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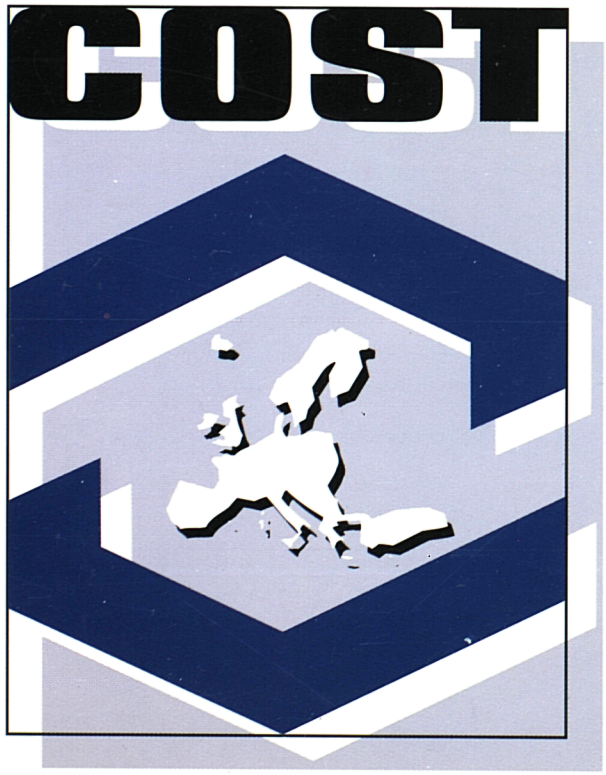

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Dominant microflora of *Picante* cheese: Effects on proteolysis and lipolysis

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Abstract

*Four species of bacteria (*Enterococcus faecium *and* E. faecalis, Lactobacillus plantarum *and* L. paracasei*) and three species of yeasts (*Debaryomyces hansenii, Yarrowia lipolytica *and* Cryptococcus laurentii*), previously isolated from* Picante *cheese, were assayed for proteolysis and lipolysis. Milk type (caprine or ovine), ripening time (0 to 65 d) and concentration of NaCl (0 to 14 %(w/v)) have been assessed in terms of their effects upon* in vitro *curdled milk. Good evidence of proteolytic and peptidolytic activities was provided 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 also observed for the other strains under study. Ripening time was statistically significant with regard to lipolysis but milk type was not. Lipolytic activities were strongly affected by presence of NaCl. According to experimental results, it is suggested that a mixed-strain starter for* Picante *cheese including* L. plantarum, E. faecium *(or* E. faecalis*) and* D. hansenii *(and/or* Y. lipolytica*) is of potential interest.*

Keywords: microorganisms, ovine milk, caprine milk, NaCl effect, ripening

Introduction

In the manufacture of artisanal cheeses, the cheesemaker often relies on lactic acid bacteria present as native microflora, or which are contributed along the process. In recent years, high quality standards for milk and cheese have been enforced, so knowledge and development of microbial additives based on indigenous microflora besides, improving the quality of raw milk and standardising technological procedures of cheesemaking and ripening, would certainly help achieve and maintain those quality standards. *Picante da Beira Baixa* cheese (or simply *Picante*), a traditional salty and spicy cheese manufactured in Portugal from mixtures of ovine and caprine milks, possesses an *Appéllation d'Origine Controllée* status since 1988, and undoubtedly falls within the scope of the aforementioned issues.

Recently published work concerning *Picante* cheese has encompassed microbiological characterisation (1, 2), physicochemical characterisation in both proteolysis (3, 4) and lipolysis (5) fields, and technological characterisation (6, 7). Although *Picante* cheese is manufactured in the absence of deliberately added 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 additives based on adventitious microflora. The microbial groups that dominate in viable numbers throughout ripening of *Picante* cheese are lactic acid bacteria and yeasts (1), above the 10^7 cfu/g_{cheese} threshold; the most abundant species of the former are enterococci (mainly *Enterococcus faecium*, *E. faecalis* and *E. durans*) and lactobacilli (mainly *Lactobacillus plantarum* and *L. paracasei*), whereas those more abundant in the latter group are *Debaryomyces hansenii* and *Yarrowia lipolytica* (2).

