Role of dominant microflora of *Picante* cheese on proteolysis and lipolysis

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Abstract

Four species of bacteria (*Enterococcus faecium* and *E. faecalis, Lactobacillus plantarum* and *Lb. paracasei*) and three species of yeasts (*Debaryomyces hansenii, Yarrowia lipolytica* and *Cryptococcus laurentii*) isolated from *Picante* cheese were assayed for proteolytic and lipolytic activities. The milk type (caprine or ovine), the ripening time (0–65 d) and the concentration of NaCl (0–14% (w/v)) have been studied in terms of their effects upon in vitro curdled milk. Proteolytic and peptidolytic activities were demonstrated to be high for *Y. lipolytica*, and at much lower levels for the other strains. Milk type, ripening time and content of NaCl appeared to be statistically significant processing factors in terms of proteolysis. Clear lipolytic activity was detected for *Y. lipolytica*, but release of free fatty acids to lesser extents was observed for the other strains under study. Ripening time was statistically significant for lipolysis but milk type was not. Lipolytic activities were strongly affected by NaCl content and the extent of fat hydrolysis was affected by the increase of NaCl from 0 to 7% (w/v) more than by change from 7 to 14% (w/v). In view of the experimental results, a mixed-strain starter for *Picante* cheese including *Lb. plantarum, E. faecium* (or *E. faecalis*) and *D. hansenii* (and/or *Y. lipolytica*) is of potential interest.

Keywords: Microorganisms; Ovine milk; Caprine milk; Proteolysis; Lipolysis

1. Introduction

Picante da Beira Baixa (or simply *Picante*) cheese is a traditional cheese manufactured in Portugal from mixtures of ovine and caprine milk and possesses an *Appéllation d'Origine Controllée* status since 1988. This variety of cheese, characterised by unique hardness, saltiness and spiciness, originates from central regions of Portugal where it is manufactured manually at the farm level only. Coagulation is usually carried out at ca. 29°C for ca. 50 min using animal rennet without deliberate addition of a starter culture; cutting is often performed with wires or, alternatively, with a large kitchen spoon; moulding and pressing are generally achieved using both hands and metallic cylindrical holders; and salting is applied on the outer surface of the cheese upon manufacture and periodically thereafter, throughout a minimum ripening period of 120 d, using crude kitchen salt.

Recently published works concerning *Picante* cheese have encompassed microbiological characterisation (Freitas, Sousa & Malcata, 1995; Freitas, Pais, Malcata & Hogg, 1996), physicochemical characterisation of both proteolysis (Freitas, Fresno, Prieto, Malcata & Carballo, 1997; Freitas, Fresno, Prieto, Franco, Malcata & Carballo, 1998) and lipolysis (Freitas & Malcata, 1998a), and technological characterisation (Freitas & Malcata, 1996, 1998b). Although *Picante* cheese is manufactured without deliberate addition of starter/non-starter cultures, it is well known that achievement and maintenance of high-quality standards for this variety of cheese will eventually require production (and consequent use) of microbial adjuncts based on adventitious microflora. The major families present throughout ripening of *Picante* cheese are lactic acid bacteria and yeasts (Freitas et al., 1995) at viable numbers above 10^7 cfu g^{-1}_{cheese}; the most abundant species of the former are enterococci (*Enterococcus faecium*, *E. faecalis* and *E. durans*) and lactobacilli (*Lactobacillus plantarum* and *Lb. paracasei*), whereas those of the latter are *Debaryomyces hansenii* and *Yarrowia lipolytica* (Freitas et al., 1996). Smaller viable numbers were also reported for *Enterobacteriaceae*, which ranged from ca. 10^7 cfu g^{-1}_{cheese} in fresh cheese to less than 10^2 cfu g^{-1}_{cheese} by 80 d of ripening, and staphylococci,

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which ranged from ca. 10^6 in fresh cheese to ca. 10^4 cfu g^{-1}_{cheese} by 180 d of ripening (Freitas et al., 1995, 1996).

The purpose of this study was to screen the dominant strains isolated from *Picante* cheese for their role in proteolysis and lipolysis of experimental, univarietal cheeses. Since such biochemical phenomena determine the progress of ripening and hence the final cheese characteristics, their monitoring, when independently caused by the microbial species present in higher viable numbers, is likely to provide important fundamental information about their contribution to the final product. In addition to the nature of the microbiological strains, the milk type (caprine or ovine), the ripening time (0, 27 and 65 d) and the concentration of NaCl (0, 7 and 14% (w/v)) were assessed for their potential effect on the physicochemical performance of the aforementioned strains. Curdled milk (rather than cheese itself) was used as an experimental model system.

2. Materials and methods

2.1. Preparation of microbial cultures

Dominant species previously isolated from *Picante* cheese and identified according to Freitas et al. (1996) were used in the present study. Four species of bacteria and three species of yeasts were isolated, namely two *Lactobacillus* strains (*Lb. plantarum* and *Lb. paracasei*) and two *Enterococcus* strains (*E. faecalis* and *E. faecium*), as well as *Debaryomyces hansenii*, *Cryptococcus laurentii* and *Yarrowia lipolytica*, respectively. Experimental inocula of lactobacilli and enterococci were obtained after growth for 18 h (which was the approximate time necessary to reach the exponential phase) in MRS broth (Lab M, Bury, UK) at 30°C; inocula of yeasts were obtained after growth for 18 h for *Y. lipolytica*, and for 48 h for *D. hansenii* and *C. laurentii* (which was the approximate time necessary to reach the exponential phase), in YM broth (Difco, Detroit MI, USA) at 30°C. Viable counts were determined for each species at proper incubation times as detailed below.

