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Influence of an adjunct culture of *Lactobacillus* on the free amino acids and volatile compounds in a Roncal-type ewe's-milk cheese

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Abstract

Free amino acids and volatile compounds were analysed in a Roncal-type cheese made from pasteurized ovine milk with and without an added adjunct culture (*Lactobacillus paracasei* + *Lactobacillus plantarum*). In both batches, the total free amino acid concentration increased 11–12-fold with ripening time, the main amino acids being Leu, Glu, Lys, Phe, Val, and Ile. At the end of ripening, significant differences were recorded for Leu, Ile, Gaba, Phe, Pser, Ser, Gin, Ala, and Orn.

A total of 73 volatile components were identified, comprising 17 hydrocarbons, 17 alcohols, 2 sulphur-containing compounds, 11 aldehydes, 12 ketones, 6 acids, and 8 esters. The cheese made with the added adjunct culture had significantly higher levels of the different volatile components after 240 days of ripening.

There appeared to be a relationship between levels of the amino acids Val and Leu and certain breakdown products of those amino acids produced by *Lactobacillus* metabolism.

Keywords: Volatile compounds; Amino acids; Ewe's-milk cheese; *Lactobacillus*

1. Introduction

Cheese flavour is an outcome of the action of the enzymes present in the source milk and the rennet, together with those contributed by microorganisms, on the milk components protein, fat, lactose, and citrate. Proteolytic enzymes from lactic acid bacteria play an important role in the degradation of casein and peptides, leading to the production of free amino acids (FAAs). The amino acids thus produced contribute directly to the basic taste of the cheese and indirectly to cheese flavour, since they are precursors for the other catabolic reactions, giving rise to volatile aroma compounds (Fox & Wallace, 1997). Catabolic reactions and side-chain modification may yield keto acids, ammonia, amines, aldehydes, acids, and alcohols, which are essential contributors to cheese taste and aroma (Hemme, Schmal, & Auclair, 1981). Non-starter lactic acid bacteria (NSLAB) possess a wide range of hydrolytic enzymes and therefore have the potential to contribute to cheese maturation (Williams & Banks, 1997). The addition of selected lactobacilli to the source milk for cheese, for the purpose of accelerating cheese development or improving cheese quality, has yielded higher FAA levels in cheese, accompanied by increased flavour intensity (Lane & Fox, 1996; Lynch, McSweeney, Fox, Cogan, & Drinan, 1997; McSweeney et al., 1994; Muir, Hunter, Banks, & Horne, 1996; Puchades, Lemeux, & Simard, 1989; Ur-Rehman, Banks, McSweeney, & Fox, 2000).

Fatty acids, esters, aldehydes, alcohols, ketones, and sulphur compounds have been reported to be important contributors to the final taste and aroma of cheese (Bosset & Gauch, 1993; Curioni & Bosset, 2002; Engels, Dekker, de Jong, Neeter, & Visser, 1997; Izco, Irigoyen, Torre, & Barcina, 2000; Urbach, 1993). Most of the volatile flavour

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components detected are formed during ripening, some being derived from catabolism of the FAAs released during proteolysis. For instance, reduction of the corresponding aldehydes formed by Strecker degradation of Val and Leu can give rise to 2-methylpropan-1-ol and 3-methylbutan-1-ol, respectively (Smit et al., 2000). In another paper, Fox and Wallace (1997) observed that the products of amino acid catabolism make a greater contribution to flavour than do the amino acids themselves. Much is now known of the enzyme systems involved in the conversion of caseins to FAAs, but only recently has attention been turned to the enzymes of starter bacteria involved in amino acid catabolism (Engels & Visser, 1996; Smit et al., 2000). Dias and Weiner (1998) reported the conversion of Met to thiols by lactococcal enzymes, catabolism of Met and Cys to sulphur compounds being essential to the formation of Cheddar cheese flavour (Urbach, 1993).

The object of this study was to investigate the effects of an adjunct culture of *Lactobacillus paracasei* + *Lb. plantarum* on the concentrations of free amino acids and volatile compounds in a ewe's-milk cheese.

2. Materials and methods

2.1. Adjunct cultures

The strains of the two species used in the adjunct culture were facultative heterofermentative lactobacillus (FHL) strains selected in earlier work (Ortigosa, 2002) with the aid of molecular techniques (RAPD and PCR), bearing in mind that FHL strains were the principal strains identified in the raw ovine milk used to manufacture Roncal cheese. In this study, 97% of the strains isolated were *Lb. paracasei* and 3% *Lb. plantarum*. Of these, 93% of the *Lb. paracasei* strains metabolized citrate, whereas none of the *Lb. plantarum* strains did. The predominant strain from each of these two species was chosen for inclusion in the adjunct culture. The strains were maintained in frozen stock cultures in skimmed milk (Difco) at 80 °C. The recovery was performed in MRS broth (Difco) at 32 °C for 24 h and, after that, they were subcultured in MRS agar (Difco) at 32 °C for 72 h. A colony was cultured in 10% skimmed milk for 8 h at 32 °C. In this culture, added to the vat, the amounts of lactobacilli were 5×10^4 cfu $100\,l^{-1}$ of citrate positive (Cit+) *Lb paracasei* and 7×10^4 cfu $100\,l^{-1}$ of citrate negative (Cit−) *Lb plantarum*.