The purpose of this research work was to screen the dominant strains isolated from *Picante* cheese for their role in experimental, univarietal pseudo-cheeses in terms of proteolysis and lipolysis. Since such pathways trigger the progress of ripening, and hence do clearly contribute to the final cheese characteristics, monitoring the evolution of those biochemical phenomena when brought about by the microbial species present in higher viable numbers in *Picante* cheese, if considered in an independent manner, is likely to provide important fundamental information about their contribution to the final product. In addition to the nature of the microbial strains, the milk type, the ripening time and the concentration of NaCl have also

been evaluated for their anticipated potential effect upon physicochemical performance of the aforementioned strains.

Material and methods

Preparation of microbial cultures

Dominant species previously isolated from *Picante* cheese, and identified according to Freitas *et al.* (2), were used in the present study. Four species of bacteria and three species of yeasts were isolated from *Picante,* namely two *Lactobacillus* strains (*L. plantarum* and *L. paracasei*) and two *Enterococcus* strains (*E. faecalis* and *E. faecium*), on the one hand, and *D. hansenii, C. laurentii* and *Y. lipolytica,* on the other. Experimental inocula of lactobacilli and enterococci for milk and curdled milk experiments were obtained after growth for 18 h (which was the average 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 of *Yarrowia lipolytica*, and for 48 h of *D. hansenii* and *C. laurentii*, in YM broth (Difco, Detroit MI, USA) at 30°C. Viable counts were determined for each species at the time of incubation as detailed below.

Preparation of curdled milk experiments

Ovine milk from the *Frízia* breed and caprine milk from the *Charnequeira* breed were thermally processed at 110 °C for 10 min; this heat treatment does not damage the casein micellar structure and the fat globule network (8, 9). Milk sterility was double-checked as absence of microorganisms on plate count agar (PCA, Lab M) incubated at 30°C for 5 d at 1:10 dilution rate.

Milk portions of 100 ml were sterilised in 250 ml-flasks. Then, 0.1 ml of each single-strain culture, and 0.2 ml of liquid animal rennet (Frabre, Monza, Italy) diluted 10 times, were added to the milk and incubated at 30 °C for 4 h (with concomitant occurrence of coagulation). Furthermore, 0.1 ml of a mixed inoculum was added to 100 ml of sterilised milk and incubated at the same conditions; the mixed inoculum (denoted hereafter as tentative starter) was previously prepared with a 2 ml-inoculum of *E. faecium*, 2 ml-inoculum of *L. plantarum* and 2-ml inoculum of *D. hansenii*. After coagulation, the temperature was reduced to 12 °C; 0, 7 or 14 %(w/v) sterile NaCl was added by 12 h and incubation was pursued for 65 d. Sterilisation of NaCl was done via heating at 100 °C for 24 h, and then checked via

dissolution in sterile peptone water (1:10) and incubation for 5 d on PCA. Animal rennet was checked for microbiological contamination in a similar fashion.

Determination of the number of viable bacteria and yeasts was done by 12 h (denoted as 0 d hereafter), as well as by 27 and 65 d of ripening, in each (milk curdled) sample and control.

Assay for physicochemical and biochemical parameters

The pH, moisture, total fat, total nitrogen, water soluble nitrogen (WSN) and non-protein nitrogen (NPN) were determined following the methods described by Freitas *et al.* (3). The measurement of the concentration of free amino acids (FAA) in milk-curdled samples containing no NaCl was carried out in an automatic amino acid analyser using sodium citrate buffer as mobile phase and an ion exchange column as stationary phase, and nynhydrin-derivatization prior to detection. Before analysis, 2.5 g of every sample was centrifuged at 4000 rpm for 15 min, and 1.4 ml of the supernatant was precipitated with 140 µl of 35 %(v/v) perchloric acid (PCA) and allowed to stand for 30 min at 4 °C (to eliminate protein interference); 110 µl of 7.0 M potassium hydroxide was then added and the mixture was centrifuged for 15 min at 4000 rpm (to eliminate PCA); 1 ml of supernatant was next 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 4000 rpm; 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; and the internal standard N-Leucine was finally added to the deproteinized 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 milk curdled samples containing no NaCl was by HPLC according to Freitas and Malcata (5). The acidity index of milk-curdled samples containing 0, 7 and 14 %(w/v) NaCl was measured according to Freitas and Malcata (5), and expressed as mg of equivalent KOH per g of fat.