2.2. Assay for proteolytic and lipolytic activities

Detection of proteolytic and lipolytic activities of the single-strain cultures was made on 30% (w/v) milk agar (Merck, Darmstad, Germany) and on 10% (w/v) Tributyrin agar (Merck), respectively, after incubation at 30°C for 3 d. Each strain was inoculated in duplicate. Cultures which did not decrease the turbid zone were considered negative (−) for proteolytic or lipolytic activity (as appropriate), and those which gave rise to a clear zone were considered positive using one of two levels: (+) for a small layer (<5 mm) and (++) for a large layer (>5 mm) of clear zone around the inoculation mass.

2.3. Preparation of milk feedstocks

Ovine milk from the *Frízia* breed and caprine milk from the *Charnequeira* breed were thermally processed at 110°C for 10 min because this heat treatment does not damage the casein micellar structure and the fat globule network (Macedo & Malcata, 1997; Gomes & Malcata, 1998). Milk sterility was double checked as absence of microorganisms on plate count agar (Lab M) incubated at 30°C for 5 d at 1 : 10 dilution rate.

2.4. Experiments with curdled milk

Milk portions of 100 ml were sterilised in 250-ml flasks using the procedure described before. Then, 0.1 ml of inoculum (from each single-strain and from the starter) and 0.2 ml of liquid animal rennet (Fabre, Monza, Italy) diluted 10 times were added to the milk and incubated at 30°C for 4 h until coagulation. Incubation temperature was then reduced to 12°C; 0, 7 or 14% (w/v) sterile NaCl was added after 12 h and incubation continued for 65 d. Sterilisation of NaCl was accomplished in 24 h at 100°C and then checked by dissolution in sterile peptone water (1 : 10) and incubation for 5 d in PCA. Sterile curdled milk samples incubated at identical experimental conditions were used as controls.

2.5. Assay for microbiological viability

Numbers of viable bacteria and yeasts were determined after 12 h (denoted as 0 d hereafter), as well as after 27 and 65 d of ripening, in each curdled milk sample and control. Animal rennet was also checked for microbiological contamination.

2.6. Assays for physicochemical and biochemical parameters

The pH, moisture, total fat, total nitrogen, water-soluble nitrogen (WSN) and non-protein nitrogen (NPN) were determined following Freitas et al. (1997). The measurement of the concentration of free amino acids (FAA) in curdled milk samples containing no NaCl was carried out in an automatic amino acid analyser Alpha Plus (Pharmacia, Uppsala, Sweden). Prior to analysis, 2.5 g of every sample was centrifuged at 4500*g* for 15 min, and 1.4 ml of the supernatant was precipitated with 140 µl of 35% (v/v) PCA and allowed to stand for 30 min at 4°C to eliminate protein interference. Then 110 µl of 7.0 M KOH was added and the mixture centrifuged for 15 min at 4500*g* to eliminate PCA; next, 1 ml of supernatant was added to 50 mg of solid 5-sulphosalycilic acid, allowed to stand for 30 min at 4°C and then centrifuged for 15 min at 4500*g*; 0.3 M lithium hydroxide was meanwhile added to the supernatant at the ratio of 1 : 1 (v/v) in

order to adjust pH to 2.0. Finally, the internal standard *N*-leucine was added to the deproteinised sample at the ratio 2 : 1 (v/v) and then filtered through a 0.2 μm membrane filter. Determination of the concentration of free fatty acids (FFA) in curdled milk samples containing no NaCl was by HPLC according to Freitas and Malcata (1998a). The acidity index of 0, 7 and 14% (w/v) NaCl curdled milk samples was measured according to Freitas and Malcata (1998a) and expressed as mg equivalent KOH per g fat.

2.7. Assessment of statistical significance of results

Analysis of variance and Scheffé's *F*-test (at the 5% significance level) were carried out on the data obtained at the various stages of ripening for the different single strains, type of milk and percentage of NaCl. All statistical analyses were performed using the StatView™ v. 4.01 Computer Software (Abacus Concepts, Berkeley CA, USA).

3. Results and discussion

The compositional characteristics of the caprine and ovine milk used are shown in Table 1. The evolution of microflora viable counts and pH in caprine and ovine curdled milks inoculated with strains isolated from *Picante* cheese, salted with either 0, 7 or 14% (w/v) NaCl and incubated at 12°C for 65 d are recorded in Tables 2 and 3, respectively. As shown in Table 2, higher contents of NaCl led to lower microbiological counts; *Picante* cheese may reach more than 12% (w/w) NaCl by the end of ripening (Freitas et al., 1995). The NaCl effect was more intense on the *Lactobacillus* than on the *Enterococcus* strains, and in some cases the numbers of the latter actually increased when the content of NaCl was increased. Note that, according to Rozes and Peres (1996), *Lb. plantarum* strains isolated from Portuguese olives were tolerant to 4% (w/v) NaCl but addition of 8% (w/v) NaCl was completely inhibitory to cell growth. The higher resistance to the presence of NaCl by the *Enterococcus* strains tested in this experiment is in agreement with the presence of enterococci as the sole constituent of the microflora in *Picante* cheese ripened for 6 months (Freitas et al., 1996). Thus, the viable numbers obtained using MRS (Table 2) suggest that *E. faecium* was probably the dominant strain when the tentative starter was employed. Scheffé's *F*-test showed no statistical difference between *E. faecium* and the tentative starter. Among the yeast strains used in the curdled milk experiment, *D. hansenii* was beyond doubt the yeast most resistant to NaCl, which agrees with results reported elsewhere (Troller, Bernard & Scott, 1984).

Table 1
Compositional characteristics of caprine and ovine milk

Parameter	Caprine milk	Ovine milk
pH (fresh milk)	6.78	6.63
pH (sterilised milk)	6.65	6.55
Total solids (% w/w)	13.50	16.65
Total protein (% w/w)	4.03	6.17
Total fat (% w/w)	4.92	6.02

In addition to the level of NaCl and the nature of the strain, milk type and ripening time were also studied as manipulated technological parameters; Scheffé's *F*-test indicated that all these processing parameters played a significant role except for pH differences between all yeasts and the control, between *E. faecium* and *Lb. plantarum*, between *E. faecium* and the tentative starter, and between *Lb. plantarum* and the tentative starter. *Lb. paracasei* was responsible for lower values of pH in samples without NaCl by 65 d of ripening (Table 3), whereas *E. faecalis* was responsible for higher values at the same conditions. Higher levels of NaCl tended to diminish pH discrepancies between lactic acid bacteria; all values were around 5.8–6.1 in samples with 14% (w/v) NaCl by 65 d of ripening, which is in agreement with pH values obtained for *Picante* cheese by 180 d of ripening and 10–12% (w/w) NaCl (Freitas et al., 1995).

