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How three adventitious lactic acid bacteria affect proteolysis and organic acid production in model Portuguese cheeses manufactured from several milk sources and two alternative coagulants

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ABSTRACT

Model cheeses were manufactured according to a full factorial experimental design to help shed light on the individual and combined roles played by 3 native lactic acid bacteria (*Lactococcus lactis* ssp. *lactis*, *Lactobacillus brevis*, and *Lactobacillus plantarum*) upon proteolysis and organic acid evolution in cheese. The model cheeses were manufactured according to a generally representative Portuguese artisanal protocol, but the (ubiquitous) adventitious microflora in the cheesemaking milk were removed via sterilization before manufacture; therefore, the specific effects of only those lactic acid bacteria selected were monitored. In addition, 2 types of coagulant (animal and plant) and 3 types of cheesemaking milk (cow, sheep, and goat) were assessed to determine their influence on the final characteristics of the model cheeses. The nature of the coagulant appeared to be essential during the first stage of proteolysis as expected, whereas the contribution of those bacteria to the pools of total free AA and organic acids was crucial afterward. This was especially so in terms of the differences observed in the metabolisms of lactic acid (in the case of *Lactococcus* spp.) as well as acetic and citric acids (in the case of *Lactobacillus* spp.).

Key words: *Lactococcus*, *Lactobacillus*, sheep milk, plant and animal coagulant

INTRODUCTION

Most Portuguese traditional cheeses bearing a Protected Denomination of Origin status are manufactured from raw whole sheep or goat milks or mixtures thereof. This permits their indigenous microflora, mainly lactic acid bacteria (**LAB**), to play a role during cheesemaking, mainly during ripening. However, this is also a major cause of variability in the organoleptic and overall quality of the final products because several poorly understood (and thus difficult to control) biochemical changes brought about by microorganisms occur in the cheese matrix. Therefore, some degree of standardization of the manufacturing practices is urged; this goal will eventually require cheesemakers to choose well-defined, specific starter cultures based on their established technological performance.

Addition of tailor-made starter cultures contributes to a higher uniformity at all stages of manufacture and ripening. The primary roles of starter cultures are to convert lactose to lactic acid, to break caseins down into medium and small peptides (and to eventually break these down into free AA), and to hydrolyze milk fat, leading to release of free fatty acids (Fox and Wallace, 1997). All of these functions contribute to the unique characteristics of traditional cheeses. Although some attention has been paid to the process of transformation of cheese curds to distinctively flavored cheeses, the ripening process of most traditional Portuguese cheeses has not yet been fully elucidated. In fact, their intrinsic and relevant variability often hampers statistical significance of the experimental data generated in loco (Macedo and Malcata, 1997a,b; Macedo et al., 1997; Tavaria and Malcata, 1998, 2000; Dahl et al., 2000; Tavaria et al., 2003).

The biochemical reactions that occur in a typical cheese matrix include lipolysis, glycolysis, and proteolysis (Fox, 1993), and the enzymes involved therein, especially those that are active in proteolysis, play a crucial role upon the final textural and sensory characteristics of each type of cheese. Proteases and peptidases are present in the coagulant or are otherwise released by microorganisms upon their lysis, and the activities thereof depend on the environmental conditions prevailing in the curd (i.e., water activity, pH, mineral contents, and redox potential), as well as on the conditions provided throughout ripening (i.e., temperature, level and mode of salt addition, and nature of the secondary microflora added or allowed to grow; Law, 1984).

The objective of this study was to characterize in depth the performance of 3 adventitious LAB via use of a model system that essentially mimics traditional

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cheesemaking practices so as to better understand proteolysis and production of organic acids in Portuguese traditional cheeses. Toward this goal, model cheeses were independently produced from sterilized sheep, goat, and cow milks using either animal or plant coagulant coupled with deliberate addition of either one or both adventitious LAB strains. These have been found to be ubiquitous in the bulk of Portuguese traditional cheeses, so their characterization (either alone or as a coculture) was in order. Glycolysis and proteolysis were monitored throughout ripening and correlated with intrinsic and extrinsic parameters that characterize cheesemaking and ripening. This research represents a considerable advance regarding previous work in the field (Pereira et al., 2008a), which pertained to cow milk solely (and thus could not be used to parallel cheesemaking of most traditional Portuguese cheeses) and which did not consider the role of adventitious strains of *Lactobacillus plantarum*.

MATERIALS AND METHODS

Cheese Manufacture

Model cheeses were manufactured from autoclaved (110°C for 10 min) cow, sheep, or goat milk, as appropriate. Thirty-six 2-L batches were thus prepared, according to a factorial design encompassing those 3 types of milk and 2 types of coagulant [from a regular animal source (i.e., an aqueous extract of calf abomasa, supplied by Lusocoalho, Montes da Senhora, Portugal) or from a commercial plant source (i.e., an aqueous extract of dried flowers of *Cynara cardunculus* L., supplied by Formulab, Maia, Portugal)] and 3 microorganisms [i.e., wild strains of *Lactococcus lactis* ssp. *lactis* (LMG S 19870), *Lactobacillus brevis* (LMG 6906), and *Lactobacillus plantarum* (LMG S 19557)]. These strains had been previously isolated from Portuguese traditional cheeses and duly deposited in the Laboratorium voor Microbiologie en Microbiele Genetica, Rijksuniversiteit (Gent, Belgium). Such microorganisms were added either as plain cultures or as 1:1 cocultures of the *Lactococcus* strain and one of the *Lactobacillus* strains, and absence of inoculum was used as control; this added up to 6 inocula. Each set of experimental conditions was made available in duplicate.

