Influence of bacterial dynamics upon the final characteristics of model Portuguese traditional cheeses

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A B S T R A C T

The microbiological profile in raw milk cheeses is typically characterized by a multitude of microbial groups, with interactions among them throughout ripening that are not fully understood to date. Incidence of undesired microorganisms in raw cheesemaking milk, as is the case of either spoilage or even pathogenic ones, is a common trait in Portuguese traditional cheeses. Hence, they will likely contribute to the physicochemical changes occurring therein and, consequently, to the characteristics of the final product. In order to gain insight into their role, model cheese systems, manufactured as far as possible according to artisanal practices (except that the initial microbial load and biodiversity were controlled), were experimentally tested. Single contaminants, or a consortium thereof, were inoculated at two levels in sterilized raw ewe's milk, and duly combined with inocula containing one or two lactic acid bacteria normally found in those traditional cheeses. The physicochemical composition, organic acid profile, and evolution of both protein breakdown and rheology were monitored throughout a 60 d-ripening period. Modifications brought about within the cheese matrix as a result of microbial metabolism, especially those arising from the interaction between lactic acid bacteria and unwanted microorganisms, included the enhanced release of peptides and free amino acids, which in turn led to higher viscoelastic moduli. The final model cheeses could be well discriminated, based on the impact of the various inocula considered upon the levels of organic acids. Conversely, proteolysis and viscoelastic properties appeared to be essentially independent of the initial microflora.

Introduction

One of the major problems associated with raw milk cheeses is the presence of undesired microorganisms throughout the manufacture and ripening processes. Use of raw milk, and thus absence of any standardizing thermal process, coupled with different milking and handling protocols, and thus variation in hygienic conditions prevailing in the farmhouses, lead to extensive and unpredictable variability. Portuguese cheeses, bearing Protected Denomination of Origin (PDO) status, are examples of such products. They are manufactured traditionally from whole raw milk and depend critically on their indigenous microflora for development of the final characteristics. The contribution of such microflora, coupled with that of milk composition, which depends on the local animals' diet and physiological condition, eventually account for the unique flavour and texture of those traditional cheeses.

Several microorganisms – including bacteria, yeasts and moulds, are present in cheese throughout ripening. Hence, they contribute to maturation, either directly via their metabolic activity, or indirectly via release of enzymes into the cheese matrix, after autolysis. Although most of the microflora of raw milk comprises lactic acid bacteria (LAB) – e.g. *Lactococcus* and *Lactobacillus* spp., passive inclusion of such undesired microorganisms as coliforms, *Staphylococcus* spp., Pseudomonads or even *Listeria* spp. raises potential public health hazards. Hence, they are of major concern towards microbiological safety of the final product (Almeida et al., 2007).

Although the aforementioned cheeses are consumed after a minimum ripening time of ca. 45 d at temperatures of ca. 10 °C (Macedo et al., 1993) – which are conditions lethal to most contaminantic microorganisms, their action while viable will eventually affect the final cheese features. Addition of a tailor-made starter culture may bring about favourable contributions to control growth of unwanted microorganisms. In particular, the action of LAB via initial fermentation of lactose and breakdown of proteins, or via more complex catabolic reactions later during ripening, is a well-known contributor to the organoleptic features perceived in the final cheese (Menéndez et al., 2000). Furthermore, yeasts have for long been recognized to play an important role in Serra da Estrela cheese (Macedo et al., 1993; Freitas and Malcata, 2000). On the other hand, much of the existing knowledge is derived from studies

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encompassing pure cultures grown and tested in synthetic media, but more specific attention is to be paid to the *in-situ* environment and the wild bacterial strains. In addition, it should be realized that actual cheese matrices do simultaneously harbour microbial cells in a diversity of physiological states, viz. growing, non-growing, dead and autolyzed (Fleet, 1999).

Therefore, this research effort was aimed at filling the aforementioned gap of knowledge, by shedding light on the role of a group of typical raw milk contaminants – viz. *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Listeria innocua*, in the presence of a putative starter culture composed by wild strains of LAB that are normally found in raw milk cheeses (viz. *Lactococcus lactis*, *Lactobacillus brevis* and *Lactobacillus plantarum*). Their combined effects upon the physicochemical characteristics developed throughout cheese ripening were elucidated via development of laboratory-scale model cheeses, which were manufactured under aseptic conditions so as to accurately control the relevant processing parameters.