3.1. Proteolytic activities

Evidence for the proteolytic and peptidolytic activities brought about by each selected strain in 30% milk agar is presented in Table 4. Hydrolysis of casein, a phenomenon that requires availability of proteinases (Macedo & Malcata, 1997), occurred to a high extent in 30% milk agar inoculated with *Y. lipolytica* and *C. laurentii*, and to a lower extent when inoculation was with either strain of enterococci or *Lb. plantarum*. Our results are in contrast to the proteolytic activity reported for *E. faecium* isolated from *Serra da Estrela* cheese (Macedo & Malcata, 1997). From the data obtained for WSN, one concludes that there were significant differences between the strains studied in terms of production of high molecular weight peptides, so evidence was generated that proteases from lactic acid bacteria and yeasts are able to breakdown proteins. Among the strains tested, the action of *Y. lipolytica* was remarkable because this species produced ca. 85% of WSN by 65 d of ripening. For the other strains, the levels of WSN ranged in 30–50%. Scheffé's *F*-test indicated that ripening time, as well as type of milk used, produced a statistically significant effect on the levels of WSN. Proteolysis was reported to be more intense in *Picante* cheese manufactured with higher contents of caprine than ovine milk, irrespective of the microflora present (Freitas & Malcata, 1996; Freitas et al., 1997); higher levels of WSN were again obtained in curdled caprine milk. Differences in terms of WSN were not

Table 2
Evolution of microflora viable counts in caprine and ovine curdled milk inoculated with strains isolated from *Picante* cheese and incubated at 12°C, using various levels of NaCl

Microorganism	Milk type	Viable number [log (cfu $g^{-1}_{curdled\ milk}$)]									
			0% (w/v) NaCl			7% (w/v) NaCl			14% (w/v) NaCl		
		0 d	27 d	65 d	SEM[a]	27 d	65 d	SEM[a]	27 d	65 d	SEM[a]
Enterococccus faecium	Caprine	8.46	9.11	8.09	0.09	8.40	8.30	0.06	7.23	7.39	0.07
	Ovine	8.73	9.04	8.89	0.14	9.42	8.56	0.10	8.74	7.34	0.10
Enterococcus faecalis	Caprine	8.01	9.07	9.46	0.07	8.22	7.95	0.09	8.16	8.41	0.08
	Ovine	7.23	9.10	9.40	0.15	7.77	7.87	0.06	7.90	7.62	0.15
Lactobacillus plantarum	Caprine	7.92	8.24	8.67	0.09	8.00	7.21	0.07	5.97	4.09	0.08
	Ovine	7.30	8.95	8.79	0.08	6.95	—[b]	—[b]	6.04	3.00	0.04
Lactobacillus paracasei	Caprine	7.62	9.09	8.66	0.08	8.63	6.52	0.08	7.55	5.12	0.11
	Ovine	7.66	9.25	9.12	0.16	7.92	7.56	0.11	7.18	3.07	0.18
Debaryomyces hansenii	prine	1.00	8.70	9.12	0.08	8.31	7.93	0.04	7.80	7.93	0.10
	Ovine	3.84	8.30	8.24	0.16	8.40	8.32	0.10	8.24	8.06	0.13
Yarrowia lipolytica	Caprine	6.01	8.51	9.10	0.26	7.87	7.68	0.09	6.36	6.53	0.11
	Ovine	5.98	8.37	8.70	0.07	7.69	7.97	0.13	5.33	5.58	0.10
Cryptococcus laurentii	Caprine	3.93	7.33	7.61	0.09	5.32	6.82	0.08	4.31	3.78	0.07
	Ovine	2.70	—[b]	—[b]	—[b]	7.75	7.76	0.15	5.57	5.55	0.05
Tentative starter	Caprine[c]	8.11	9.04	8.58	0.08	8.48	—[b]	—[b]	8.17	7.83	0.11
	Caprine[d]	5.11	6.22	5.70	0.07	8.09	—[b]	—[b]	7.69	7.26	0.05
	Ovine[c]	8.22	9.24	8.88	0.08	8.56	8.81	0.05	7.92	9.01	0.23
	Ovine[d]	3.44	6.86	5.83	0.06	8.56	8.42	0.05	7.78	8.18	0.07
Control	Caprine	0.00	0.00	0.00	0.00	n/d[e]	n/d[e]	n/d[e]	n/d[e]	n/d[e]	n/d[e]
	Ovine	0.00	0.00	0.00	0.00	n/d[e]	n/d[e]	n/d[e]	n/d[e]	n/d[e]	n/d[e]

[a] Standard error of the mean.
[b] Contaminated samples.
[c] Enumeration performed on MRS agar.
[d] Enumeration performed on PDA agar.
[e] Not determined.

significant between both types of enterococci, but the reverse was observed for the two types of lactobacilli, which is consistent with results reported by Requena, Pelaez and Fox (1993) regarding caseinolytic activity of the same species. In terms of yeasts, the three strains studied proved that they affect proteolysis to a statistically significant extent. According to Roostita and Fleet (1996), *Candida lipolytica* isolated from cheese exhibited a strong proteolytic action on UHT-treated milk, whereas *D. hansenii* demonstrated a low proteolytic action. In terms of the tentative starter, Scheffé's *F*-test indicated no statistically significant difference for WSN between *E. faecium* and *D. hansenii*, on the one hand, and the tentative starter, on the other, so once again the action of *E. faecium* seems to predominate over the action of *Lb. plantarum*.