Assessment of statistical significance of results

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

Results and discussion

The evolution of microflora viable counts and pH in caprine and ovine curdled milks inoculated with strains previously isolated from *Picante* cheese, salted with either 0, 7 or 14 %(w/v) NaCl and incubated at 12° C throughout 65 d are depicted in Tables 1 and 2. From inspection of these tables, it is clear that higher contents of NaCl lead to lower microbiological counts (note that *Picante* cheese may reach more than 12 %(w/w) NaCl by the end of ripening (1).

Table 1. Evolution of microflora viable counts in caprine and ovine curdled milk inoculated with strains previously isolated from *Picante* cheese, using incubation at 12 °C with various levels of NaCl.

		Viable number log (cfu/$g_{curdled\ milk}$)									
	Milk		0% NaCl			7% NaCl			14% NaCl		
Microorganism	Type	0 d	27 d	65 d	SEM[a]	27 d	65 d	SEM[a]	27 d	65 d	SEM[a]
E. 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
E. 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
L. 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]	0.05	6.04	3.00	0.04
L. 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
D. hansenii	Caprine	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
Y. 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
C. 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]	-	7.75	7.76	0.15	5.57	5.55	0.05
tentative	Caprine[c]	8.11	9.04	8.58	0.08	8.48	-[b]	-	8.17	7.83	0.11
starter	[d]	5.11	6.22	5.70	0.07	8.09	-[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
	[d]	3.44	6.86	5.83	0.06	8.56	8.42	0.05	7.78	8.18	0.07
Control	Caprine	<0	<0	<0	<0	-	-	-	-	-	-
	Ovine	<0	<0	<0	<0	-	-	-	-	-	-

[a] Standard error of the mean; [b] Contaminated samples; [c] Enumeration performed on MRS agar; [d] Enumeration performed on PDA agar. No control was performed for 7 and 14 %(w/v) NaCl samples since NaCl was sterilized.

The NaCl effect was more intense on the *Lactobacillus* strains than on the *Enterococcus* counterparts, and in some cases the numbers of the latter actually increased when the content of NaCl was increased. According to Rozes and Peres (10), *L. plantarum* strains isolated from

Portuguese olives were tolerant to 4 %(w/v) NaCl, yet addition of 8 %(w/v) NaCl was completely inhibitory to cell growth; this observation could provide evidence for the claim that the wild strains of *L. plantarum* isolated from *Picante* cheese could be dramatically more resistant to NaCl. The higher resistance of the *Enterococcus* strains to the presence of NaCl 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 mo (2). In view of this rationale, the viable numbers obtained using MRS (Table 1) suggest that *E. faecium* was probably the dominating strain when the tentative starter was employed.

Table 2. Evolution of pH in caprine and ovine curdled milk inoculated with strains previously isolated from *Picante* cheese, using incubation at 12 °C with various levels of NaCl.

		pH									
	Milk		0 %(w/v) NaCl			7 %(w/v) NaCl			14 %(w/v) NaCl		
Microorganism	type	0 d	27 d	65 d	SEM[a]	27 d	65 d	SEM[a]	27 d	65 d	SEM[a]
E. 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
E. 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
L. 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]	0.02	6.03	6.05	0.02
L. 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
D. 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
Y. 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
C. 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]	-	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]	-	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	-	-	-	-	-	-
	Ovine	6.57	6.27	6.34	0.01	-	-	-	-	-	-

[a] Standard error of the mean; [b] Contaminated samples; [c] Enumeration performed on MRS agar; [d] Enumeration performed on PDA agar. No control was performed for 7 and 14 %(w/v) NaCl samples since NaCl was sterilized.

Scheffé's *F*-test conveyed no statistical validity to the apparent 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 more resistant to NaCl, which agrees with results reported elsewhere (11).

In addition to the level of NaCl and nature of 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 yeasts and the control, between *E. faecium* and *L. plantarum*, between *E. faecium* and the tentative starter, and between *L. plantarum* and the tentative starter.

Values for the water soluble nitrogen (WSN) and free amino acids (FAA) produced via action of each strain on both types of milk by 0, 27 and 65 d of ripening, in the absence of NaCl, are depicted in Table 3.

Table 3. Water soluble nitrogen (WSN) and overall free amino acids (FAA) in caprine and ovine curdled milk inoculated with strains, previously isolated from *Picante* cheese, using incubation at 12 °C in the absence of NaCl.