Materials and methods

Experimental design

Eighty-eight batches, of 1 L each, were prepared and duly autoclaved at 110 °C for 10 min. Putative contaminants – *S. aureus* ATCC 6538, *P. aeruginosa* (obtained from our own collection), *L. innocua* NCTC 11846 and *E. coli* ATCC 8739, were combined with specific LAB strains – *L. brevis* LMG 6906, *L. plantarum* LMG S 19557 and *L. lactis* LMG S 19870 (all from the collection held by Laboratorium voor Microbiologie en Microbiele Genetica, Rijksuniversiteit, Gent, Belgium), at two viable levels in model cheeses, as detailed in the flow chart depicted in Fig. 1. Each contaminant bacterium was first studied as a single culture, and later in a consortium of contaminants that was combined with either individual or mixed LAB, to mimic as far as possible the microbial environment prevailing in cheese. The contaminant bacteria were chosen so as to parallel the major groups that normally contaminate traditional cheeses. *S. aureus* represents the enterotoxin-producing staphylococci (Gaya et al., 1988), *E. coli* the *Enterobacteriaceae* associated with poor sanitary conditions, *P. aeruginosa* the psychrotrophic bacteria prevailing throughout refrigeration of raw ewe's milk, and *L. innocua* the *Listeria* group at large. Furthermore, the two distinct initial viable levels were

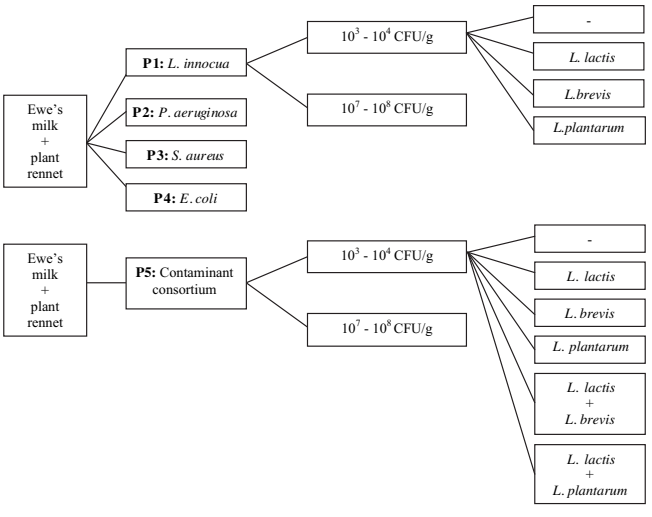

Fig. 1. Experimental layout; P1–P5: model cheeses manufactured in different days and from different milk batches.

intended to simulate an extreme scenario characterized by a heavy microbial contamination, and a more reasonable one characterized by only a light contamination. Each cheese batch was produced twice for each set of conditions. When appropriate, the LAB bacterial culture was added at 1%(v/v), in order to attain a log of 6–8 of viable numbers by 0 d in the model cheeses.

Model cheese manufacture and ripening

Model cheeses were manufactured with ovine milk provided by ANCOSE – Associação Nacional de Criadores de Ovinos Serra da Estrela (Oliveira do Hospital, Portugal), and clotted using a commercial plant rennet previously obtained from the dried flowers of the wild thistle, *Cynara cardunculus* L. (Formulab, Portugal). Each contaminant inoculum was used at a time in 5 different production days, thus encompassing 5 distinct cheese batches.

Cheese manufacture followed traditional practices as described elsewhere (Macedo et al., 1993). Their major steps are coagulation of raw ewe's milk with an aqueous extract from dry cardoon flowers containing proteases, and addition of this crude rennet directly to the milk vat. The milk is then allowed to rest in a closed pan at temperatures between 17 and 40 °C, and for time intervals in the range 30–90 min. The cutting of the curd consists of manually stirring the coagulum for 10–15 s with a knife or a kitchen spoon. Draining of the whey is carried out via pressing the curd. Salting is often done via addition of crude, unrefined kitchen salt to the raw milk prior to coagulation, although it may also be rubbed on the top and bottom surfaces of the pressed cheese curd.