2.2. Cheese samples

Two different cheese vats were manufactured, one vat (S) made from pasteurized milk using a commercial starter comprising *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* (Ezal from Texel, Dangé Saint Romain, France) and a second vat (SC), made in the same way as S but with the addition of an adjunct culture comprising *Lactobacillus paracasei* (Cit+) and *Lactobacillus plantarum* (Cit−), with doses corresponding to levels found in raw milk. Two replicate manufacturing runs were carried out, yielding duplicates of each vat.

Samples were prepared according to the procedure laid down in the regulations issued by the Regulatory Board of the Roncal Cheese Appellation of Origin (BOE, 1991), though it should be noted that the regulations do not allow the use of pasteurized milk in the manufacture of Roncal cheese. Raw ewe's milk was collected from a sheep flock on the morning of cheese-making and transported to the pilot plant, where it was pasteurized in an electric model ATA pasteurizer ($250\,l\,h^{-1}$) (Tecnología Alimentaria, S.L.) at 72 °C for 20 s. The starter culture was added to each of the milk vats at 20 °C, and commercial rennet (Laboratorios Arroyo, Santander, Spain) was added in the proportion of 1:10,000 at 32 °C. Coagulation took 45–60 min. Amounts of $10\,ml\,100\,l^{-1}$ $Cacl_2$ and $14\,ml\,100\,l^{-1}$ of lysozyme were also added to each vat. The whey was drained off from the curd at 38 °C, and the curds were moulded and pressed. The chesses were later removed from the moulds and places in saturated salt brine at 11 °C for 18 h. The cheeses were then stored for two weeks in the first ripening chamber at 11 °C and a relative humidity of 75%. They were then transferred to a second ripening chamber and stored at 11 °C and a relative humidity of 85% until sampling.

For each manufacturing process, two cheeses were taken from each vat after 1,15, 30, 120, and 240 days of ripening for analysis; this amounts to 40 cheeses (2 manufacturing × 2 vat × 5 ripening × 2 duplicates). All the analysis carried out was duplicated so that eight analyses were performed for each type cheese on each of the collection dates.

2.3. Chemical analysis

2.3.1. Dry matter

Total dry matter (DM) was determined according to FIL-IDF standard no. 4 (International Dairy Federation, 1986).

2.3.2. Free amino acids (FAAs)

FAA extraction from the cheese samples and RP-HPLC analysis of the FAAs were performed according to the method used previously by Izco et al. (2000). The HPLC equipment (Waters Corporation, Milford, MA, USA) consisted of two M510 pumps connected to an ULTRA WISP 715 injector. The Waters Pico-Tag C_{18} reversed-phase column was heated in a temperature control module at 46 °C, and the resolved peaks were detected using an M966 photodiode array detector set at 254 nm and an NEC PowerMate 486/33i computer, employing Millennium 2010 software for quantification. For identification and quantification of FAAs, a master solution of amino acids (Sigma, St. Louis, MO, USA) was used, to which methionine sulfone (Sigma) was added as an internal standard.

A gradient with two solvents was used to run the samples: solution A comprised 70 mM sodium acetate adjusted

to pH 6.55 with acetic acid and treated with 2.5% acetonitrile and solution B was 45% acetonitrile, 40% water and 15% methanol. Before each injection, the column was equilibrated with solvent A for 20 min.

2.3.3. Volatile compounds

The extraction of the volatile compounds by the purge and trap method and separation and identification by GC-MS were done as previously described by Ortigosa, Torre, and Izco (2001). Briefly, 10 g of finely grated cheese were mixed with 10 g of anhydrous sodium sulphate and 100 μl of 13 mM L-Borneol (Sigma, St. Louis, MO, USA) as internal standard (IS) and placed in a suitable U vial. Volatile compounds were extracted using an automatic model 4460 A purge and trap sample concentrator system (O.I. Analytical, College Station, TX, USA) equipped with a Tenax trap (O.I. Analytical). The purge and trap system was connected to an HP Series 6890 GC System coupled to a model 5973 mass selective detector (Hewlett Packard, Palo Alto, CA, USA). Peak identification was based on comparison of the MS spectra with the HP Wiley 275 library and with the spectra of injected standards as well as on the retention times of the standards, where available. The peak area: IS peak area ratio was used as an arbitrary unit for calculating the quantities of each volatile compound.

2.4. Statistical processing

The SPSS computer programme (version 11.5, SPSS Inc., Chicago, IL, USA) was used for statistical processing. Statistical differences between the cheese batches made with and without the added adjunct culture during ripening were assessed by means of analysis of variance (ANOVA) using 95% confidence intervals run on the volatile components analysed and on the FAAs.

3. Results and discussion

3.1. General

The physicochemical parameters did not vary during the cheese manufacturing process between the 2 vats (data not shown).