Starter cultures were added to the aforementioned 2 L of autoclaved milk at a 1% (vol/vol) level. As for the remaining process, a typical traditional manufacture protocol was followed (Macedo et al., 1993; Freitas and Malcata, 2000) as much as possible, except that the entire process was carried out in a sterile flow chamber using sterile tools and equipment. Sterile common table salt was added to bulk milk at a rate of 20 g/L, and inoculation was followed by addition of a solution (Orange Scientific, Belgium) of animal or plant coagulant (0.6 or 4 mL, respectively), sterilized by filtration through a 0.22-μm filter, as appropriate. The resulting mixture was distributed into 200-mL sterile flasks, each with a diameter of 6 cm, and incubated for 1 h at 30°C to bring about clotting. Perpendicular vertical cuts were then made in the curd, and another incubation period of 90 min followed. Finally, the curd was pressed to help in removing residual whey. Cheeses were kept for up to 60 d at 8°C under controlled air humidity (85%).

Physicochemical Characterization

Model cheeses were assayed for moisture throughout ripening by oven drying (IDF, 1982). The pH of cheeses was measured directly with a pH meter (Micro pH 2002, Crison, Barcelona, Spain). Fat, salt, and total protein contents were determined by Fourier-transform infrared spectroscopy using a LactoScope Advanced FTIR (Delta Instruments, Drachten, the Netherlands) after previous calibration.

Microbial Enumeration

Model cheeses were sampled at 0, 7, 14, 30, 45, and 60 d. *Lactobacillus brevis* and *L. plantarum* were enumerated on Rogosa agar (Merck, Whitehouse Station, NJ), and *Lc. lactis* ssp. *lactis* were enumerated on M17 agar (Merck). In the case of the mixed culture, a defined differential medium (which produces colonies with different colors for lactobacilli and lactococci) was used (i.e., lactose sulfite agar, which is often used to enumerate bacteria in yogurt; Imprensa Nacional—Casa da Moeda, 1998). Plates were incubated aerobically at 30°C for 48 h; in the case of Rogosa agar, they were incubated at the same temperature for 5 d.

Glycolysis Assessment

Cheese samples were taken at 0, 7, 14, 30, 45, and 60 d of ripening. A 2-g aliquot was added to 10 mL of 13 mmol/L sulfuric acid and homogenized with an Ultra-Turrax homogenizer (LaboControle, Linda-a-Velha, Portugal) for 3 min at 16,128 × *g*. The mixture was centrifuged (Universal 32R, Hettich, Kirchlengern, Germany) at 3,584 × *g* and 4°C for 10 min and then filtered through no. 42 filter paper (Whatman, Kent, UK). Immediately before HPLC analysis, samples were sterilized through 0.22-μm filters (Orange Scientific). The HPLC system (Lachrom, Merck Hitachi, Darmstadt, Germany) was composed of an ion exchange aminex HPX 87H column (300 × 7.8 mm), which was maintained at 65°C, and 2 detectors (refractive index

Table 1. Variation of gross composition in model cheeses throughout ripening

Milk source	Ripening time (d)	Moisture (%)	Total protein per DM (%)	Fat per DM (%)	Salt per DM (%)	pH
Goat	0	86.2 ± 0.7[a]	33.5 ± 2.2[a]	20.1 ± 6.2[a]	22.5 ± 4.3[a]	6.18 ± 0.03[a]
	30	82.3 ± 2.9[b]	36.2 ± 1.6[b]	24.5 ± 4.3[a]	18.5 ± 2.4[b]	4.64 ± 0.64[b]
	60	82.3 ± 2.8[b]	36.6 ± 3.1[b]	23.2 ± 6.5[a]	18.7 ± 3.2[b]	4.36 ± 0.47[c]
Sheep	0	76.5 ± 1.2[a]	37.9 ± 1.9[a]	43.7 ± 2.8[a]	7.7 ± 2.1[a]	6.15 ± 0.06[a]
	30	73.7 ± 2.7[b]	38.5 ± 2.2[a]	38.7 ± 4.0[b]	9.7 ± 1.5[b]	4.97 ± 0.71[b]
	60	71.5 ± 4.4[c]	38.3 ± 2.0[a]	37.5 ± 3.7[b]	10.4 ± 1.2[c]	4.83 ± 0.68[b]
Cow	0	86.2 ± 1.5[a]	35.3 ± 2.6[a]	22.0 ± 6.2[a]	20.4 ± 4.5[a]	5.95 ± 0.04[a]
	30	82.6 ± 1.6[b]	35.7 ± 3.4[a]	28.8 ± 6.6[b]	15.3 ± 3.3[b]	4.61 ± 0.66[b]
	60	82.8 ± 1.1[b]	35.5 ± 2.3[a]	23.0 ± 7.3[a]	17.9 ± 4.1[b]	4.49 ± 0.51[b]

[a–c]Means (considering 12 batches produced per type of milk; n = 24) within the same column for each type of milk without a common superscript are significantly different ($P < 0.05$).

and spectrophotometry, 220 nm) in series. The mobile phase used was 13 mmol/L sulfuric acid, pumped at a flow-rate of 0.8 mL/min; the running time was 30 min, and the injection volume was 50 μL.