Since sterility was strictly required for full validation of our fundamental work, the whole cheesemaking process was carried out in a sterile flow chamber, using sterile tools and equipments as well. Every milk batch was added with sterile kitchen salt (20 g/L) and an aqueous suspension of rennet, after filter-sterilization through 0.22 μm filters (Orange Scientific, Belgium). Each batch was then distributed over 100 mL-sterile flasks (5 cm in diameter), which were immediately stoppered. Incubation took place in a water bath for 1 h at 30 °C. Following coagulation, the curd was aseptically cut using a sterile spatula. Finally, another incubation period of 90 min was provided to finalize syneresis. The curd was then slightly and aseptically compressed with a sterile spatula, to aid in whey expulsion. Cheeses were afterwards stored up to 60 d, at 8 °C and 85% relative humidity.

Gross composition assessment

Throughout ripening, model cheeses were assayed for moisture, fat, salt and total protein contents using Fourier Transformed Infra-Red spectroscopy, in a LactoScope Advanced FTIR (Delta Instruments, Drachten, The Netherlands), following preliminary calibration. The pH of cheeses was measured directly with a pH meter (Micro pH 2002, Crison, Spain).

Sugar and organic acid quantification

Model cheeses, ripened for 0, 30 and 60 d, were assessed in terms of consumption of sugars and production of organic acids using a Lachrom HPLC (Merck Hitachi, Germany). Seven organic compounds, viz. lactose and glucose, as well as citric, acetic, succinic, lactic and formic acids, were resolved, identified and quantified in the same chromatographic run. A 2 g-aliquot was added to 10 mL of 13 mM sulphuric acid, and homogenized with an Ultra-Turrax (LaboControle, Portugal) for 3 min at 18,000 rpm. The mixture was centrifuged with a Universal 32R apparatus (Hettich, Germany) at 4000 rpm for 10 min (4 °C), and filtered

through No. 42 filter paper (Whatman, UK). Immediately before analysis, samples were filtered through 0.22 μm (Orange Scientific, Belgium). The isocratic HPLC system was composed of: an ion exchange aminex HPX 87H, 300 × 7.8 mm column (BioRad, USA) maintained at 65 °C, and two detectors in series, refractive index and spectrophotometry (220 nm). The mobile phase used was 13 mM sulphuric 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

Model cheeses by 0, 30 and 60 d of ripening were duly sampled, and then assayed for proteolysis. Nitrogen soluble in water, WSN, and nitrogen soluble in 12%(w/v) trichloroacetic acid (Merck, Germany), TCASN, were determined following extraction as described elsewhere (Kuchroo and Fox, 1982), except that a Sorvall Omni-mixer (Dupont, USA) was used for homogenization, and that the supernatant obtained was filtered through No. 42 filter paper (Whatman). Nitrogen soluble in 5%(w/v) phosphotungstic acid (Merck), PTASN, was also determined by Kjeldahl, according to Stadhouders (1960) – except that extracts were also prepared as described above. Using our experimental data, three proteolytic indices were calculated as ratios between those soluble fractions to total nitrogen, TN: ripening extension index, WSN/TN; ripening depth index, TCASN/TN; and free amino acid index, PTASN/TN.

Rheology assessment

Model cheeses ripened for 0, 30 and 60 d were subject to dynamic rheological tests, performed at low strain amplitude (1%) and a frequency of 0.1 Hz, within the linear viscoelastic range, using a controlled stress Rheometer CS-50 (Bohlin Instruments, UK) fitted with a plane-and-plate device (gap of 2000 μm and diameter of 2 cm). At least two independent replicates were produced.

Statistical analyses

All analytical results (encompassing gross composition, organic compounds, soluble nitrogen fractions and rheology), obtained at various stages during ripening, were analyzed using SPSS v. 16.0.0 software (SPSS, USA) to assess statistical differences at the 5% level, between the effects caused by the various LAB and contaminant inocula and the levels thereof. A categorical principal component analysis (CATPCA) was carried out to correlate the levels of organic compounds with the ripening time and the microbiological groups considered. A stepwise discriminant analysis was also performed to ascertain the physicochemical parameters most useful to classify the model cheeses according to ripening time, type of contaminant inoculum, type of LAB inoculum and initial contaminant inoculum, using Wilks' lambda (Λ) test as criterion for statistical selection.