The adjunct culture implanted well in the cheeses. The levels of FHL obtained in the cheeses SC were of

Table 1
Amino acids (mg 100 g^{-1} DM) in cheeses manufactured from pasteurized milk (S) and pasteurized milk + adjunct starter (SC)

	1 day			15 day			30 day			120 day			240 day		
	S	SC	*p*	S	SC	*p*	S	SC	*p*	S	SC	*p*	S	SC	*p*
PSER	1.43	1.58	Ns	1.77	0.38	**	3.92	1.16	**	9.04	5.30	**	16.5	7.50	***
Asp	7.32	7.23	Ns	9.25	9.72	Ns	12.5	13.9	Ns	21.1	29.0	**	40.5	47.3	Ns
Glu	75.2	77.4	Ns	76.4	74.8	Ns	189	170	Ns	316	361	Ns	644	668	Ns
Hypro	38.7	35.4	Ns	24.9	25.5	Ns	37.8	37.0	Ns	30.9	30.0	Ns	26.8	27.4	Ns
Ser	5.46	5.35	Ns	14.6	12.3	Ns	21.8	15.4	*	44.8	41.7	Ns	75.4	66.1	*
Gly	1.54	1.54	Ns	4.91	6.41	Ns	9.44	8.49	Ns	25.3	31.4	Ns	46.2	47.1	**
Gln	8.22	8.97	Ns	36.1	44.6	Ns	50.5	58.9	Ns	84.1	117	**	142	166	**
Tau	12.5	12.5	Ns	13.4	12.7	*	12.9	13.8	Ns	13.5	12.7	*	12.5	12.7	Ns
Hys	8.41	8.98	Ns	12.8	13.5	Ns	16.0	17.9	Ns	37	47.3	*	96.9	114	Ns
Gaba	7.13	3.37	***	26.2	41.5	**	29.3	48.2	***	54.9	74.3	***	60.4	89.1	***
Thr	13.1	10.9	Ns	20.4	18.5	Ns	24.1	24.0	Ns	43.5	46.8	Ns	62.5	63.6	Ns
Ala	6.90	6.50	Ns	21.9	21.9	Ns	30.5	30.9	Ns	62.6	75.5	*	97.0	110	**
Arg	0.04	0.08	Ns	3.73	3.07	Ns	9.10	8.22	Ns	11.4	11.8	Ns	12.5	11.5	Ns
Pro	36.7	38.6	*	46.0	52.1	Ns	52.3	63.5	Ns	113	134	Ns	201	213	Ns
Tyr	16.1	17.5	*	21.5	23.1	Ns	32.3	32.7	Ns	65.0	73.3	Ns	97.9	114	*
Val	8.86	9.37	Ns	33.9	38.9	Ns	66.4	74.4	Ns	178	202	Ns	280	298	Ns
Met	2.38	3.45	*	13.1	15.1	Ns	21.9	23.8	Ns	66.3	75.5	Ns	114	123	Ns
Cyst B	0.00	0.14	Ns	0.24	0.22	Ns	2.03	1.07	Ns	1.54	0.67	***	1.08	0.50	**
Cys	0.56	0.57	Ns	2.06	2.27	Ns	1.79	2.19	Ns	12.5	10.3	**	7.14	6.07	Ns
Ile	6.69	7.50	Ns	30.3	33.2	Ns	53.6	56.5	Ns	146	175	Ns	270	298	**
Leu	26.8	24.1	Ns	90	109	Ns	162	184	Ns	421	498	Ns	659	747	**
1H-Lys	12.1	10.5	Ns	17.7	16.9	Ns	14.7	14.7	Ns	16.7	16.8	Ns	11.5	12.1	Ns
2H-Lys	1.39	1.90	Ns	3.94	3.58	Ns	5.12	3.42	***	4.03	3.22	Ns	2.19	2.58	Ns
Phe	9.81	10.5	Ns	32.0	37.1	Ns	68.7	71.6	Ns	180	202	Ns	271	304	**
Trp	5.30	5.96	Ns	6.57	7.27	Ns	6.29	7.89	*	11.0	13.6	Ns	21.7	22.2	Ns
Orn	9.06	9.39	Ns	25.8	27.9	Ns	34.9	42.8	Ns	56.9	93.4	***	81.6	128	***
Lys	35.4	36.4	Ns	63.5	73	Ns	103	113	Ns	272	336	Ns	538	578	Ns
Total	358	357	Ns	654	726	Ns	1073	1140	Ns	2185	2719	Ns	3892	4277	Ns

Ns = non-significant.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

10^6 cfu ml^{-1}, whilst those microorganisms were not found in the cheeses S. With regards to the levels of lactobacilli and lactococci, SC had double the amount of microorganisms of S at the end of ripening. Table 1 presents the concentrations of the FAAs quantified in the samples from cheese vats S and SC, together with the ANOVA results for each sampling date during ripening. In both vats, the total FAA concentration (calculated as the sum of the individual amino acids considered) increased with ripening time and, at the end of ripening, was 11–12 times higher than the total FAA level in the cheeses on day 1.