Microorganism	Milk type	WSN (%TN)			FAA (mg/kg$_{TS}$)		
		27 d	65 d	SEM[a]	27 d	65 d	SEM[a]
E. faecium	Caprine	36.70	51.10	0.70	1200.0	1973.7	86.9
	Ovine	35.82	43.58	0.50	735.4	1759.0	14.4
E. faecalis	Caprine	38.45	46.38	1.95	742.1	772.6	52.4
	Ovine	29.36	35.15	0.43	392.4	2069.2	107.0
L. plantarum	Caprine	40.58	40.77	0.85	915.8	3089.6	62.3
	Ovine	36.02	38.92	0.07	1226.0	2961.0	138.5
L. paracasei	Caprine	29.84	34.58	0.98	2216.0	2475.6	94.8
	Ovine	24.41	27.44	0.25	1481.2	1577.4	2.0
D. hansenii	Caprine	29.50	47.88	0.78	628.6	1307.9	145.4
	Ovine	28.94	41.50	0.53	668.0	2549.9	2.0
Y. lipolytica	Caprine	73.13	83.57	0.86	4283.3	22385.1	901.3
	Ovine	77.91	87.07	0.39	3149.9	18142.1	127.8
C. laurentii	Caprine	34.10	39.82	0.86	71.6	1300.5	5.9
	Ovine	-[b]	-[b]	-	-[b]	-[b]	-
tentative starter	Caprine	46.29	48.09	0.76	1639.2	2417.5	198.1
	Ovine	36.32	47.31	0.65	1253.7	5360.2	50.3
Control	Caprine	25.00	32.20	0.71	360.6	445.0	0.3
	Ovine	19.00	26.32	0.30	132.3	153.5	2.1

[a] Standard error of the mean; [b] Contaminated samples. Values for WSN at 0 d for the caprine and ovine curdled milk control were 16.43 and 15.24% of TN, respectively. Values for FAA at 0 d for the caprine and ovine curdled milk control were 251.0 and 104.5 mg /kg$_{TS}$, respectively.

In order to ascertain the effect of NaCl upon proteolysis, the non-protein nitrogen fraction (NPN) in all samples was measured for three levels of salt (see Table 4). According to Furtado and Partridge (12), WSN is a ripening extension index which correlates well with rennet activity, and thus with production of high molecular weight peptides. Our results, which show that there are significant differences between the strains studied in terms of WSN, do agree with Green and Foster (13), who reported that rennet and proteases from lactic acid bacteria exhibit in cheeses patterns of protein breakdown that are similar to one another.

Table 4. Non protein nitrogen (WSN) in caprine and ovine curdled milk inoculated with strains previously isolated from *Picante* cheese, using incubation at 12 °C, with various levels of NaCl.

		NPN (%TN)								
	Milk	0 %(w/v) NaCl			7 %(w/v) NaCl			14 %(w/v) NaCl		
Microorganism	Type	27 d	65 d	SEM[a]	27 d	65 d	SEM[a]	27 d	65 d	SEM[a]
E. faecium	Caprine	12.09	17.06	0.88	12.48	13.35	0.81	11.38	12.50	0.81
	Ovine	9.90	16.74	0.14	8.33	10.99	0.13	8.04	9.56	0.06
E. faecalis	Caprine	11.11	15.33	0.82	10.07	12.29	0.82	10.66	12.57	0.85
	Ovine	10.67	19.85	0.32	8.40	10.46	0.06	7.47	9.51	0.43
L. plantarum	Caprine	13.24	17.72	0.81	12.01	14.03	0.82	11.28	12.11	0.81
	Ovine	11.03	13.19	0.07	7.66	-[b]	-	7.73	7.59	0.06
L. paracasei	Caprine	17.64	22.76	0.83	14.70	17.27	0.81	9.05	13.64	0.85
	Ovine	13.88	17.83	0.09	9.84	14.80	0.10	8.51	9.10	0.11
D. hansenii	Caprine	10.40	15.19	0.81	9.23	11.69	0.94	9.26	10.69	0.82
	Ovine	10.85	17.82	0.05	8.10	9.97	0.15	8.50	8.02	0.09
Y. lipolytica	Caprine	57.42	85.30	0.83	24.53	43.09	0.87	15.82	17.99	0.81
	Ovine	62.49	88.68	2.10	21.03	40.51	0.57	9.10	10.59	0.10
C. laurentii	Caprine	9.90	15.06	0.81	9.87	12.20	0.97	10.79	11.06	0.81
	Ovine	11.16	-[b]	0.13	8.60	9.90	0.15	7.45	9.36	0.06
tentative	Caprine	25.62	31.43	0.81	10.05	-[b]	-	9.21	11.36	0.81
starter	Ovine	16.82	35.95	0.47	8.23	11.52	0.11	7.37	8.66	0.14
Control	Caprine	9.28	11.74	0.82	-	-	-	-	-	-
	Ovine	8.20	10.05	0.07	-	-	-	-	-	-

[a] Standard error of the mean; [b] Contaminated samples. Values for NPN at 0 d for the caprine and ovine curdled milk control were 7.15 and 7.65 % TN, respectively.