Proteolysis Assessment

Cheese samples were taken at 0, 7, 14, 30, 45, and 60 d of ripening. Water-soluble nitrogen (**WSN**) and nitrogen soluble in 12% (wt/vol) trichloroacetic acid were determined as described by Kuchroo and Fox (1982), except that a Sorvall Omni Mixer (Dupont, TX) was used for homogenization and the supernatant obtained was filtered through no. 42 filter paper. Nitrogen soluble in 5% (wt/vol) phosphotungstic acid was also determined by Kjeldahl according to Stadhouders (1960), except that extracts were prepared as described above.

Statistical Analyses

The averages and standard deviations were calculated for each type of experimental results (encompassing gross composition and microbiology, as well as organic compound and proteolysis profiles) at the various ripening times and were analyzed using SPSS software (v. 16.0.0, SPSS, Chicago, IL) to assess statistically significant differences (at the 5% level) among the 6 microbial inocula, the 2 types of coagulant, and the 3 types of milk. Because the original experimental data failed to meet the normal distribution assumption, nonparametric tests were applied to each set of experimental data.

RESULTS AND DISCUSSION

Physicochemical Characterization

The moisture, pH values, and total protein, fat, and salt contents throughout ripening (0, 30, and 60 d) of model cheeses manufactured from each of the 3 milk sources and the 2 types of coagulants are shown in Table 1. The evolution in moisture content and pH in sheep milk model cheeses differed ($P < 0.05$) from those manufactured with cow or goat milks, which revealed trends similar to each other. This phenomenon could be attributed to the lower buffering capacity of cow and goat milks, derived from their lower protein contents (Kervina et al., 1981; Simos et al., 1991). As expected,

Table 2. Variation of microbiological composition [log (cfu/g)] in model cheeses throughout ripening

		Ripening time (d)					
Reference strain	Type of culture[1]	0	7	14	30	45	60
Lactococcus lactis ssp. *lactis*	Alone	6.95 ± 0.30[a]	9.19 ± 0.23[a]	8.87 ± 0.75[a]	7.87 ± 0.66[a]	7.17 ± 0.91[a]	6.80 ± 1.00[a]
	+ *L. brevis*	6.02 ± 0.54[b]	9.18 ± 0.29[a]	8.77 ± 0.34[a]	7.63 ± 0.63[a]	6.75 ± 0.35[a]	5.91 ± 0.48[b]
	+ *L. plantarum*	6.06 ± 0.46[b]	9.09 ± 0.27[a]	8.78 ± 0.52[a]	7.48 ± 0.49[a]	6.27 ± 0.74[a]	5.14 ± 1.01[c]
Lactobacillus brevis	Alone	5.99 ± 0.30[b]	8.29 ± 0.16[b]	8.34 ± 0.23[b]	8.38 ± 0.25[b]	8.29 ± 0.23[b]	8.24 ± 0.34[d]
	+ *Lc. lactis* ssp. *lactis*	5.64 ± 0.32[b]	8.09 ± 0.45[b]	8.24 ± 0.36[b]	8.14 ± 0.38[b]	7.97 ± 0.43[b]	8.01 ± 0.22[d]
Lactobacillus plantarum	Alone	5.87 ± 0.39[b]	8.35 ± 0.23[b]	8.61 ± 0.33[b]	8.68 ± 0.26[c]	8.49 ± 0.41[c]	8.47 ± 0.37[d]
	+ *Lc. lactis* ssp. *lactis*	5.57 ± 0.33[b]	8.12 ± 0.31[b]	8.26 ± 0.34[b]	8.14 ± 0.58[b]	8.13 ± 0.24[b]	8.17 ± 0.29[d]

[a–d]Means (considering the 3 milks and the 2 coagulants; n = 12) within the same column without a common superscript are significantly different ($P < 0.05$).

[1]The two sets of rows, 2 and 5 and 3 and 7, refer to the same starter mixture but list viable data of only the reference strain indicated; e.g., row 2 refers to viable numbers of *Lc. lactis* ssp. *lactis*, and row 5 refers to viable numbers of *L. brevis* in the starter mixture *Lc. lactis* ssp. *lactis* + *L. brevis*.