The total FAA concentration in the vat made with the added adjunct culture of *Lb. paracasei* + *Lb. plantarum* was higher from day 15 of ripening, though the differences between the vats were not statistically significant (Table 1). Other studies have also found that addition of an adjunct culture of lactobacilli resulted in higher total FAA levels (Fenelon, O'Connor, & Guinee, 2002; Lane & Fox, 1996; Lynch, McSweeney, Fox, Cogan, & Drinan, 1996; McSweeney et al., 1994; Mendía, Ibáñez, Torre, & Barcina, 2000).

The main amino acids on day 240 accounted for about 70% of the total FAAs. These were Leu (about 18%), Glu (about 16%), Lys (about 14%), and Phe, Val, and Ile (about 7% each). Certain of these amino acids have been regarded as indicators of cheese ripening in certain ovine cheeses, namely, Roncal (Muñoz, Ortigosa, Torre, & Izco, 2003), Ossau-Iraty (Izco et al., 2000), and Idiazábal (Mendía, 2000). Leu was the most abundant amino acid in both cheese vats at 658 and 746 mg 100 g^{-1} DM at the end of ripening (Table 1), with branched-chain amino acids (Leu, Ile, Val), aromatic amino acids (Phe, Tyr, Trp) and Met being the main precursors of key aroma compounds (Yvon & Rijnen, 2001). Leu and Ile levels in the two vats were significantly higher in vat SC at the end of ripening. Glu was the second most abundant amino acid in both vats. Also, catabolism of Glu by decarboxylases can produce γ-aminobutyric acid (GABA) (McSweeney & Sousa, 2000). However, we observed a significant correlation between the concentrations of both these amino acids ($r = 0.827$, $p < 0.01$). GABA concentration has been correlated with a larger number of eyes but has no direct or indirect impact on cheese flavour (Christensen, Dudley, Pederson, & Steele, 1999). GABA concentrations differed significantly between the two vats, being higher in vat SC. However, sensory analysis performed by a trained panel (data not shown) did not produce any differences for the attribute "eyes" for the two cheese vats considered.

The Phe concentration was significantly higher in the vat SC cheeses on day 240 (271 and 304 mg 100 g^{-1} DM). Breakdown of this amino acid by Strecker degradation can yield phenylethanol and phenylacetaldehyde, though neither of these compounds was observed in the cheeses with the technique used in this study. High concentrations of these breakdown products in Cheddar cheese contribute to astringent, and bitter taste sensations (Christensen et al., 1999). Tyrosine is another amino acid which, along with tyramine, can generate phenyl derivatives by a typical Strecker degradation (McSweeney & Sousa, 2000). Like Phe, the concentration of Tyr was also higher in the cheeses in vat SC (97.9 and 114 mg 100 g^{-1} DM) and could influence the organoleptic characteristics of the cheese. In the sensory analysis, the cheeses in vat SC earned slightly higher scores than the cheeses in vat S; hence the possible higher levels of phenylethanol and phenylacetaldehyde did not adversely affect the sensory evaluation results (Ortigosa, 2002).

Ornithine levels in the two vats were significantly different ($p < 0.001$) from day 120, with higher levels in vat SC. This agreed with the findings reported by Laht, Kaska, Eliasc, Adamberg, and Paalme (2002), who observed that the accumulation of Orn in ripening cheese was related to the growth of the NSLAB, which produced Orn from Arg via the arginine deiminase pathway. Lactobacilli are the most common NSLAB that continue to grow during ripening, and populations can reach levels of about 10^7 cfu g^{-1} (Mannu, Comunian, & Scintu, 2000) in a variety of cheeses. Other work (Mendía et al., 2000; Muñoz et al., 2003) has reported higher concentrations of Orn in cheeses manufactured in summer because of the higher concentrations of NSLAB at that time of year.

Addition of the adjunct culture with the two *Lactobacillus* strains acted to decrease pSer and Ser levels in the cheeses. This finding agreed with the report by Vescovo, Torriani, Dellaglio, and Botazzi (1993) that adding *Lb. plantarum* lowered Ser concentrations, resulting in deamination of this amino acid to yield pyruvate and ammonium.

Thr breakdown by LAB is initiated by threonine aldolase, an enzyme widely present in LAB. Threonine aldolase converts Thr to acetaldehyde and Gly (Marshall & Cole, 1983). Thr and acetaldehyde levels (see below) in the two vats were similar over the entire ripening period. Gly concentrations in the two vats were also quite similar, though by the end of ripening vat SC exhibited a significantly higher level.

3.2. Volatile compounds in cheese

The volatile components present in these Roncal-type cheese samples consisted of 17 hydrocarbons (Table 2), 17 alcohols (Table 3), 2 sulphur-containing compounds (Table 4), 11 aldehydes (Table 5), 12 ketones (Table 6), 6 acids (Table 7), and 8 esters (Table 8). Thus, 73 volatile components were identified in all. Most of these components had already been reported in other types of cheeses (Bosset, Gauch, Mariaca, & Klein, 1995; Fernández-García, 1996; Izco & Torre, 2000; Ortigosa et al., 2001). Certain of the volatile components present in the cheeses in this study increased during ripening, while others decreased or fell to undetectable levels.