It is well known that lactic acid bacteria are nutritionally fastidious microorganisms with complex requirements in terms of free amino acids and possess, in general, a proteolytic system that is able to hydrolyse milk proteins to free amino acids (FAA) (Fox & Law, 1991). The results listed in Table 4 for FAA indicate that *Y. lipolytica* exhibited the highest peptidolytic activity; the differences between the concentrations of FAA obtained with *Y. lipolytica* and all other strains studied were statistically significant. Much more moderate peptidolytic activity was observed for the other strains with the exception of *E. faecalis* and *C. laurentii*: the differences between total FAA in samples inoculated with these strains vs. the corresponding controls were not statistically significant. Differences in total concentrations of FAA between both species of enterococci and

Table 3
Evolution of pH in caprine and ovine curdled milk inoculated with strains isolated from *Picante* cheese and incubated at 12°C, using various levels of NaCl

Microorganism	Milk type	pH									
			0% (w/v) NaCl			7% (w/v) NaCl			14% (w/v) NaCl		
		0 d	27 d	65 d	SEM[a]	27 d	65 d	SEM[a]	27 d	65 d	SEM[a]
Enterococccus faecium	Caprine	6.46	5.48	4.89	0.03	5.51	5.66	0.01	5.77	5.79	0.03
	Ovine	6.50	5.12	4.89	0.03	5.70	5.60	0.05	5.94	5.84	0.04
Enterococcus faecalis	Caprine	6.67	5.63	5.30	0.02	6.35	6.16	0.02	6.08	6.10	0.05
	Ovine	6.57	5.74	5.28	0.03	6.12	6.16	0.02	6.12	6.09	0.02
Lactobacillus plantarum	Caprine	6.48	4.92	4.46	0.03	5.13	5.03	0.02	5.99	5.86	0.04
	Ovine	6.53	4.86	4.49	0.03	6.14	—[b]	—[b]	6.03	6.05	0.02
Lactobacillus paracasei	Caprine	6.54	3.77	3.68	0.04	4.24	4.85	0.06	6.26	5.98	0.04
	Ovine	6.65	3.90	3.75	0.05	5.78	5.10	0.06	6.03	6.06	0.02
Debaryomyces hansenii	Caprine	6.63	6.01	5.69	0.02	5.51	5.66	0.01	6.22	6.04	0.04
	Ovine	6.72	6.11	5.66	0.02	6.02	5.66	0.02	6.03	6.02	0.03
Yarrowia lipolytica	Caprine	6.75	6.62	7.28	0.04	6.25	6.80	0.03	6.34	5.91	0.01
	Ovine	6.51	6.91	6.81	0.02	6.50	6.82	0.01	6.15	6.09	0.04
Cryptococcus laurentii	Caprine	6.78	6.60	6.18	0.03	6.44	6.40	0.03	6.19	6.27	0.03
	Ovine	6.66	—[b]	—[b]	—[b]	6.34	6.24	0.04	6.15	6.15	0.01
Tentative starter	Caprine[c] [d]	6.42	4.46	4.26	0.02	5.19	—[b]	—[b]	5.92	5.83	0.03
	Ovine[c] [d]	6.46	4.63	4.46	0.06	5.65	5.53	0.05	5.93	5.86	0.05
Control	Caprine	6.71	6.54	6.54	0.02	n/d[e]	n/d[e]	n/d[e]	n/d[e]	n/d[e]	n/d[e]
	Ovine	6.57	6.27	6.34	0.01	n/d[e]	n/d[e]	n/d[e]	n/d[e]	n/d[e]	n/d[e]

[a]Standard error of the mean.
[b]Contaminated samples.
[c]Enumeration performed on MRS agar.
[d]Enumeration performed on PDA agar.
[e]Not determined.

between both species of lactobacilli were also not statistically significant; however, Requena et al. (1993) reported significant differences in aminopeptidase activities between *Lb. casei* and *Lb. plantarum*. The aminopeptidase activity of *D. hansenii*, measured as total FAA, was not statistically different from that of *E. faecium* and from those of both *Lactobacillus* strains.

The different profiles of FAA (without Asn and Glu) obtained for each strain by 65 d of ripening at 12°C are plotted in Fig. 1. Different FAA profiles are apparent as the yeasts, especially *D. hansenii* and *Y. lipolytica*, produced a higher diversity of FAA by 65 d of ripening. The major FAA detected in *Picante* cheeses manufactured with several ratios of caprine and ovine milk throughout 180 d of ripening were Val, Leu and Phe (Freitas et al., 1998); Ile and Lys appeared as dominant FAA in some experimental *Picante* cheeses. The fractions of Val, Leu and Phe released by each strain are in the range observed in *Picante* cheese; *Y. lipolytica*, which is responsible for high levels of FAA and large FAA diversity, was one of the least significant contributors to these major FAA. Bearing this in mind, the tentative starter presented a profile that at first sight appeared desirable for *Picante* cheesemaking. It is interesting to observe that, besides the amounts of Arg produced by *L. plantarum* and *D. hansenii*, only traces of this FAA were produced by the tentative starter; Arg was reported to be responsible for unpleasant bitter-sweet taste (Lemieux & Simard, 1992). However, it cannot be forgotten that traces of γ-aminobutyric acid were released by this mixed starter; according to Choisy, Desmazeaud, Gripon, Lamberet, Lenoir and Tourneur (1987), this acid is present in low-quality cheeses and exists in small concentrations in *Picante* cheese (Freitas et al., 1998).