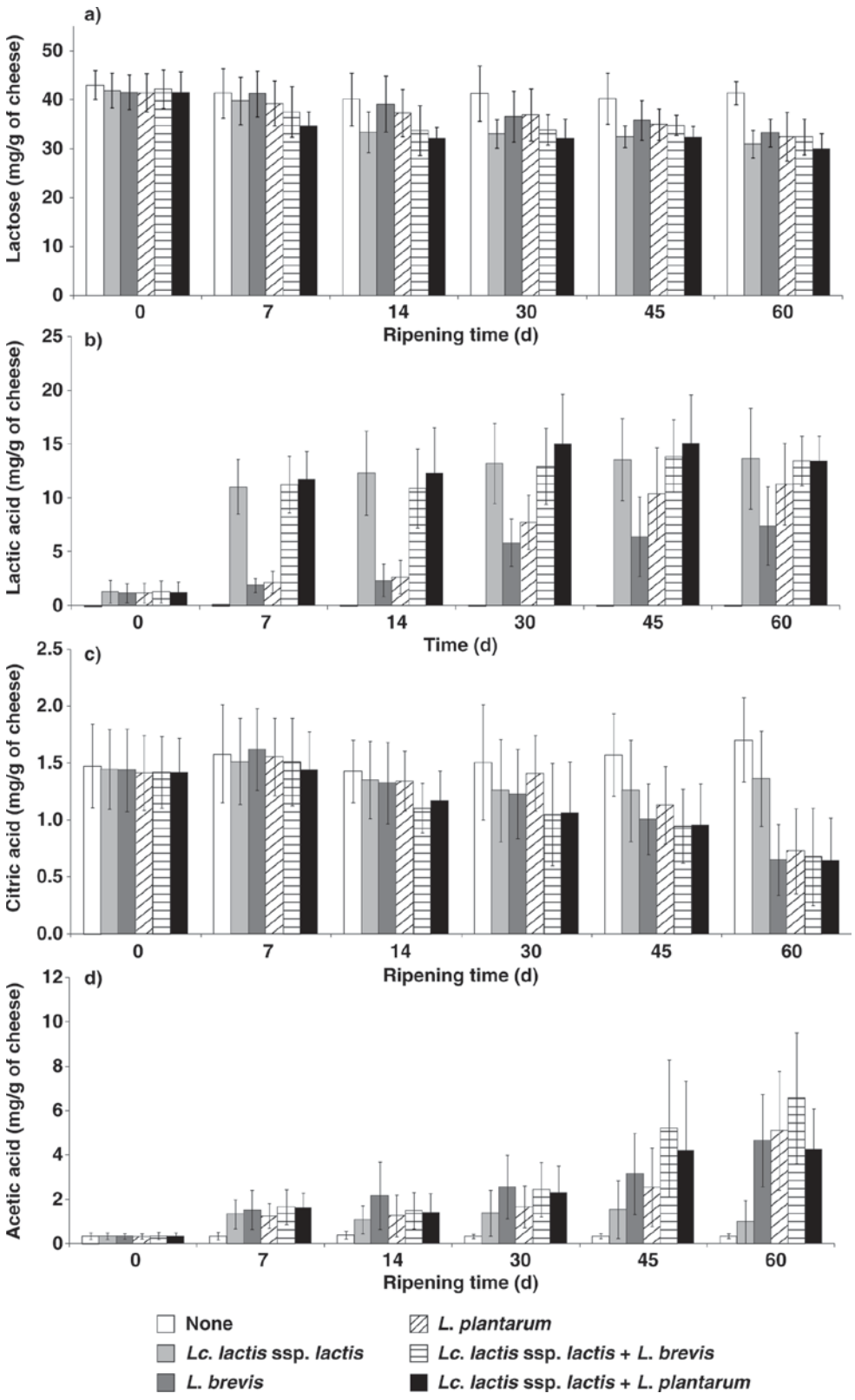

Figure 1. Evolution of a) lactose and b) lactic, c) citric, and d) acetic acid levels in model cheeses throughout ripening according to type of inoculum (no inoculum, *Lactococcus lactis* ssp. *lactis*, *Lactobacillus brevis*, *Lactobacillus plantarum*, mixture of *Lactococcus lactis* ssp. *lactis* with *Lactobacillus brevis,* and mixture of *Lactococcus lactis* ssp. *lactis* with *Lactobacillus plantarum*). Error bars represent standard deviations of the means.

the pH was lowest by the end of ripening, irrespective of type of milk. The largest pH decrease was observed in cheeses manufactured with goat milk, although a sharper decrease occurred during the first rather than the second half of the 60-d ripening period.

The total protein content remained essentially constant throughout the ripening period considered except in model cheeses manufactured from goat milk. Similarly, the fat and salt contents underwent considerable variation depending on the type of cheesemaking milk. Mallatou et al. (1994) and Pappas et al. (1994) reported that the moisture content of cheeses increased as the casein:fat ratio in cheese milk increased. This is in agreement with our observations: the higher protein:fat ratios of goat and cow model cheeses were observed at the higher moisture contents (Table 1).

Salt content variations were likely related to the whey lost by model cheeses throughout ripening, particularly in the case of cow and goat model cheeses (which lost whey at different rates throughout ripening). Conversely, the salt in DM became apparently more concentrated in sheep model cheeses, probably because some fat was also lost suspended in whey (as is often observed, owing to the higher concentration of fat in sheep milk than in cow and goat milk).

Although physicochemical data seemed somewhat erratic throughout the ripening period, samples from 2 independent cheese replicates were assayed at each sampling time (i.e., a given cheese was sampled only once throughout the entire experimental program, although each pair of cheese replicates was originated from a common milk batch for the same type of milk, coagulant, and inoculum). Note that the analytical data pertaining to each cheese were averaged so as to generate a single datum point.