Hydrocarbons are a family of secondary products of lipid autoxidation (Barbieri et al., 1994). These components do not make a major contribution to aroma, although they

Table 2
Hydrocarbons in cheeses manufactured from pasteurized milk (S) and pasteurized milk + an adjunct culture (SC)[a]

Peak no.	Volatile Compound[b]	1 day			120 days			240 days		
		S	SC	*p*	S	SC	*p*	S	SC	*p*
1	Pentane	6003	3145	NS	29,280	37,082	*	6586	9491	*
2	2-Methylpentane (T)	169	221	NS	1045	3939	*	502	606	NS
3	3-Methylpentane				501	592	NS	326	360	NS
4	Hexane	5355	7673	NS	22,878	58,262	*	19,507	18,099	NS
6	Cyclopentane (T)				2439	2255	NS	801	500	*
7	Cyclopenta-1,3-diene (T)	79	89	NS	614	918	NS	220	212	NS
8	Methyl cyclopentane (T)	163	183	NS	1050	2229	*	538	586	NS
9	Heptane	132	38	*	248	322	NS	318	242	NS
12	1,2-Dimethylcyclopentane					528		187	197	NS
13	Cyclohexane (T)				356	298	NS			
14	Heptane				64	99	NS	33	50	*
16	Methylcyclohexane (T)	56	63	NS	222	478	*	309	171	NS
21	3-Octene (T)	101	142	NS	196	238	NS	182	240	NS
22	Octen-2-ene (T)	32	44	NS	58	96	NS	55	63	NS
31	Benzene	546	561	NS	1175	1274	*	924	1422	*
41	Toluene (T)	208	320	NS	494	691	*	351	413	*
50	*p*-Xilene (T)	25	23	NS	23	34	NS		33	

NS = $P > 0.05$.

[a] Quantities of volatile compounds expressed in arbitrary units (1000 × peak area/IS peak area); each value being the mean of eight determinations (four cheeses per ripening date × two replicate samples). With very few exceptions, standard error values ranged between 1% and 30%, on average.

[b] Identification of all compounds was effected by comparison with the retention times and mass spectra for the actual substances, except for: (T) = Tentatively identified on the basis of the Wiley library and the literature.

* $P = 0.05$.

Table 3
Alcohols in cheeses manufactured from pasteurized milk (S) and pasteurized milk + an adjunct culture (SC)[a]

Peak no.	Volatile compound[b]	1 day			120 days			240 days		
		S	SC	*p*	S	SC	*p*	S	SC	*p*
29	Propan-2-ol (T)	1098	604	NS	803	852	NS	638	766	NS
30	Ethanol	44,004	36,676	NS	46,313	55,895	*	27,882	35,196	*
42	Propan-1-ol	213	397	*	38	41	NS			
48	2-Methylpropan-1-ol	59	62	NS	126	169	NS	119	206	*
49	Pentan-2-ol				198	225	NS	320	378	NS
51	Butan-1-ol	38	29	NS	62	132	*	82	123	NS
52	1-Penten-3-ol				89	78	NS	28	40	NS
56	3-Methylbutan-1-ol	30	27	NS	103	143	NS	93	180	*
57	2-Ethoxyethanol (T)	29	38	NS	196	222	NS	262	351	*
59	Heptanol	52	40	NS	106	108	NS	60	70	NS
60	3-Methylbuten-3-ol	27	25	NS	59	74	NS	59	75	NS
63	Heptan-2-ol				41	58	*	57	51	NS
64	3-Methylbuten-2-ol (T)	29	32	NS	60	67	*	51	62	NS
65	Hexanol	40	63	NS	23	51	*	18	34	*
66	2-Methylpentan-3-ol (T)	195	168	NS	236	291	NS	167	204	NS
68	Pentan-3-ol (T)				165	164	NS			
83	2-(2-Ethoxyethoxy)ethanol (T)				135	154	NS	755	780	NS

Ns = non-significant.

[a] Quantities of volatile compounds expressed in arbitrary units (1000 × peak area/IS peak area); each value being the mean of eight determinations (four cheeses per ripening date × two replicate samples). With very few exceptions, standard error values ranged between 1% and 30%, on average.

[b] Identification of all compounds was effected by comparison with the retention times and mass spectra for the actual substances, except for: (T) = Tentatively identified on the basis of the Wiley library and the literature.

* $P = 0.05$.

may serve as precursors for the formation of other aromatic compounds (Arora, Cormier, & Lee, 1995). On the whole, the different hydrocarbons were present in greater quantities at 120 than at 240 days of ripening. Significantly higher levels of benzene and toluene were recorded in the cheeses of vat SC. Although phenyl derivatives can be formed from amino acids like Phe and Tyr by Strecker degradation (McSweeney & Sousa, 2000), certain workers (Dimos, Urbach, & Miller, 1996) have claimed that toluene is a normal component of milk of unknown origin, while

Table 4
Sulphur-containing compounds in cheeses manufactured from pasteurized milk (S) and pasteurized milk + an adjunct culture (SC)[A]

Peak no.	Volatile compound[B]	1 day			120 days			240 days		
		S	SC	*p*	S	SC	*p*	S	SC	*p*
15	Dimethylsulphide	26[a]	23[a]	NS	60	61	NS	50	68	NS
45	Dimethylsulphide				510	266	*	331	318	NS

Ns = non-significant.
[A] Quantities of volatile compounds expressed in arbitrary units (1000 × peak area/IS peak area); each value being the mean of eight determinations (four cheeses per ripening date × two replicate samples). With very few exceptions, standard error values ranged between 1% and 30%, on average.
[B] Identification of all compounds was effected by comparison with the retention times and mass spectra for the actual substances, except for: (T) = Tentatively identified on the basis of the Wiley library and the literature.
* $P = 0.05$.