Results

Gross composition

As described above, model cheeses were assayed for total protein, salt, fat and moisture contents, as well as pH changes. The results of such a characterization are shown in Table 1.

Initial values within each contaminant set comprising the LAB inocula did not in general differ significantly ($P < 0.05$), although ripening time produced a strong influence upon its variation (statistical results not shown). Irrespective of being inoculated alone or in the presence of LAB, the various contaminants, either isolated or as a consortium, had no significant impact upon pH

Table 1
Physicochemical characterization of model cheeses, inoculated with various contaminant and lactic acid bacteria (LAB), at the beginning and end of the ripening period.[a]

Pathogens	LAB	pH		Moisture %(m/m)		SDM[b] %(m/m)		FDM[c] %(m/m)		PDM[d] %(m/m)	
		0 d	60 d	0 d	60 d	0 d	60 d	0 d	60 d	0 d	60 d
L. innocua	–	6.20 ± 0.02^{1}	6.00 ± 0.03^{1}	77.7 ± 1.2^{1}	77.4 ± 0.5^{1}	11.7 ± 1.1^{1}	11.9 ± 0.6^{1}	40.4 ± 2.0^{1}	41.6 ± 1.2^{1}	34.3 ± 0.3^{1}	33.4 ± 0.6^{1}
	L. lactis	6.20 ± 0.05^{1}	4.20 ± 0.09^{2}	76.8 ± 2.0^{1}	73.1 ± 0.8^{1}	10.2 ± 3.1^{1}	9.0 ± 0.3^{1}	42.3 ± 3.4^{1}	42.7 ± 0.9^{1}	34.5 ± 1.0^{1}	34.7 ± 0.2^{1}
	L. brevis	6.20 ± 0.02^{1}	4.70 ± 0.24^{3}	78.1 ± 0.5^{1}	74.1 ± 5.7^{1}	11.7 ± 0.6^{1}	7.9 ± 1.5^{1}	40.3 ± 1.0^{1}	46.9 ± 5.1^{1}	34.1 ± 0.2^{1}	33.2 ± 1.4^{1}
	L. plantarum	6.20 ± 0.02^{1}	4.30 ± 0.05^{2}	77.1 ± 0.8^{1}	71.5 ± 0.6^{1}	10.6 ± 1.4^{1}	8.0 ± 0.9^{1}	43.6 ± 3.9^{1}	48.7 ± 1.8^{1}	32.8 ± 2.0^{1}	33.4 ± 0.8^{1}
P. aeruginosa	–	6.20 ± 0.02^{1}	5.30 ± 0.18^{1}	79.2 ± 1.1^{1}	72.6 ± 1.5^{1}	12.5 ± 0.4^{1}	8.6 ± 0.5^{1}	40.7 ± 1.3^{1}	47.2 ± 2.4^{1}	32.3 ± 0.4^{1}	34.0 ± 1.4^{1}
	L. lactis	6.20 ± 0.01^{1}	4.30 ± 0.03^{2}	79.0 ± 0.4^{1}	70.7 ± 2.5^{1}	12.1 ± 0.3^{1}	7.4 ± 1.3^{1}	41.1 ± 0.4^{1}	47.9 ± 3.1^{1}	32.3 ± 0.3^{1}	35.4 ± 1.1^{1}
	L. brevis	6.20 ± 0.02^{1}	4.80 ± 0.04^{3}	79.3 ± 0.7^{1}	73.3 ± 4.1^{1}	12.0 ± 1.0^{1}	8.6 ± 2.2^{1}	40.3 ± 1.9^{1}	47.0 ± 3.4^{1}	32.9 ± 0.6^{1}	33.7 ± 1.6^{1}
	L. plantarum	6.20 ± 0.03^{1}	4.40 ± 0.02^{2}	78.4 ± 0.4^{1}	73.6 ± 3.9^{1}	11.7 ± 0.6^{1}	8.2 ± 0.6^{1}	42.6 ± 0.8^{1}	45.7 ± 4.3^{1}	31.8 ± 0.2^{1}	35.2 ± 2.8^{1}