Glycolysis Assessment

The organic acid quantification and the associated (qualitative) profile can be used for cheese classification in terms of ripening extent (Akalin et al., 2002) and are often regarded as microbial metabolism indicators. Hence, in attempts to more accurately ascertain the effect of the LAB strains considered, the evolution in viable numbers of individual microorganisms in model cheeses is presented in Table 2. Note that rows 2 and 5 refer to the same starter mixture but convey viable data of only the reference strain indicated therein; the same is true for rows 3 and 7.

The evolution of several organic compounds (lactose and lactic, citric, and acetic acids) throughout ripening for the various types of inocula is shown in Figure 1. The data presented should not be analyzed as absolute values, but regarded in a relative fashion to pinpoint

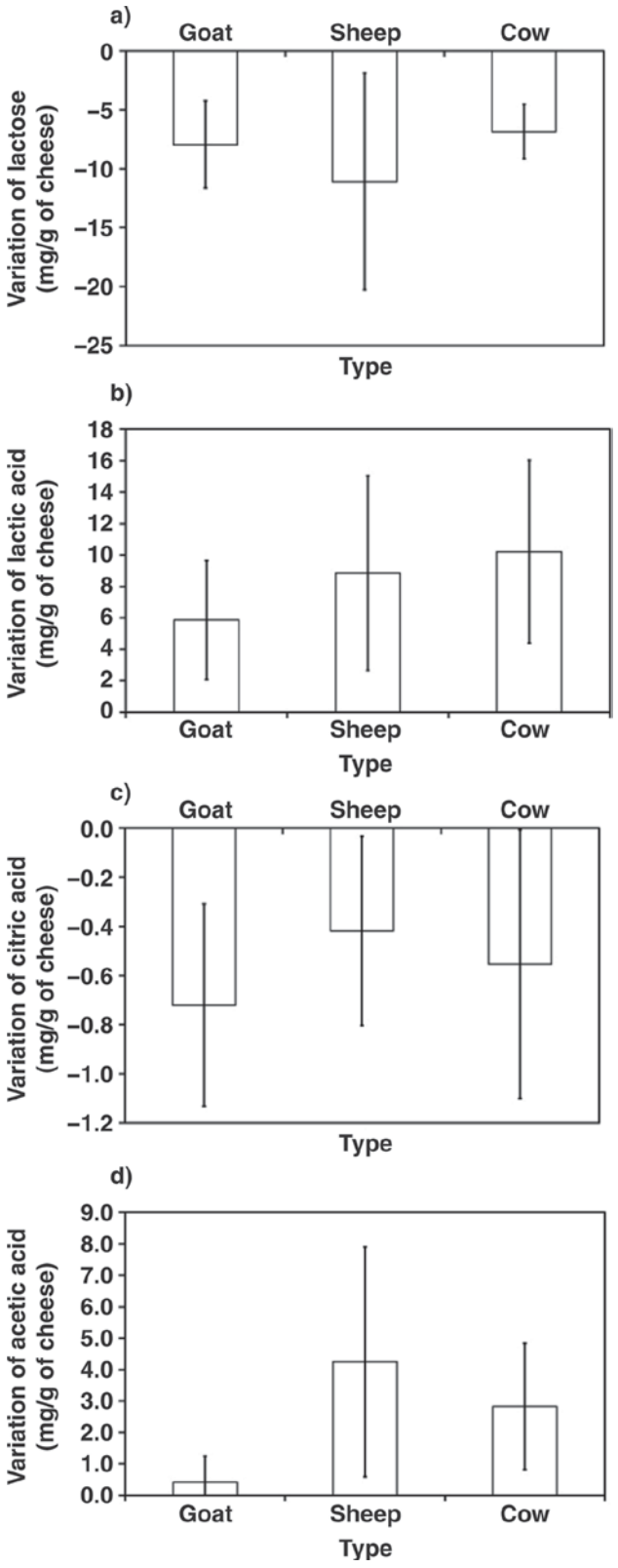

Figure 2. Variation of a) lactose and b) lactic, c) citric, and d) acetic acid levels in model cheeses throughout ripening according to type of cheesemaking milk. Error bars represent standard deviations of the means.

trends and observe the influence of the various factors tested. Moreover, one cannot expect the values for the parameters assayed to be identical to those found in an actual cheese matrix because the initial microbial charge encompassed only 2 species of LAB, the milk used was subjected to preliminary thermal treatment, and the model cheeses were ripened in sealed flasks.

As expected, the 0 d levels of the aforementioned organic acids were similar. These values remained constant throughout ripening in control cheeses, whereas the 3 LAB strains studied (*Lc. lactis* ssp. *lactis*, *L. brevis,* and *L. plantarum*) produced distinct metabolic patterns when incorporated in the model cheese matrix. In regard to lactose consumption and consequent lactic acid formation, model cheeses inoculated with *Lc. lactis* ssp. *lactis* (either plain or coculture) exhibited the largest variations, which became significant ($P < 0.05$) right after 7 d of ripening; this is in agreement with the concomitant model cheese acidification until 45 d (data not shown). Such an observation can tentatively be explained by the homofermentative character of *Lc. lactis* ssp. *lactis* (Drinan et al., 1976), which converts lactose directly into lactic acid, thus making the rate of lactic acid formation higher than that via *Lactobacillus*-mediated fermentation. In fact, metabolism by single strains of lactobacilli was slower; production of lactic acid was gradual, but never achieved the levels obtained upon inoculation with *Lc. lactis* ssp. *lactis* ($P < 0.05$). This piece of evidence is consistent with the exponential growth observed for every inoculum, an approximate 3 log-cycle increase during the first 7 d of ripening, when their maximum viable numbers were reached (Table 2).