Table 5
Aldehydes in cheeses manufactured from pasteurized milk (S) and pasteurized milk + an adjunct culture (SC)[a]

Peak no.	Volatile compound[b]	1 day			120 days			240 days		
		S	SC	*p*	S	SC	*p*	S	SC	*p*
11	Acetaldehyde	1841	1757	NS	2355	2184	NS	2799	2466	NS
17	Propanal (T)	381	337	NS	2193	1652	NS	718	849	NS
18	2-Methylpropanal (T)	35	49	NS	271	222	NS	545	306	*
23	Butanal (T)	31	31	NS	106	113	NS	61	77	NS
28	2-Methylbutanal (T)				145	87	NS	204	164	NS
29	3-Methylbutanal				346	306	NS	688	351	*
34	Pentanal	83	75	NS	484	545	NS	343	410	NS
47	Hexanal	122	120	NS	930	858	NS	353	537	NS
55	Heptanal	41	33	NS	101	108	NS	49	66	NS
74	Nonanal	40	35	NS	41	36	NS	14	19	NS
79	Decanal (T)	22	24	NS	27	27	NS			

Ns = non-significant.
[a] Quantities of volatile compounds expressed in arbitrary units (1000 × peak area/IS peak area); each value being the mean of eight determinations (four cheeses per ripening date × two replicate samples). With very few exceptions, standard error values ranged between 1% and 30%, on average.
[b] Identification of all compounds was effected by comparison with the retention times and mass spectra for the actual substances, except for: (T) = Tentatively identified on the basis of the Wiley library and the literature.
* $P = 0.05$.

Table 6
Ketones in cheese manufactured form pasteurized milk (s) and pasteurized milk + an adjunct culture (SC)[a]

Peak no.	Volatile compound[b]	1 day			120 days			240 days		
		S	SC	*p*	S	SC	*p*	S	SC	*p*
19	Propan-2-one	4706	4723	NS	9509	12,856	*	8492	12,557	*
27	Butan-2-one	1006	851	NS	2238	3816	*	1486	2461	*
33	Pentan-2-one	81	76	NS	6751	8547	*	7970	10,018	*
35	Butan-2,3-dione	13,198	15,994	NS	3780	4904	*	2164	2536	*
37	4-Methylpentan-2-one (T)	73	77	NS	111	97	NS	50	59	NS
44	Pentan-2,3-dione (T)				208	144	*	86	93	NS
46	Hexan-2-one				63	99	*	73	85	NS
54	Heptan-2-one	15	24	NS	1306	1864	NS	1162	1282	NS
61	3-Hydroxybutan-2-one	59,233	60,672	NS	56,740	69,740	*	37,484	50,487	*
62	1-Hydroxypropan-2-one (T)	32	37	NS	143	306	*	159	293	
70	4-Hydroxy-4-methylpentan-2-one(T)	76	58	NS	73					
73	Nonan-2-one				297	302	NS	178	233	NS

Ns = non-significant.
[a] Quantities of volatile compounds expressed in arbitrary units (1000 × peak area/IS peak area); each value being the mean of eight determinations (four cheeses per ripening date × two replicate samples). With very few exceptions, standard error values ranged between 1% and 30%, on average.
[b] Identification of all compounds was effected by comparison with the retention times and mass spectra for the actual substances, except for: (T) = Tentatively identified on the basis of the Wiley library and the literature.
* $P = 0.05$.

others (Johnson, Nursten, & Self, 1969) have expressed the view that benzene and toluene could originate from the degradation of the carotene in the milk or could be related to the use of solvents or other substances during analysis (Molimard & Spinnler, 1996). Aromatic hydrocarbons, such as toluene, *p*-xylene, and benzene, have already been

Table 7
Acids in cheeses manufactured from pasteurized milk (S) and pasteurized milk + an adjunct culture (SC)[a]

Peak no.	Volatile compound[b]	1 day			120 days			240 days		
		S	SC	*p*	S	SC	*p*	S	SC	*p*
78	Acetic acid	38	52	NS	1659	1974	*	7267	9439	*
80	Propanoic acid	7			45	58	NS	59	48	NS
81	2-Methylpropanoic acid	883	912	NS	2271	3201	*	2065	2851	*
84	Butanoic acid	731	695	NS	2755	4078	*	4592	5825	*
85	3-Methylbutanoic acid	1236	1295	NS	4627	6491	*	6297	8546	*
86	Hexanoic acid	8	7	NS	28	36	NS	55	62	NS

Ns = non-significant.
[a] Quantities of volatile compounds expressed in arbitrary units (1000 × peak area/IS peak area); each value being the mean of eight determinations (four cheeses per ripening date × two replicate samples). With very few exceptions, standard error values ranged between 1% and 30%, on average.
[b] Identification of all compounds was effected by comparison with the retention times and mass spectra for the actual substances, except for: (T) = Tentatively identified on the basis of the Wiley library and the literature.
* $P = 0.05$.