S. aureus	–	6.30 ± 0.02^{1}	6.30 ± 0.21^{1}	80.8 ± 1.3^{1}	80.0 ± 0.6^{1}	15.0 ± 1.2^{1}	14.3 ± 0.4^{1}	36.4 ± 2.2^{1}	37.0 ± 1.3^{1}	32.5 ± 0.5^{1}	33.4 ± 0.5^{1}
	L. lactis	6.30 ± 0.05^{1}	4.30 ± 0.04^{2}	81.6 ± 0.5^{2}	79.1 ± 3.4^{2}	15.3 ± 0.1^{2}	13.0 ± 2.4^{2}	35.8 ± 0.8^{2}	36.0 ± 7.2^{2}	32.0 ± 0.3^{1}	35.8 ± 2.1^{1}
	L. brevis	6.30 ± 0.05^{1}	5.10 ± 0.08^{3}	80.3 ± 1.2^{1}	73.9 ± 2.7^{1}	14.7 ± 0.3^{1}	9.8 ± 1.2^{1}	37.7 ± 1.2^{1}	46.7 ± 4.3^{1}	32.0 ± 0.4^{1}	32.5 ± 1.6^{1}
	L. plantarum	6.30 ± 0.03^{1}	4.40 ± 0.09^{2}	79.1 ± 1.1^{2}	75.3 ± 2.3^{2}	13.1 ± 1.4^{2}	10.9 ± 1.0^{2}	39.8 ± 1.9^{2}	42.8 ± 3.6^{2}	32.5 ± 0.3^{1}	34.3 ± 1.1^{1}
E. coli	–	6.00 ± 0.10^{1}	5.60 ± 0.26^{1}	78.7 ± 0.3^{1}	75.8 ± 2.9^{1}	12.0 ± 0.3^{1}	10.1 ± 1.3^{1}	42.1 ± 0.8^{1}	44.3 ± 3.4^{1}	31.7 ± 0.3^{1}	33.2 ± 1.7^{1}
	L. lactis	6.00 ± 0.30^{1}	4.40 ± 0.05^{2}	79.7 ± 1.1^{1}	74.8 ± 1.9^{1}	12.5 ± 0.6^{1}	9.2 ± 0.7^{1}	40.3 ± 1.8^{1}	44.0 ± 3.4^{1}	32.1 ± 0.8^{1}	35.0 ± 2.3^{1}
	L. brevis	6.00 ± 0.06^{1}	4.70 ± 0.02^{3}	78.2 ± 0.8^{1}	74.0 ± 0.8^{1}	11.8 ± 0.1^{1}	9.4 ± 0.2^{1}	42.8 ± 1.0^{1}	45.5 ± 1.4^{1}	31.7 ± 0.3^{1}	33.9 ± 0.7^{1}
	L. plantarum	6.00 ± 0.10^{1}	4.40 ± 0.04^{2}	78.0 ± 1.0^{1}	74.4 ± 1.9^{1}	11.1 ± 1.2^{1}	9.1 ± 1.4^{1}	43.5 ± 2.0^{1}	43.8 ± 4.6^{1}	31.7 ± 0.2^{1}	35.5 ± 2.1^{1}
Contaminant consortium	–	6.10 ± 0.02^{1}	5.30 ± 0.6^{1}	77.2 ± 2.4^{1}	74.0 ± 3.0^{1}	12.2 ± 2.4^{1}	9.6 ± 1.9^{1}	36.5 ± 2.4^{1}	43.9 ± 4.2^{1}	37.7 ± 1.6^{1}	35.2 ± 1.1^{1}
	L. lactis	6.10 ± 0.03^{1}	4.50 ± 0.07^{2}	78.9 ± 0.3^{1}	72.8 ± 1.1^{1}	14.1 ± 0.4^{1}	9.4 ± 0.6^{1}	36.8 ± 1.2^{1}	45.3 ± 1.7^{1}	34.7 ± 0.7^{1}	34.8 ± 0.7^{1}
	L. brevis	6.10 ± 0.04^{1}	4.90 ± 0.06^{3}	79.7 ± 0.6^{1}	73.0 ± 4.3^{1}	14.7 ± 0.5^{1}	9.9 ± 2.2^{1}	35.0 ± 1.7^{1}	42.5 ± 6.7^{1}	35.2 ± 0.6^{1}	36.6 ± 2.1^{1}
	L. plantarum	6.10 ± 0.02^{1}	4.60 ± 0.05^{2}	79.0 ± 0.6^{1}	72.5 ± 0.8^{1}	14.6 ± 0.6^{1}	9.5 ± 0.7^{1}	35.9 ± 2.2^{1}	44.9 ± 2.1^{1}	35.0 ± 1.0^{1}	35.2 ± 1.1^{1}
	L. lactis + *L. brevis*	6.10 ± 0.01^{1}	4.40 ± 0.05^{2}	77.1 ± 1.1^{1}	71.9 ± 1.1^{1}	12.0 ± 0.7^{1}	8.3 ± 0.3^{1}	32.8 ± 3.3^{1}	45.7 ± 2.7^{1}	41.5 ± 2.3^{1}	35.7 ± 1.8^{1}
	L. lactis + *L. plantarum*	6.10 ± 0.02^{1}	4.30 ± 0.1^{2}	77.5 ± 1.3^{1}	71.9 ± 1.0^{1}	11.9 ± 1.0^{1}	8.5 ± 0.3^{1}	33.0 ± 3.3^{1}	45.4 ± 3.3^{1}	41.1 ± 1.3^{1}	35.9 ± 2.4^{1}