Inspection of Figure 1 shows a low consumption rate of citric acid in model cheeses inoculated with *Lactobacillus* strains (plain or coculture) and a consequently slow production of acetic acid. Only the control and the model cheeses inoculated with *Lc. lactis* ssp. *lactis* did not undergo depletion; the former lacked inoculum, and the latter was homofermentative. Because of heterofermentation, model cheeses inoculated with lactobacilli were differentiated in terms of production of acetic acid, owing to utilization of lactose and metabolism of citrate (Califano and Bevilacqua, 2000). Conversely, *Lc. lactis* ssp. *lactis* was associated with a slight increase in acetic acid levels within the first 7 d, which remained low throughout the remainder of ripening.

The variation in the concentrations of lactose and of each organic acid with ripening time and for each type of milk is shown in Figure 2. These variations were calculated as the difference between the mean values of all experimental cheeses (i.e., cheese produced via 2 coagulants × 6 inocula × 2 replicates) at 60 d and at 0 d for each type of milk.

The evolution in lactose (Figure 2a) and citric acid (Figure 2c) levels were essentially similar among the 3 types of milk used. However, differences ($P < 0.05$) were found between sheep and cow milks in terms of lactose depletion and between sheep and goat milks in terms of citric acid variation. In regard to lactic and acetic acids production levels, cheeses manufactured with goat milk led to the lowest levels ($P < 0.05$) of both acids, especially of the latter; cow and sheep milks presented levels similar to each other.

The combined data of Figures 1 and 2 and Table 2 imply that the source of milk itself did not significantly affect the viable numbers of each strain. The initial lactose content, which ranged from 40 to 46 mg/g for the 3 types of milk tested, may have accounted for this implication given the intrinsic variability of the data produced. The differences in the organic acid profile throughout ripening were thus a result of the different metabolic rates of lactose uptake by the strains in the distinct media rather than a result of their growth rates.

The amounts, as recorded throughout ripening, of the various organic compounds assayed were lower than those typically reported in the literature as pertaining to traditional cheeses. Strict aseptic conditions prevented the model cheeses from contacting the environment; because sterile, closed flasks were used, the development of the typical final characteristics in cheese was hindered. Furthermore, the pool of organic compounds produced in our experimental cheeses developed from milks that were previously subjected to thermal treatment and thus deprived of their full load of adventitious microorganisms, which are known to mediate the catabolic routes of such organic acids as lactic and citric acids (Grappin and Beuvier, 1997; McSweeney and Sousa, 2000).

Proteolysis Assessment

Based on the experimental data generated, 3 proteolytic indices were calculated as ratios between each soluble nitrogen fraction to total nitrogen (**TN**): 1) ripening extension index, WSN:TN (a measure of proteolytic activity); 2) ripening depth index, trichloroacetic acid:TN (a measure of aminopeptidase activity; Furtado and Partridge, 1988); and 3) free AA index, phosphotungstic acid:TN (Aston et al., 1983). As emphasized earlier, proteolytic indices should be considered just for comparative purposes because the microbial charge was restricted to only 2 species of wild LAB, the milk used was autoclaved, and the model cheeses were ripened in sealed flasks.

The evolution of the ripening extension index over time is depicted in Figure 3a–c. As expected, the WSN