Among the strains tested, the action of *Y. lipolytica* is remarkable because this species produced ca. 85% of WSN by 65 d of ripening. For the other strains, the levels of WSN ranged in 30-40% for *E. faecalis*, *L. plantarum* and *L. paracasei*, and 40-50% for *E. faecium*, *D. hansenii*, *C. laurentii* and the tentative starter. 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 (3, 6); higher levels of WSN were again obtained in curdled caprine milk. Differences in terms of WSN were not 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 *et al.* (14) regarding caseinolytic activity of identical species. In terms of yeasts, the three strains studied proved that they affect proteolysis in statistically different ways. According to Roostita and Fleet (15), *Candida lipolytica* isolated from cheese exhibited a strong proteolytic action on UHT-treated milk, whereas *D. hansenii* demonstrated a weak proteolytic action. In terms of tentative starter, Scheffé's *F*-test does not unfold any statistically significant difference in terms of WSN between *E. faecium* and either *D. hansenii* or the tentative starter, so once again the action of *E. faecium* seems to override the action of *L. plantarum* in the tentative starter.

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 down to free amino acids (16). The results listed in Table 3 for FAA indicate that *Y. lipolytica* exhibits the highest peptidolytic activity, which is in accordance with Scheffé's *F*-test that indicated as statistically significant the differences between the values of FAA obtained with *Y. lipolytica* and all other strains studied. Differences that were not statistically significant in terms of total concentrations of FAA were found between both species of enterococci and between both species of lactobacilli; however, Requena *et al.* (14) reported differences in terms of aminopeptidase activity between *L. casei* and *L. plantarum*. *D. hansenii* led to values of total FAA that were not statistically different from those generated by *E. faecium* and *Lactobacillus* spp.

As expected, the concentration of NaCl influenced substantially the extent and rate of release of NPN, 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 for 65 d of ripening), except with regard to *Y. lipolytica* (which was extremely affected by NaCl content). The relatively higher contents of NPN in samples possessing lower NaCl levels can be attributed to a considerably higher activity of the microflora under those environmental conditions, which is consistent with data reported in Table 1. All the processing factors under scrutiny played significant roles, yet Sheffé *F*-test indicated that the differences between *E. faecium*, *E. faecalis*, *L. plantarum* and *D. hansenii* were not significant

in terms of NPN levels. Although it is not possible to directly compare results obtained *in vitro* using pure, single cultures with those obtained *in vivo* using cheese itself because a multitude of simultaneous and interactive phenomena occur during cheese ripening, *in vitro* results provide, nevertheless, trends and bases for comparison. Comparison between data obtained for *Picante* cheese manufactured with caprine and ovine milks by 83 d of ripening, i.e. 9 to 11 % NPN, ca. 8 %(w/w) NaCl and pH 5.3 (3), and data obtained for inoculated curdled milk by 65 d showed good approximation for the case of curdled caprine and ovine milks with 7% (w/v) NaCl inoculated with *E. faecium* (11 to 13 % NPN and pH 5.6 to 5.7), with *L. plantarum* (ca. 14 % NPN and pH 5.0), with *D. hansenii* (10 to 12 % NPN and pH 5.7) and with the tentative starter (ca. 12 % NPN and pH 5.5).

Table 5 encompasses the total concentration of free fatty acids (FFA) in caprine and ovine curdled milks, in the absence of NaCl, by 27 and 65 d of ripening. 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 produced by *Y. lipolytica*, on the one hand, and by all other strains under study, on the other, were statistically significant. Although to a much lesser degree, *E. faecium* and *L. plantarum* were also able to hydrolyse milk fat, followed by *E. faecalis*, *L. paracasei* and *D. hansenii*. According to Macedo and Malcata (8), milk fat was significantly hydrolysed by *E. faecium* but not by *L. paracasei* ssp. *paracasei*. In terms of contribution to the final organoleptic profile of *Picante* cheese, the action of *E. faecium* and *L. plantarum* seems potentially important since these strains were present in *Picante* cheese throughout the whole ripening period. Lipolytic activities, measured as fat acidity index, were strongly affected by the NaCl content (see 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 (15). Unusually high values were found by 27 and 65 d of ripening for the fat acidity index when *L. paracasei* was inoculated in salt-free curdled milk.