Table 8
Esters in cheeses manufactured from pasteurized milk (S) and pasteurized milk + an adjunct culture (SC)[a]

Peak no.	Volatile compound[b]	1 day			120 days			240 days		
		S	SC	*p*	S	SC	*p*	S	SC	*p*
20	Methanoic acid, ethyl ester(T)				107	187	*	110	206	*
25	Acetic acid, ethyl ester	29	44	NS	192	371	*	259	525	*
34	Methanoic acid, butyl ester(T)							190	305	*
41	Butanoic acid, ethyl ester				70	117	*	122	269	*
53	Acetic acid, pentyl ester(T)				79	106	NS	52	72	NS
58	Hexanoic acid, ethyl ester				20	34	NS	21	51	NS
72	Heptanoic acid, ethyl ester				78	158	*	43	50	NS
77	Octanoic acid, ethyl ester		5		33	44	*		10	

Ns = non-significant.
[a] Quantities of volatile compounds expressed in arbitrary units (1000 × peak area/IS peak area); each value being the mean of eight determinations (four cheeses per ripening date × two replicate samples). With very few exceptions, standard error values ranged between 1% and 30%, on average.
[b] Identification of all compounds was effected by comparison with the retention times and mass spectra for the actual substances, except for: (T) = Tentatively identified on the basis of the Wiley library and the literature.
* $P = 0.05$.

identified in Roncal cheese (Izco et al., 2000; Ortigosa et al., 2001) and in other cheeses (Molimard & Spinnler, 1996).

Styrene has been described as a trace element in several cheeses (e.g., Camembert) and can be produced from Phe (Molimard & Spinnler, 1996). However, styrene was not detected in any of the samples in this study.

Alcohols (Table 3) made up the largest group of aroma components, accounting for 25% of all the volatiles identified. Certain of these components, namely pentan-2-ol, 1-penten-3-ol, heptan-2-ol, and pentan-3-ol, were not detected in the cheeses on the first day of ripening but were found to be present after 120 days of ripening.

Table 3 also shows that the concentrations of certain alcohols in the two cheese vats decreased from day 120 to day 240.

Ethanol was the most abundant of the volatile compounds on all the ripening dates sampled, and the cheeses made with the adjunct culture had higher levels of this alcohol. Ethanol can be formed from Ala by Strecker degradation, and the results of this study suggested a relationship between the two, in that concentrations of both Ala and ethanol were higher in vat SC from 120 days of ripening.

Concentration of 2-methylpropan-l-ol and 3-methylbutan-l-ol were significantly higher in vat SC after 240 days of ripening. Certain primary alcohols, e.g. 2-methylpropan-l-ol and 3-methylbutan-l-ol, can form by reduction of the aldehydes produced by Strecker degradation of the amino acids Val and Leu, respectively (Larsen, 1998). As shown in Table 1, concentrations of Leu and Val were higher in vat SC on day 240.

Levels of propan-1-ol, an alcohol derived from Met metabolism (Collin, Osman, Delcambre, El Zayat, & Dufour, 1993), were significantly higher in the cheeses in vat SC on the first day of ripening, with no significant differences observed on day 120. On day 240, this substance was no longer detectable. Met concentrations were significantly higher in vat SC on day 1 and similar in both vats on days 120 and 240, but levels of this amino acid did

increase over the entire ripening period. Met can also be converted by lactobacilli into sulphur compounds, such as dimethylsulphide (DMS) (Thierry & Maillard, 2002) and dimethyldisulphide (DMDS). Breakdown of the sulphur-containing amino acids Met and Cys during cheese ripening produces such sulphur compounds as hydrogen sulphide and methanethiol. Oxidative reactions may then bring about the formation of such other components as DMDS (Engels et al., 1997). However, the sulphur compounds present in cheeses must originate principally from Met, because the concentration of Met in the caseins is higher than that of Cys (McSweeney & Sousa, 2000). In the present study, Cys concentrations were significantly higher in vat S on day 120 (Table 1), coinciding with significantly higher levels of DMDS in that same vat on that date (Table 4). While sulphur-containing components are considered indispensable to the characteristic aromas of such cheeses as Cheddar, Emmental, Camembert, and Gruyère, they may not be particularly important in the development of Roncal cheese aroma (Izco et al., 2000).