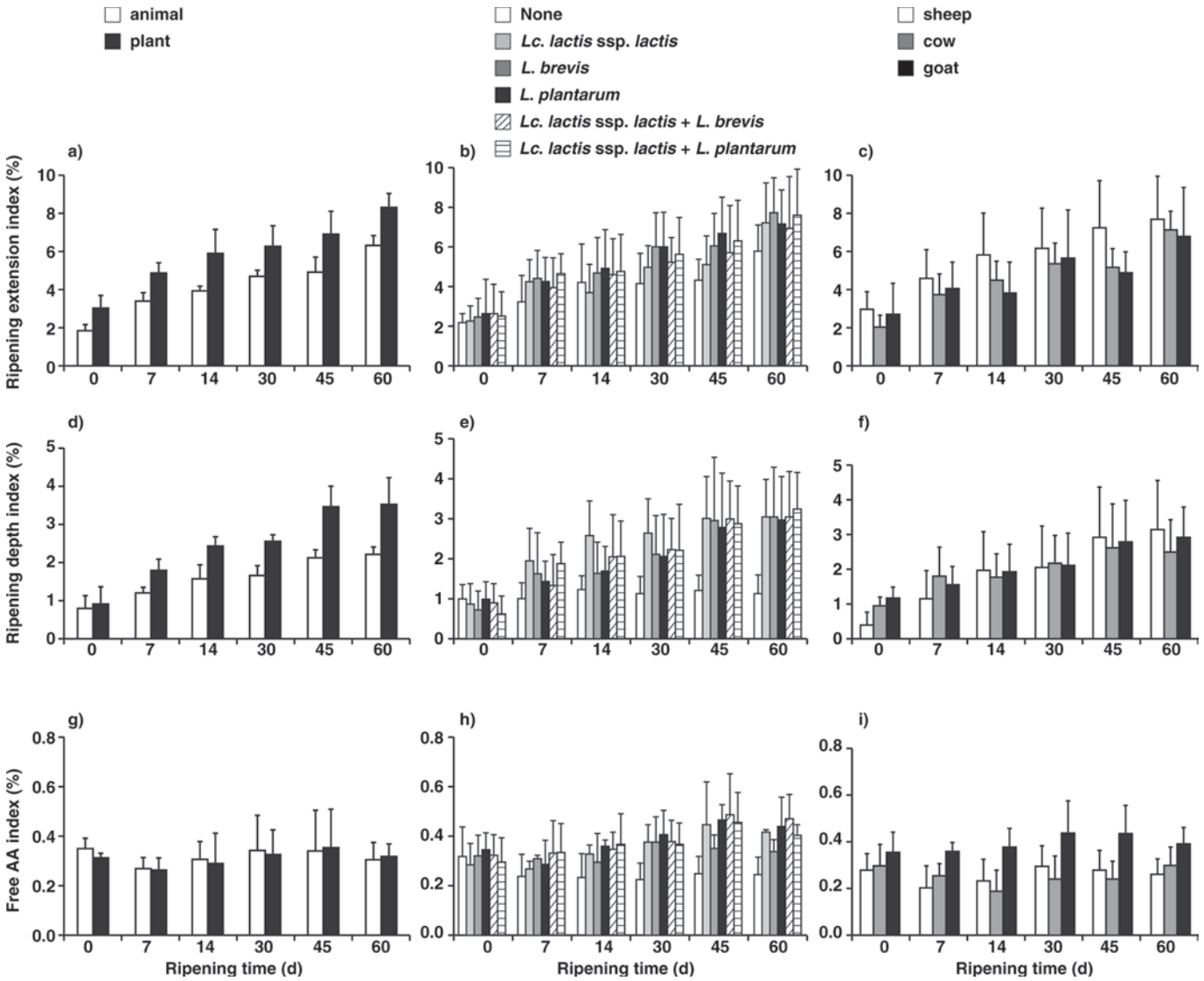

Figure 3. Variation of proteolysis indices in model cheeses throughout ripening. Panels a–c: ripening extension index (water-soluble nitrogen:total nitrogen); panels d–f: ripening depth index (trichloroacetic acid soluble nitrogen:total nitrogen); panels g–i: free AA index (phosphotungstic acid soluble nitrogen:total nitrogen). Panels a, d, g show the effect of animal coagulant and plant coagulant; panels b, e, h show the effect of no inoculum, *Lactococcus lactis* ssp. *lactis*, *Lactobacillus brevis*, *Lactobacillus plantarum*, mixture of *Lactococcus lactis* ssp. *lactis* with *Lactobacillus brevis,* and mixture of *Lactococcus lactis* ssp. *lactis* with *Lactobacillus plantarum*; and panels c, f, i show the effect of sheep milk, cow milk, and goat milk. Error bars represent standard deviations of the means.

content increased over time ($P < 0.01$). Although lower than those for actual cheeses (Tavaria et al., 2003; Ong et al., 2007), the trends encompassing the WSN:TN values were consistent with those reported elsewhere (Pereira et al., 2008b) that also encompassed model cheeses manufactured from autoclaved milk. The effect of coagulant was crucial during this stage of proteolysis because its enzymes were able to hydrolyze casein into water-soluble peptides of lower molecular weight. Bacterial enzymes have accordingly little effect at this stage, as shown in Figure 3b; inoculated model cheeses did not differ from the control cheeses.

In our study, model cheeses manufactured with plant coagulant underwent higher proteolytic degradation than their animal coagulant counterparts (Figure 3a), which is in agreement with the literature (Fernández-Salguero and Sanjuán, 1999). Plant coagulant appeared to be the most important factor upon increase of WSN:TN throughout ripening ($P < 0.01$). Coagulant proteases retained in the curd following manufacture continued to degrade casein as ripening time elapsed.

During the aforementioned primary proteolysis, a few differences in the extent of release of water-soluble peptides were apparent according to type of milk used

(Figure 3c). As observed elsewhere (Sousa and Malcata, 1997a), cheeses from cow and goat milks did not differ throughout the ripening period, whereas sheep milk appeared, at least at some intermediate moments of ripening, to have higher levels of casein breakdown into peptides of lower molecular weight ($P < 0.01$). The higher protein content of this type of milk, as well as a (putatively) greater affinity of plant coagulant for sheep caseins (Fernández-Salguero and Sanjuán, 1999), may at least partially account for this realization.

