-butanal reached their highest levels in R10 cheeses (Table 2). R5 and R10 cheeses exhibited a higher pH on day 1 than S5 and S10 cheeses. This may be attributed to a lower acid producing activity of the FC culture when milk was inoculated before overnight storage (vats 1 and 2) compared with that of the fresh lactic culture added to milk on the day of manufacture (vats 3 and 4). This fact probably permitted a better growth of wild lactococcal strains able to convert isoleucine and leucine into 2-methyl-1-butanal and 3-methyl-1-butanal (Morgan et al. 1966; Morales et al. 2003) during maturation of cheeses from ripened milk. Branched-chain aldehydes are normally

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found in cheese, and in some varieties they are even considered key flavour compounds (Bosset & Gauch, 1993; Engels et al. 1997; Curioni & Bosset, 2002).

Ketones not influenced by milk treatment (mean values for 60 d-old cheeses in brackets) were 2,3-butanedione (19·69), 2-pentanone (4·00), 2-hexanone+hexanal (0·56), 2-heptanone (1·01) and 2-nonanone (0·11). Acetone, 2-butanone and 2,3-heptanedione were influenced by milk treatment (Table 2). The highest relative abundances of acetone and 2-butanone were found in R10 cheeses, probably due to a better growth of wild microbiota, and 2,3-heptanedione was only detected in R5 and R10 cheeses. Relative abundances of 2-butanone and 2,3-heptanedione were higher in raw milk than in pasteurised milk Manchego cheese (Fernández-García et al. 2002).

Alcohols not influenced by milk treatment (mean values for 60 d-old cheeses in brackets) were 1-propanol (15·51), 2-propen-1-ol (17·72) 2-butanol (331·5), 2-pentanol (7·00), 1-methoxy-2-propanol (0·22), 1-butanol (5·04) and 1-hexanol (0·48). Ethanol, 2-propanol, 2-methyl propanol, 3-methyl-1-butanol, 2-heptanol and 1-pentanol+2-methyl-3-buten-1-ol were influenced by milk treatment (Table 2). Ethanol is formed during lactose fermentation by heterofermentative lactic acid bacteria and coliforms, and from amino acid metabolism, aldehyde degradation or acetaldehyde reduction (Cogan, 1995; Urbach, 1995). In the present work, ethanol reached its maximum levels in R10 cheeses (Table 2), which also exhibited the highest coliform counts (Table 1). The lower activity of the FC culture in R5 and R10 cheeses with respect to the fresh lactic culture in S5 and S10 cheeses permitted a better growth and metabolism of coliforms, and probably of wild heterofermentative lactic acid bacteria, in R10 cheeses resulting in high levels of ethanol. Ethanol was more abundant in raw milk than in pasteurized milk Manchego cheese (Fernández-García et al. 2002).

The highest levels of 2-methyl-1-propanol and 3-methyl-1-butanol were reached in R10 cheeses, followed by R5 cheeses (Table 2), most probably due to the lower activity of the lactic culture in those cheeses, which permitted growth of wild lactococci able to produce branched-chain alcohols (Morales et al. 2003). A significant correlation was found in the present work between levels of 3-methyl-1-butanol and 3-methyl-1-butanal (r=0·891) in the different cheeses. Branched-chain alcohols are formed as the reduction products of aldehydes derived from branched-chain amino acids, the reduction taking place mostly during ripening (Ayad et al. 2000), and although they are generally recognized as off-flavours in some cheese varieties they are considered key flavour compounds (Bosset & Gauch, 1993; Barbieri et al. 1994; Engels et al. 1997).

Relative abundances of 2-propanol and 2-heptanol were significantly higher for S5 and S10 cheeses than for R5 and R10 cheeses (Table 2), but there were no significant differences for 2-butanol, which was the most abundant alcohol in our cheeses. Lower levels of 2-propanol and 2-butanol were formed by wild strains of lactococci producing high levels of branched-chain aldehydes and alcohols than by wild or commercial strains producing low levels of those branched-chain compounds (Morales et al. 2003).

Esters with no significant differences due to milk treatment (mean values for 60 d-old cheeses in brackets) were ethyl pentanoate (0·08), butyl acetate (0·22), 2-butyl butyrate (0·22), 2-butyl caproate (0·24) and methyl caproate+heptanal (0·26). The relative abundances of 11 esters were influenced by milk treatment (Table 2), with higher levels for R10 cheeses than for R5 cheeses and for S10 cheeses than for S5 cheeses. Esters are formed through enzymic or chemical reactions of short- to medium-chain fatty acids with alcohols. Acetic acid comes from the metabolism of heterofermentative lactobacilli and coliforms or from oxidative deamination of amino acids, butyric acid from lipolysis, butyric fermentation or amino acid breakdown, and other volatile fatty acids may derive from lactate (Fox & Wallace, 1997). Esterases from lactic acid bacteria and lipases from gram-negative psychrotrophic bacteria also contribute to the formation of free fatty acids in cheese. The high ethanol concentration together with the high counts of gram-negative bacteria may help to explain the high levels of ethyl esters found in R10 cheeses (Table 2). Higher levels of ethyl esters were reported for raw milk than for pasteurized milk Manchego cheese (Fernández-García et al. 2002). Ethyl esters possess pleasant sweet and fruity notes and contribute to the aroma of many cheese varieties (Barbieri et al. 1994; Carbonell et al. 2002).

Other volatile compounds not influenced by milk treatment (mean values for 60 d-old cheeses in brackets) were: heptane (0·43), octane (1·49), 3-methyl-1-heptene (0·80), carbon disulphide (0·14), dimethyl disulphide (0·49), α-pinene (1·65) and toluene (1·61). Terpenes such as α-pinene usually derive from pastures (Dumont & Adda, 1978), whereas toluene, of unknown origin, seems to be a normal constituent of milk.

Sensory characteristics

Flavour quality of 60 d-old cheeses ranged from 6·6 to 6·8, and flavour intensity from 6·8 to 7·0, with no significant differences between cheeses (data not shown). In spite of the potent volatile profiles and the high proteolysis values of R10 cheeses, panellists attributed flavour quality and intensity scores to R10 cheeses not significantly different from those of R5, S5 or S10 cheeses. However, it cannot be assumed that similar flavour quality scores corresponded to similar flavour characteristics, since no detailed sensory analysis using flavour descriptors was carried out. On the other hand, cheeses made in our experiments showed a strong background flavour, more traceable to proteolysis and lipolysis caused by raw milk microbiota and enzymes than to the formation of volatile compounds, which probably forced uniformity in panel scores for global flavour attributes. Low correlations of volatile compounds and proteolysis with flavour quality

and intensity were generally found (data not shown), which might be explained by the background flavour of cheeses.

Ripening of raw milk with lactic cultures is considered to be useful in controlling growth of psychrotrophic gram-negative bacteria during refrigerated storage. In the present work, however, gram-negative bacteria were able to grow independently of lactic culture inoculation during overnight storage of milk at 5 or 10 °C, reaching counts over 10^7 cfu/ml after 18 h. The higher pH values found on day 1 for cheeses from ripened milk favoured the survival of gram-negative bacteria during early ripening, with counts over 10^7 cfu/g persisting on day 15 in those cheeses. The highest populations of gram-negative bacteria and coliforms, and the highest levels of ketones, esters and proteolysis, were found in cheeses made from milk ripened at 10 °C. Although there were significant differences between cheeses in the levels of volatile compounds and proteolysis, panel scores for flavour quality and intensity of cheeses made from milk ripened overnight with a lactic culture were similar to those made from refrigerated milk inoculated with the lactic culture at the time of cheese manufacture.

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