Means within the same column for each pathogen inoculum, without a common superscript, are significantly different ($P < 0.05$).
[a] Results are expressed as mean ± standard deviation.
[b] SDM – Salt per dry matter.
[c] FDM – Fat per dry matter.
[d] PDM – Protein per dry matter.

(statistical results not shown). Conversely, the LAB strains employed exerted a strong influence on pH as time elapsed. The two LAB genera, viz. *Lactococcus* and *Lactobacillus*, showed statistically different behaviours in terms of acidification when used as single cultures ($P < 0.05$). By the end of ripening, model cheeses containing *L. lactis* and *L. plantarum* exhibited similar pH values ($P > 0.05$). The final pH when *L. lactis* was used alone was similar to that obtained when mixed inocula were considered. Moisture, salt and fat contents were affected to a significant extent ($P < 0.05$) by ripening time, probably due to dissolution of salt in whey coupled with concentration of fat owing to whey loss. However, the total protein remained essentially unchanged up to 60 d of ripening.

Sugars and organic acids

Categorical principal component analysis (CATPCA) was performed on data encompassing organic acids and sugars in attempts to simplify them via quantification of categorical variables, while reducing the dimensionality of the data. Initially, the whole data set (n = 264) was subject to that analysis, in order to determine whether model cheese ripening could be classified according to the concentrations of each of the organic compounds considered. Two principal components resulted, which together could explain 65.3% of the total variance; dimensions 1 and 2 explained 41.2% and 24.1% of said variance, respectively. These results are depicted in Fig. 2a as a two-dimensional plot. As expected, the lactose content correlated negatively with lactic and acetic acid contents, whereas succinic and citric acids were strongly associated therewith. Obvious changes in quantitative organic compounds throughout ripening were perceived. Higher concentrations of sugars, viz. lactose and glucose, and succinic and citric acids were associated with fresh model cheeses, whereas high amounts of lactic, acetic and formic acids, which depart from sugars, were related to ripened ones.

A second CATPCA was applied to data pertaining to 60 d-model cheeses (n = 88), with grouping either by contaminants or LAB inocula. These results are depicted in Fig. 2b and c as two-dimensional plots. The two principal components justify ca. 60% of the total variance.

High lactose concentrations, associated with model cheeses inoculated with *P. aeruginosa* and *E. coli*, and absence of LAB, correlated inversely with production of lactic acid. This realization is clearly apparent in model cheeses inoculated with *L. innocua* and the contaminant consortium (Fig. 2b), as well as with any of the LAB (Fig. 2c). They also correlated inversely with formation of acetic acid, which was somehow associated with presence of *S. aureus* and *Lactobacillus* strains (Fig. 2b and c, respectively). The more complex microbiota – i.e. the contaminant consortium coupled with homofermentative *L. lactis*, were clearly associated with high citric and succinic acid concentrations. Heterofermentative *L. brevis* and *L. plantarum* were associated with high levels of acetic acid. The mixture of LAB comprising *L. lactis* and one *Lactobacillus* formed a small cluster that was consistently located between lactic and acetic acids. This is a clue to the balance between homo- and heterofermentative behaviours of those strains. The attempt to perform a similar analysis, aimed at pinpointing differences between the 2 initial viable levels of contaminants, was not however successful. This realization confirms that such a parameter does not influence the pool of organic compounds considered in this work.