Eleven aldehydes were identified (Table 5). Acetaldehyde is one of the most common aldehydes found in cheese (Bosset et al., 1995; Fernández-García, Serrano & Núñez, 2002), and acetaldehyde levels increased, together with Thr levels, in all the cheeses during ripening. Branched aldehydes are produced from the catabolism of branched amino acids, but they do not accumulate in cheese because they are in turn quickly converted into the corresponding alcohols. Oumer, Fernández-García, Garde, Medina, and Núñez (2001) reported a decrease in the concentration of the acetaldehyde in Hispánico cheese with ripening time. 3-Methylbutanal, derived from Leu, was also quantified in the samples. However, there was no variation in the levels of this aldehyde or in those of the corresponding alcohol (3-methylbutanol) in vat S during ripening. On day 240, 3-methylbutanal appeared to have accumulated in the cheeses in vat S without being converted into the alcohol. The same finding was made for 2-methylpropanal. The lactobacilli present in the adjunct culture may thus have played a role by contributing to conversion of these two aldehydes into the corresponding alcohols.

The rest of the aldehydes in the samples (propanal, butanal, pentanal, hexanal, heptanal, nonanal, and decanal, formed by β-oxidation of unsaturated fatty acids) did not exhibit any significant differences, but they are known to contribute to cheese flavour because of their low perception thresholds and rapid conversion to the corresponding alcohols.

As Ortigosa et al. (2001) had previously reported, most of the ketones present in these Roncal-type cheeses were methyl ketones (Table 6). Formation of these components in cheese results mainly from the lipolytic action of the microflora present and is a typical attribute of mould-ripened cheeses, e.g. blue cheeses, contributing to the characteristic pungent aroma. Levels of propan-2-one, butan-2-one, and pentan-2-one were significantly higher in vat SC from day 120.

Diacetyl is produced as a consequence of lactococcal lactose and citrate metabolism, in particular by *Lactococcus lactis* ssp *lactis* bio-variety *diacetylactis* (Crow, 1990). Diacetyl can be reduced to acetoin, which in turn can be reduced to butan-2,3-diol, then to butan-2-one, and finally to butan-2-ol. According to Urbach (1993), production of diacetyl and acetoin and reduction of the latter to butan-2,3-diol can be attributed to the starter bacteria, but subsequent reduction to butan-2-one and then to butan-2-ol is brought about by the NSLAB.

Diacetyl, acetoin, and 2-butanone levels were all significantly higher in cheese SC on days 120 and 240, suggesting that their levels were related. On the other hand, 2-butanol was not detected in either of the cheese vats. Pasteurization of the milk may have affected the NSLAB components responsible for the final conversion in the series.

Fatty acids are released by lipolysis of the fat. While butanoic acid, propanoic acid, and acetic acid may also be produced by fermentation of the lactose and the lactic acid, the acids formed from the branched-chain acids 2-methylpropanoic (isobutyric) acid and 3-methylbutanoic (isovaleric) acid are the result of metabolism of the amino acids Val and Leu, respectively (Molimard & Spinnler, 1996). In this study, levels of acetic acid, 2-methylpropanoic acid, butanoic acid and 3-methylbutanoic acid were significantly higher in vat SC on days 120 and 240 (Table 7). There appeared to be a relationship between 2-methylpropanoic (isobutyric) acid and 3-methylbutanoic (isovaleric) acid and Val and Leu, since the concentrations of these two amino acids were also higher in the cheeses in cheese SC (Table 1).

Eight esters were identified, and they were all present in all the samples (Table 8). Again, concentrations of the four ethyl esters were higher in cheese SC, probably because these cheeses had more ethanol available for esterification (Table 3). The important contribution of esters to cheese aroma is not in doubt, since short-chain esters have a perception threshold 10 times lower than the alcohols from which they are derived. Like Manchego cheese (Martínez-Castro, Sanz, Amigo, Ramos, & Martín Alvarez, 1991) ethyl esters were the most prevalent esters in this cheese, as reported previously (Izco et al., 2000; Ortigosa et al., 2001). Large quantities of ethanol and esters are associated with fruity flavours (Urbach, 1993), and the contribution of these ester components to the aroma of the Roncal-type cheese considered here also appears to be very important (Izco et al., 2000).

4. Conclusions

Addition of an adjunct culture of *Lb. plantarum* + *Lb. paracasei* affected the levels of certain amino acids and volatile compounds in a Roncal-type cheese made from pasteurized ewe's milk over the course of the ripening period.

At the end of ripening, vat SC exhibited significantly higher levels of Leu, Ile, GABA, Phe, Gin, Ala, Orn, benzene, toluene, ethanol, 2-methylpropanol, 3-methylbuta-

nol, 2-methylpropanal, 3-methylbutanal, 2-propanone, 2-butanone, 2-pentanone, diacetyl, and acetoin, acetic acid, 2-methyl propanoic acid, butanoic acid, 3-methylbutanoic acid, the ethyl ester of methanoic acid, the ethyl ester of acetic acid, the butyl ester of methanoic acid, and the ethyl ester of butanoic acid.

Addition of the lactobacilli increased cheese proteolysis, in that the cheeses to which the adjunct culture was added had higher concentrations of amino acids, which in turn played a role in the different metabolic reactions taking place in the cheese during ripening, contributing to formation of the various volatile components. The lactobacilli present in the adjunct culture may thus have played a role by contributing to conversion of 3-methylbutanal and 2-methylpropanal (derived from Leu and Val) into the corresponding alcohols.

Finally, possible relationships between the different amino acids and the respective volatile components potentially formed by catabolism of those same amino acids were observed.

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