Table 4
Proteolytic activity on milk agar, and consequent effect on the water-soluble nitrogen (WSN), the overall free amino acid concentration (FAA) and the non-protein nitrogen (NPN), in caprine and ovine curdled milk inoculated with strains isolated from *Picante* cheese and incubated at 12°C

Microorganism	30% milk agar[d]	Milk type	WSN (% TN)[a]			FAA (mg kg^{-1} TS)[b]			NPN (% TN)[c]								
			0% (w/v) NaCl			0% (w/v) NaCl			0% (w/v) NaCl			7% (w/v) NaCl			14% (w/v) NaCl		
			27 d	65 d	SEM[e]	27 d	65 d	SEM[e]	27 d	65 d	SEM[e]	27 d	65 d	SEM[e]	27 d	65 d	SEM[e]
Enterococccus faecium	+	Caprine	36.70	51.10	0.70	1200.02	1973.74	86.92	12.09	17.06	0.88	12.48	13.35	0.81	11.38	12.50	0.81
		Ovine	35.82	43.58	0.50	735.43	1759.08	14.42	9.90	16.74	0.14	8.33	10.99	0.13	8.04	9.56	0.06
Enterococcus faecalis	+	Caprine	38.45	46.38	1.95	742.12	772.61	52.43	11.11	15.33	0.82	10.07	12.29	0.82	10.66	12.57	0.85
		Ovine	29.36	35.15	0.43	392.40	2069.21	107.04	10.67	19.85	0.32	8.40	10.46	0.06	7.47	9.51	0.43
Lactobacillus plantarum	+	Caprine	40.58	40.77	0.85	915.84	3089.64	62.32	13.24	17.72	0.82	12.01	14.03	0.82	11.28	12.11	0.81
		Ovine	36.02	38.92	0.07	1226.00	2961.03	138.52	11.03	13.19	0.07	7.66	—[f]	—[f]	7.73	7.59	0.06
Lactobacillus paracasei	−	Caprine	29.84	34.58	0.98	2216.03	2475.63	94.84	17.64	22.76	0.83	14.70	17.27	0.81	9.05	13.64	0.85
		Ovine	24.41	27.44	0.25	1481.24	1577.40	2.06	13.88	17.83	0.09	9.84	14.80	0.10	8.51	9.10	0.11
Debaryomyces hansenii	−	Caprine	29.50	47.88	0.78	628.62	1307.93	145.43	10.40	15.19	0.81	9.23	11.69	0.94	9.26	10.69	0.82
		Ovine	28.94	41.50	0.53	667.90	2549.93	2.05	10.85	17.82	0.05	8.10	9.97	0.15	8.50	8.02	0.09
Yarrowia lipolytica	+ +	Caprine	73.13	83.57	0.86	4283.32	22385.14	901.35	57.42	85.30	0.83	24.53	43.09	0.87	15.82	17.99	0.81
		Ovine	77.91	87.07	0.39	3149.93	18142.14	127.83	62.49	88.68	2.10	12.03	40.51	0.57	9.10	10.59	0.10
Cryptococcus laurentii	+ +	Caprine	34.10	39.82	0.86	71.62	1300.53	5.95	9.90	15.06	0.81	9.87	12.20	0.97	10.79	11.06	0.81
		Ovine	—[f]	—[f]	—[f]	—[f]	—[f]	—[f]	11.16	—[f]	0.13	8.60	9.90	0.159	7.45	9.36	0.06
Tentative starter	n/d[g]	Caprine	46.29	48.09	0.76	1639.24	2417.52	198.12	25.62	31.43	0.81	10.05	—[f]	—[f]	9.21	11.36	0.81
		Ovine	36.32	47.31	0.65	1253.70	5360.24	50.32	16.82	35.95	0.47	8.23	11.52	0.11	7.37	8.66	0.14
Control	—	Caprine	25.00	32.20	0.71	360.63	445.03	0.32	9.28	11.74	0.82	n/d[g]	n/d[g]	n/d[g]	n/d[g]	n/d[g]	n/d[g]
		Ovine	19.00	26.32	0.30	132.34	153.52	2.14	8.20	10.05	0.07	n/d[g]	n/d[g]	n/d[g]	n/d[g]	n/d[g]	n/d[g]

[a]Values for WSN at 0 d for the caprine and ovine curdled milk control were 16.43 and 15.24% TN, respectively.
[b]Values for FAA at 0 d for the caprine and ovine curdled milk control were 251.02 and 104.54 mg kg^{-1} TS, respectively.
[c]Values for NPN at 0 d for the caprine and ovine curdled milk control were 7.15 and 7.65% TN, respectively.
[d]Cultures which do not decrease the turbid zone (−), decrease the turbid zone via a small layer (<5 mm) (+) and decrease the turbid zone via a large layer (>5 mm) (++).
[e]Standard error of the mean.
[f]Contaminated samples.
[g]Not determined.