Table 5. Overall free fatty acid concentration (FAA) and fat acidity index of caprine and ovine curdled milk inoculated with strains previously isolated from *Picante* cheese, using incubation at 12 °C, in the absence of NaCl, or in the presence of various levels of NaCl, respectively.

		FFA ($mg/kg_{dry\ fat}$)			Acidity index of fat ($mg_{equiv\ KOH}/kg_{dry\ fat}$)					
	Milk	0 %(w/v) NaCl			0 %(w/v) NaCl		7 %(w/v) NaCl		14 %(w/v) NaCl	
Microorganism	type	27 d	65 d	SEM[a]	27 d	65 d	27 d	65 d	27 d	65 d
E. faecium	Caprine	266.0	346.6	1.0	1302.8	1980.9	579.4	623.4	249.8	467.6
	Ovine	248.3	315.2	4.1	1587.3	1827.0	746.3	747.4	639.5	681.1
E. faecalis	Caprine	163.4	188.7	2.1	877.3	1206.8	365.5	331.1	183.1	190.4
	Ovine	130.9	-[c]	-	882.2	-[c]	503.0	545.2	425.2	434.8
L. plantarum	Caprine	233.7	466.7	2.3	1627.6	3184.3	495.5	727.1	230.4	188.0
	Ovine	161.5	302.0	6.1	1099.8	2443.7	273.0	-[b]	229.8	258.2
L. paracasei	Caprine	121.1	191.6	1.5	8531.0	8973.3	556.3	727.4	219.2	226.1
	Ovine	92.9	146.2	0.6	6792.8	7647.4	480.1	614.0	333.8	334.0
D. hansenii	Caprine	93.4	119.6	0.6	379.8	459.8	74.9	319.6	166.6	324.2
	Ovine	147.7	187.9	4.3	538.8	1302.2	315.8	479.7	526.7	49.61
Y. lipolytica	Caprine	36489.1	44680.8	22.1	6364.6	10137.9	2094.2	2063.4	750.6	1381.0
	Ovine	26850.5	40622.7	23.9	6051.2	8872.0	2491.9	2050.5	253.5	675.6
C. laurentii	Caprine	250.6	388.9	0.9	1222.1	2368.7	696.2	251.2	243.8	228.7
	Ovine	-[b]	-[b]	-	-[b]	-[b]	468.8	525.1	341.8	230.2
tentative	Caprine	182.1	220.9	2.7	1081.5	1718.7	607.2	-[b]	274.1	354.6
starter	Ovine	187.9	234.2	2.7	1104.0	1897.4	550.1	750.2	445.6	428.5
Control	Caprine	182.4	211.7	1.9	272.4	434.2	-	-	-	-
	Ovine	140.8	177.0	3.2	262.7	373.8	-	-	-	-

[a] Standard error of the mean; [b] Contaminated samples; [c] No sample left for analysis. Values of FFA at 0 d for the caprine and ovine curdled milk control were 160.5 and 121.3 $mg/kg_{dry\ fat}$. Acidity index of fat at 0 d for the caprine and ovine curdled milk control were 191.0 and 198.4 $mg_{equiv\ KOH}/kg_{dry\ fat}$. No standard error of the mean was calculated for acidity index of fat since no replications were performed.

In conclusion, our experimental results produced indicate that a potential starter for *Picante* cheese should include, besides *L. plantarum*, one species of enterococci (*E. faecium* seems the more appropriate) and one species of yeast (*D. hansenii* and/or *Y. lipolytica*). Lee and Lim (17, 18) studied the inclusion of *D. hansenii* and *Candida lipolytica* in a cheese starter, whereas Deiana *et al.* (19) manufactured *Pecorino* cheese with a starter which included *D. hansenii,* and both groups of researchers reported that yeasts developed well in cheese and promoted faster proteolysis. Although relevant in themselves, the conclusions of this study should be extrapolated with caution to practical cheesemaking because the curdled milk system is somewhat different from a true cheese system, and because the action of each selected strain was studied in an dependent manner (except for the tentative starter) when in cheese they operate in a conjugate fashion.

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