The evolution of the ripening depth index is illustrated in Figure 3d–f. This index responds to small peptides with chain lengths of 2 to 20 AA residues (Macedo and Malcata, 1997a). The increase ($P < 0.01$), with ripening time, of the medium and small peptide levels is the result of microbial enzymes acting upon large peptides that were previously released by coagulant enzymes. In our model cheeses, the effect of inoculum (Figure 3e) appeared to be the driving force for release of TCA-soluble peptides. This behavior was validated throughout ripening time via monitoring of the evolution of control model cheeses (i.e., those without addition of inoculum) in which only the enzymes pertaining to coagulant were present (Figure 3e).

The various sets of model cheeses manufactured yielded similar ripening depths at early stages of ripening, yet the inoculated cheeses became higher in that index than the controls during the second half of the ripening period ($P < 0.01$). The synergisms between coagulant and bacterial enzyme action toward proteolysis may account for this observation; coagulant is responsible for release of large, water-soluble peptides, which will be further hydrolyzed by bacteria and enzymes released thereby. At this level of proteolysis, no differences were detected among the various inocula tested. However, it should be emphasized that the ripening depth index, being roughly half of the ripening extension index, is likely explained by the lower affinity of coagulant enzymes for short peptides (Macedo and Malcata, 1997a). From early stages of ripening on, model cheeses manufactured with plant coagulant showed higher values ($P < 0.01$) for the ripening depth index than their animal coagulant counterparts; furthermore, such a difference became more and more distinct as ripening time elapsed, especially between 45 and 60 d (Figure 3d). Following the trend previously observed for the ripening extension index, model cheeses manufactured with sheep milk were significantly different from those manufactured with cow and goat milks ($P < 0.01$) during the first week of storage (Figure 3f) concerning the release of smaller peptides.

Fractionation with 5% PTA is able to extract only peptides with low molecular weight (i.e., <600 Da) and free AA (Sousa and Malcata, 1996). The free AA index progression throughout the 60-d ripening period is depicted in Figure 3g–i; a significant increase ($P < 0.05$) in the free AA levels over time is apparent. Bacterial enzymes had a particularly intense involvement during this stage of proteolysis (coagulant enzymes cannot hydrolyze peptides with low molecular weight into free AA) and especially so in the second half of the ripening period (30–60 d). On the other hand, the strains inoculated had a proteinase–peptidase system that is able to hydrolyze peptides into free AA, a realization that can also be validated by the gradual increase in PTA-soluble nitrogen in the inoculated model cheeses as opposed to the essentially steady values found in control cheeses. This difference became significant ($P < 0.05$) only by 30 d of ripening; however, the relatively slow appearance of free AA may also be attributable to their consumption as a nitrogen source during exponential growth of the inoculated LAB (Table 2) as a consequence of the higher demand for nutrients during this period (Sousa and Malcata, 1997a).

No synergisms were detected for the mixtures of bacteria relative to their plain counterparts. *Lactobacillus* strains were not different from the *Lactococcus* strain concerning release of free AA, even though the viable numbers of *Lc. lactis* ssp. *lactis* (either inoculated alone or in coculture with a *Lactobacillus* strain) decreased gradually until the end of ripening, differing from the latter by 2 to 3 log cycles (Table 2). This could indicate the occurrence of autolysis, which would result in the release of their intracellular enzymes and thus contribute to their differentiation in terms of free AA release. As expected (Macedo and Malcata, 1997a), the coagulant played no significant role in the evolution of this index (Figure 3g). Unlike the aforementioned 2 indices, model cheeses manufactured with goat milk exhibited higher ($P < 0.05$) AA levels throughout most of the ripening period; this type of evidence was also reported elsewhere (Sousa and Malcata, 1997b).

As mentioned previously, the microflora present in model cheeses were not as diverse as in actual cheeses because the microbial consortium was restricted to only 2 species of LAB that were inoculated in milk previously subject to thermal processing and allowed to ripen for only 60 d. Although it has been claimed (Swearingen et al., 2001) that native nonstarter LAB (i.e., lactobacilli) need more than 3 mo of ripening to significantly improve overall quality and consistency of cheeses, the average ripening period of traditional cheesemaking in Portugal does not typically go beyond 2 mo. As also referred to above, severe heating affects milk properties considerably via inactivation or activation of enzymes and partial denaturation of whey proteins and disruption of their interaction with casein (Buffa et al., 2004). Nevertheless, the qualitative and relative quantitative

profiles are close to those typically found in actual cheeses, so the significance of our approach toward elucidation of the biochemical phenomena throughout ripening is apparent.

CONCLUSIONS

The originality of this study relies on the dedicated and reproducible application of a model cheese system to study the effect of various milk sources and wild LAB strains, besides types of coagulant, under conditions that allow a fundamental analysis of traditional cheesemaking. Based on its intrinsic enhancement of release of nonprotein nitrogen, which is known to contribute to development of the desired final characteristics in actual cheeses, sheep milk appears to be the most promising for cheesemaking (as is usually accepted). The type of coagulant employed (*C. cardunculus* aqueous extracts) produces a unique effect upon primary proteolysis. The bacterial strains tested produce biochemical changes in the cheese matrix that will eventually contribute to the complexity of the final flavor of the actual cheeses. No synergism exists between strains regarding proteolytic features, yet glycolytic features are favored by coculturing *Lactococcus* with *Lactobacillus* strains.

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