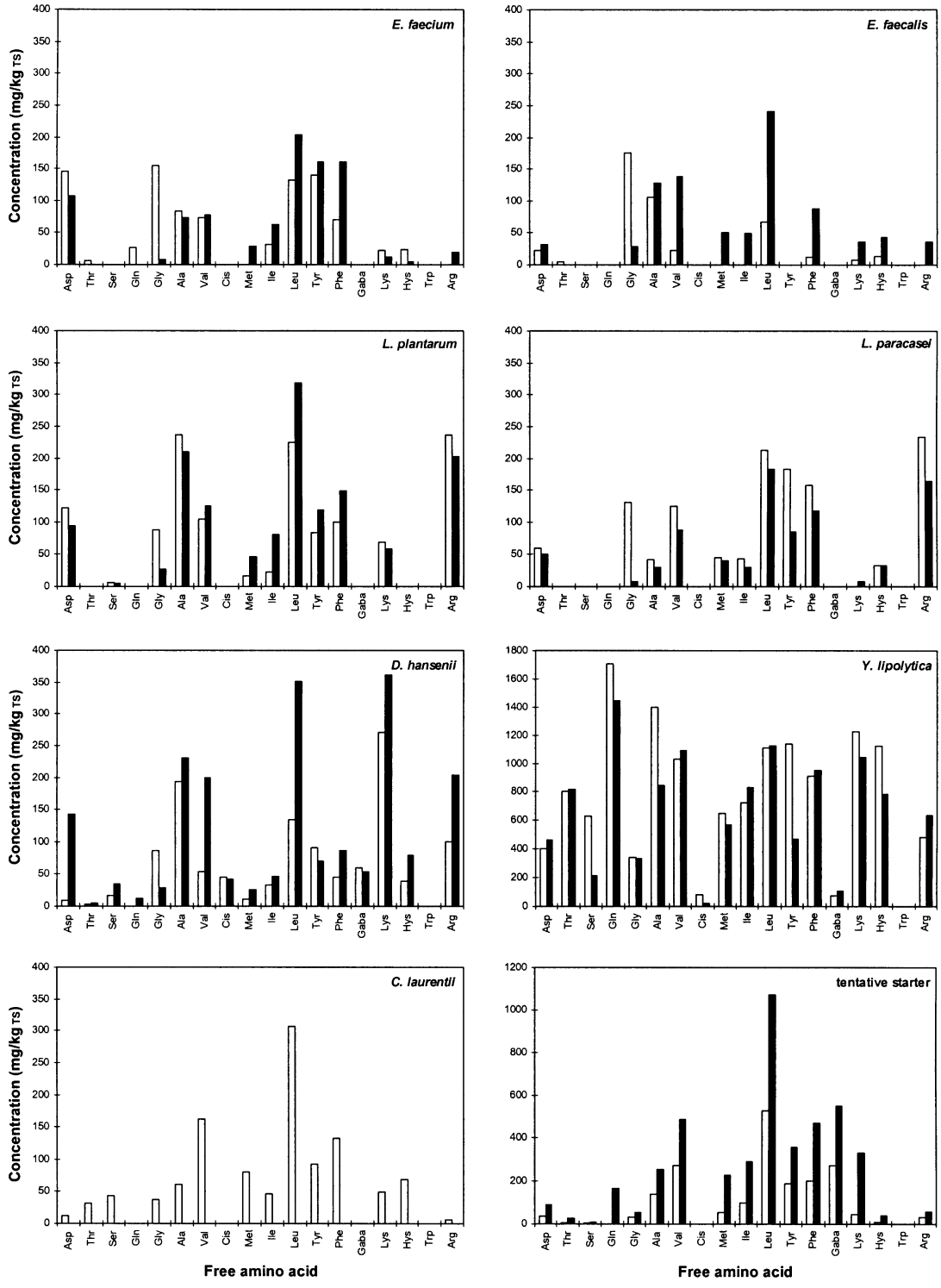

Fig. 1. Changes in the concentration of free amino acids (FAA) in curdled caprine milk (□) and curdled ovine milk (■) inoculated with each strain without NaCl and ripened for 65 d at 12°C.

In order to ascertain the effect of NaCl on proteolysis, the NPN fractions in all samples were quantified for the three levels of NaCl (Table 4). As expected, the concentration of NaCl influenced substantially the extent and rate of release of FAA, but it was remarkable that the drop in NPN values in curdled milk was steeper between 0 and 7% (w/v) NaCl than between 7 and 14% (w/v) NaCl (see Table 4), except for data from *Y. lipolytica* (which showed an extreme effect of NaCl content). The relatively higher contents of NPN in samples with lower NaCl levels can

be attributed to a considerably higher activity of the microflora under those environmental conditions, in agreement with microbiological data reported in Table 2. Besides the NaCl factor, the other two factors (milk type and ripening time) played overall significant roles in terms of NPN levels. However, Scheffé's *F*-test indicated that the differences between *E. faecium*, *E. faecalis*, *Lb. plantarum* and *D. hansenii* were not significant in terms of NPN levels. Although it is not possible to directly compare results obtained using pure, single-cultures with those obtained using cheese itself because a multitude of simultaneous phenomena occur during cheese ripening, the single-culture results provide trends and bases for discussion. Comparison between data obtained for *Picante* cheese manufactured with caprine and ovine milk by 83 d of ripening (i.e. 9–11% NPN, ca. 8% (w/w) NaCl and pH 5.3; Freitas et al., 1997), and data obtained for inoculated curdled milk by 65 d, indicates that a good approximation is obtained for the case of curdled caprine and ovine milk with 7% (w/v) NaCl inoculated with *E. faecium* (11–13% NPN and pH 5.6–5.7), with *Lb. plantarum* (ca. 14% NPN and pH 5.0), with *D. hansenii* (10–12% NPN and pH 5.7) and with the tentative starter (ca. 12% NPN and pH 5.5).

3.2. Lipolytic activities

The lipolytic activity, as assessed by the rate of release of butyric acid from tributyrin, is depicted in Table 5. It is apparent from inspection of this table that only *Y. lipolytica* and *C. laurentii* were able to hydrolyse this fat. According to Stadhouders and Veringa (1973),

Table 5
Lipolytic activity on tributyrin agar, and consequent effect on overall free fatty acid concentration (FAA) and fat acidity index of caprine and ovine curdled milk inoculated with strains isolated from *Picante* cheese and incubated at 12°C

Microorganism	10% tributyrin agar[c]	Milk type	FFA (mg $kg_{dry\ fat}^{-1}$)[a]			Acidity index of fat ($mg_{eq.KOH}$ $kg_{dry\ fat}^{-1}$)[b]					
			0% (w/v) NaCl			0% (w/v) NaCl		7% (w/v) NaCl		14% (w/v) NaCl	
			27 d	65 d	SEM[d]	27 d	65 d	27 d	65 d	27 d	65 d
Enterococccus faecium	+	Caprine	266.03	346.63	1.03	1302.83	1980.92	579.40	623.40	249.81	467.62
		Ovine	248.34	315.24	4.13	1587.34	1827.04	746.34	747.46	639.53	681.10
Enterococcus faecalis	+	Caprine	163.45	188.73	2.13	877.32	1206.84	365.52	331.13	183.12	190.43
		Ovine	130.96	—[e]	—[e]	882.24	—[e]	503.01	545.23	425.28	434.82
Lactobacillus plantarum	−	Caprine	233.72	466.74	2.32	1627.63	3184.32	495.54	727.16	230.43	188.02
		Ovine	161.55	302.05	6.14	1099.80	2443.70	273.03	—[f]	229.82	258.23
Lactobacillus paracasei	+	Caprine	121.12	191.65	1.53	8531.02	8973.31	556.32	727.46	219.23	226.12
		Ovine	92.93	146.83	0.62	6792.83	7647.44	480.14	614.03	333.84	334.04
Debaryomyces hansenii	−	Caprine	93.43	119.65	0.61	379.80	459.80	74.95	319.64	166.62	324.23
		Ovine	147.75	187.92	4.33	538.82	1302.20	315.86	479.73	526.73	496.10
Yarrowia lipolytica	++	Caprine	36489.13	44680.83	22.12	6364.62	10137.91	2094.23	2063.42	750.62	1381.03
		Ovine	26850.52	40622.75	23.94	6051.22	8872.03	2491.94	2050.50	253.54	675.63
Cryptococcus laurentii	++	Caprine	250.63	388.93	0.92	1222.14	2368.73	696.26	251.22	243.82	228.72
		Ovine	—[f]	—[f]	—[f]	—[f]	—[f]	468.84	525.14	341.84	230.24
Tentative starter	n/d[g]	Caprine	180.12	220.93	2.73	1081.52	1718.70	607.25	—[f]	274.10	354.61
		Ovine	187.94	234.22	2.70	1104.03	1897.42	550.19	750.23	445.60	428.50
Control	—	Caprine	182.43	211.72	1.92	272.43	434.20	n/d[g]	n/d[g]	n/d[g]	n/d[g]
		Ovine	140.82	177.03	3.24	262.74	373.83	n/d[g]	n/d[g]	n/d[g]	n/d[g]

[a] Values of FFA at 0 d for the caprine and ovine curdled milk control were 160.53 and 121.32 mg $kg_{dry\ fat}^{-1}$, respectively.
[b] Acidity index of fat at 0 d for the caprine and ovine curdled milk control were 191.03 and 198.43 $mg_{eq.KOH}$ $kg_{dry\ fat}^{-1}$, respectively.
[c] Cultures which do not decrease the turbid zone (−), decrease the turbid zone via a small layer (<5 mm) (+) and decrease the turbid zone via a large layer (>5 mm) (++).
[d] Standard error of the mean.
[e] No sample left for analysis.
[f] Contaminated samples.
[g] Not determined.

mesophilic lactobacilli that normally occur in hard cheeses do not have a noticeable effect on the release of FFA from fat throughout ripening. Data reported by Wessels, Jooste and Moster (1990) have shown that enterococci are proteolytic rather than lipolytic. According to Roostita and Fleet (1996), *Candida lipolytica* displays strong lipolytic action whereas very weak lipolytic action is observed for *D. hansenii*. Table 5 also gives the total concentration of FFA in caprine and ovine curdled milk in the absence of NaCl by 27 and 65 d of ripening, whereas the FFA profile generated by each strain is shown in Fig. 2. Ripening time was a statistically significant factor, whereas milk type was not; in terms of microbial species, Scheffé's *F*-test revealed that only the differences between the values obtained by *Y. lipolytica* vs. all other strains under study were statistically

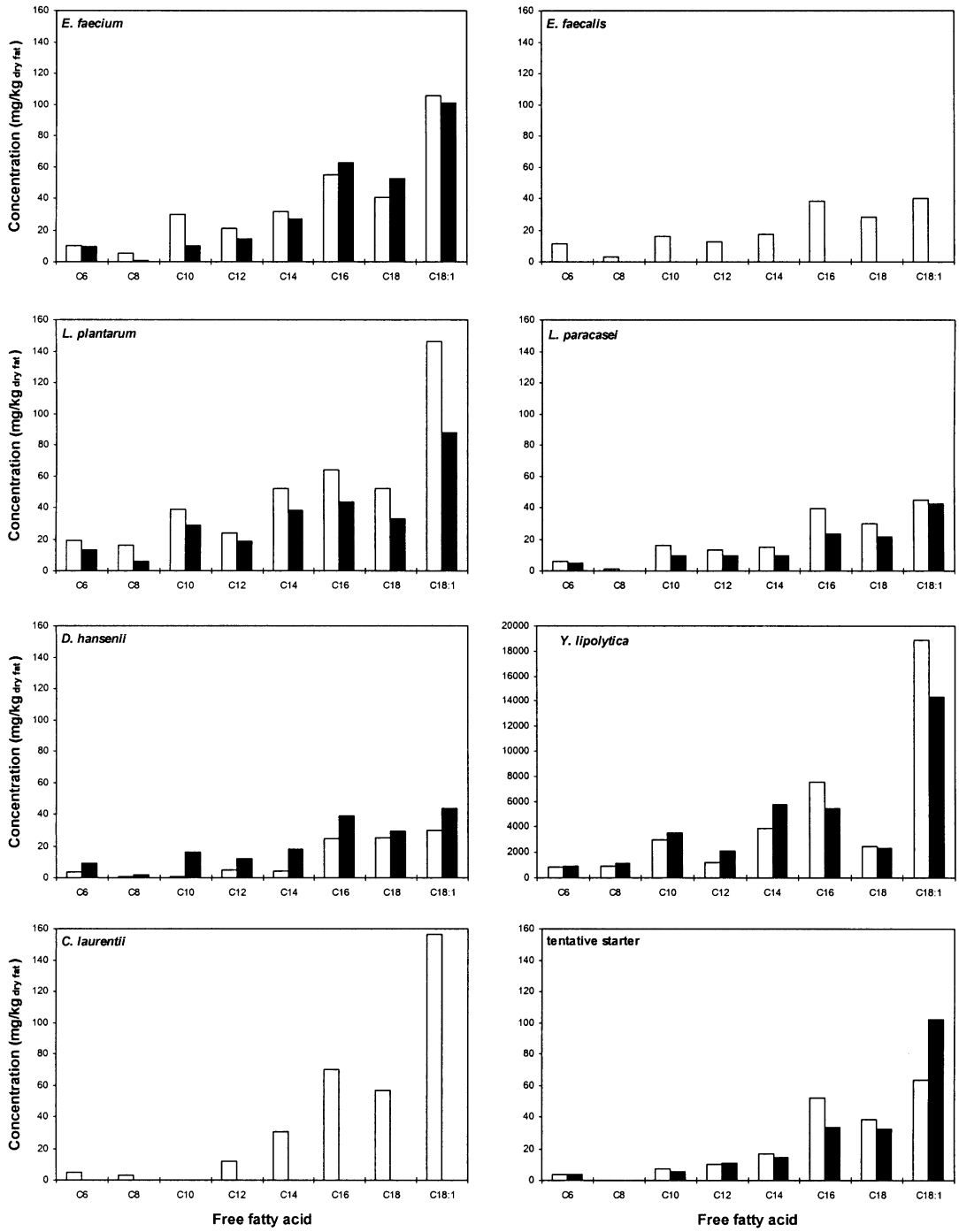

Fig. 2. Changes in the concentration of free fatty acids (FFA) in curdled caprine milk (□) and curdled ovine milk (■) inoculated with each strain without NaCl and ripened for 65 d at 12°C.

significant. The dominant FFA produced by *Y. lipolytica* by 65 d of ripening (see Fig. 2) were oleic, palmitic, myristic and capric acids: with the exception of capric acid, these are among the predominant FFA reported by Roostita and Fleet (1996) in UHT-treated milk cheese and, with the exception of myristic acid, they are also among the predominant FFA present in ripened *Picante* cheese (Freitas & Malcata, 1998a). Although to a much lesser degree, *E. faecium* and *L. plantarum* were also able to hydrolyse milk, whereas the lipolytic activity for *E. faecalis*, *L. paracasei* and *D. hansenii* was very low or absent. According to Macedo and Malcata (1997), milk fat was significantly hydrolysed by *E. faecium* but not by *Lb. paracasei* ssp. *paracasei*.

Since the lipolytic activities of *E. faecalis*, *L. paracasei* and *D. hansenii* were very low or even absent, the FFA profiles produced in the samples inoculated with these strains were likely the result of the action of milk lipases. The dominant FFA resulting from milk lipase action are palmitic, stearic and oleic (each one representing 17–27% of the total FFA, and all together representing 64–85% of the total FFA), followed by myristic, caprylic and lauric (each one representing 5–10% of the total FFA, and all together representing 23–28% of the total FFA). Within some variability, this profile was also observed in the curdled milk samples where the lipolytic action by the microflora is evident; the main difference that can be observed is the higher levels of oleic acid (32–50% of the total FFA) when compared with the levels of palmitic and stearic (6–23% of the total FFA). No significant differences were observed for the short- or medium-chain fatty acids. The dominant FFA produced by the strains by 65 d of ripening (see Fig. 2) were oleic, palmitic, myristic and capric: these FFA are among the predominant FFA reported by Roostita and Fleet (1996) for UHT-treated milk cheese inoculated with *C. lipolytica*, with the exception of capric acid.

The results obtained for each single strain, or even for the mixed starter, do not show any clear contribution to the FFA profile of *Picante* cheese, which is characterised by $18 \pm 2\%$ of short-, $27 \pm 1\%$ of medium- and $54 \pm 2\%$ of long-chain fatty acids (Freitas & Malcata, 1998a). However, *Y. lipolytica*, and to a lesser extent, *E. faecium* and *L. plantarum*, revealed to be important contributors to high levels of lipolysis (which are characteristic of *Picante* cheese). In terms of contribution to the final sensory profile of *Picante* cheese, the action of *E. faecium* and *L. plantarum* seems to be potentially relevant since these strains are present in *Picante* cheese throughout the whole ripening period.

Lipolytic activities, measured as fat acidity index, were strongly affected by the NaCl content (Table 5). As happened with the release of FAA, the extent of fat hydrolysis was much more affected by the increase of NaCl from 0 to 7% (w/v) than by its increase from 7 to 14% (w/v), possibly because salt affects the specificity of lipolytic enzymes (Roostita & Fleet, 1996). Unusually high values were found by 27 and 65 d of ripening for the fat acidity index when *Lb. paracasei* was inoculated in salt-free curdled milk; these values were certainly affected by the high acidity promoted by this strain.

4. Conclusions

Milk type, ripening time and NaCl content were statistically significant with respect to proteolysis, whereas ripening time was a statistically significant factor but milk type was not in terms of lipolysis; peptidolytic and lipolytic activities were strongly affected by NaCl content, and extent of hydrolysis was affected by increase in NaCl from 0 to 7% (w/v) much more than from 7 to 14% (w/v).

Data obtained for ripened *Picante* cheese manufactured with caprine and ovine milk are relatively similar to data obtained for curdled milk inoculated with *E. faecium*, *L. plantarum*, *D. hansenii* and the tentative starter. In terms of lipolysis, despite the lower values obtained for fat acidity index by all strains in the presence of 7% (w/v) NaCl, the action of *E. faecium* and *L. plantarum* is apparently important because these strains appear in *Picante* cheese throughout the whole 6 months of ripening.

These experimental results indicate that a potential starter for *Picante* cheese should include, besides *Lb. plantarum*, one species of *Enterococcus* (*E. faecium* seems to be appropriate) and one species of yeast (possibly *D. hansenii*). Lee and Lim (1988a, b) studied the inclusion of *D. hansenii* and *C. lipolytica* in a cheese starter, whereas Deiana et al. (1984) manufactured *Pecorino* cheese with a starter which included *D. hansenii*, and reported that yeasts developed well in cheese and caused faster proteolysis.

Although relevant by themselves, the conclusions of this study should be used with caution in practical cheesemaking because the curdled milk system is different from a true cheese system and because the action of each selected strain was studied independently, except for the tentative starter.

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