Proteolysis

The evolution of the ripening extension index, the ripening depth index and the free amino acid index in model cheeses, as affected by type of contaminant inoculum and LAB inoculum, is depicted in Fig. 3.

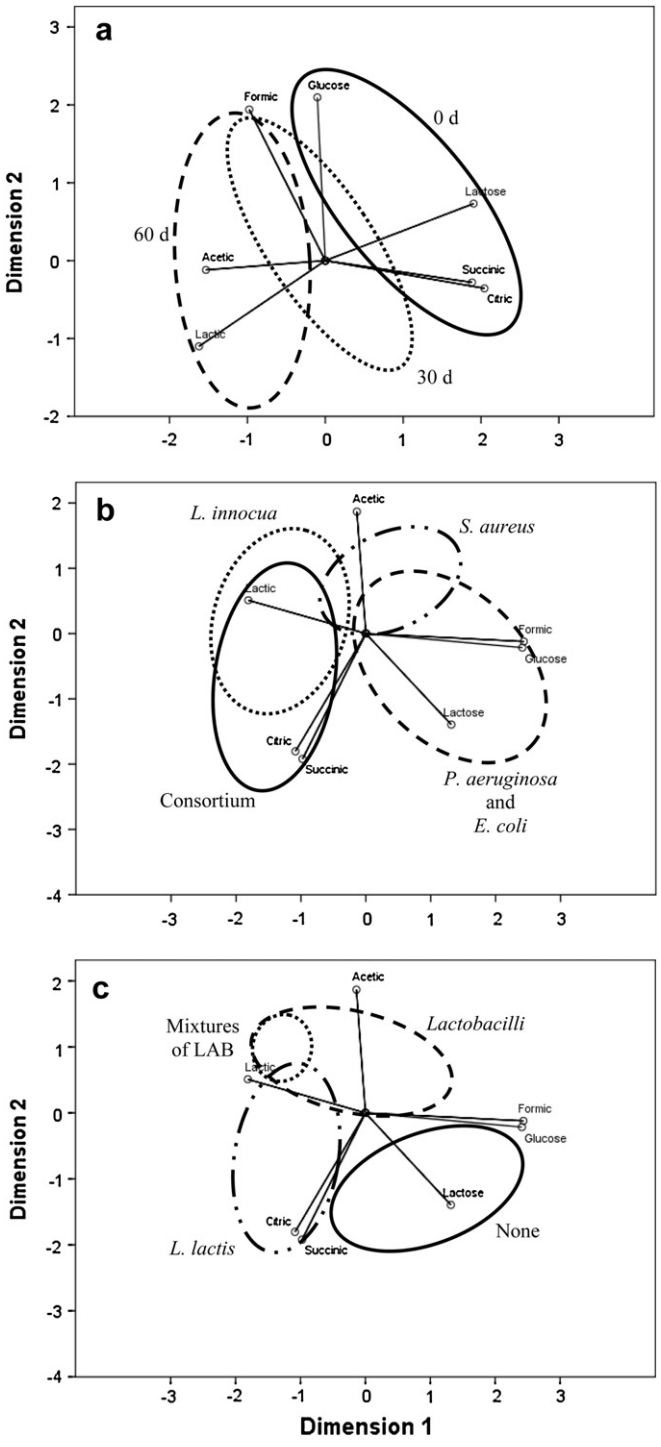

Fig. 2. Categorical principal component biplots, encompassing the relationships between lactose and glucose, and citric, lactic, succinic, formic and acetic acid concentrations, in (a) model cheeses throughout ripening or (b) 60 d-model cheeses, inoculated with the various (b) contaminants or (c) LAB inocula.

As expected, the various soluble nitrogen fractions increased throughout ripening time ($P < 0.05$, statistical results not shown), although the initial contaminant load did not influence those parameters to a significant extent ($P > 0.05$, statistical results not shown). The ripening extension index (Fig. 3a and b) was not significantly affected by the various contaminants or LAB tested,

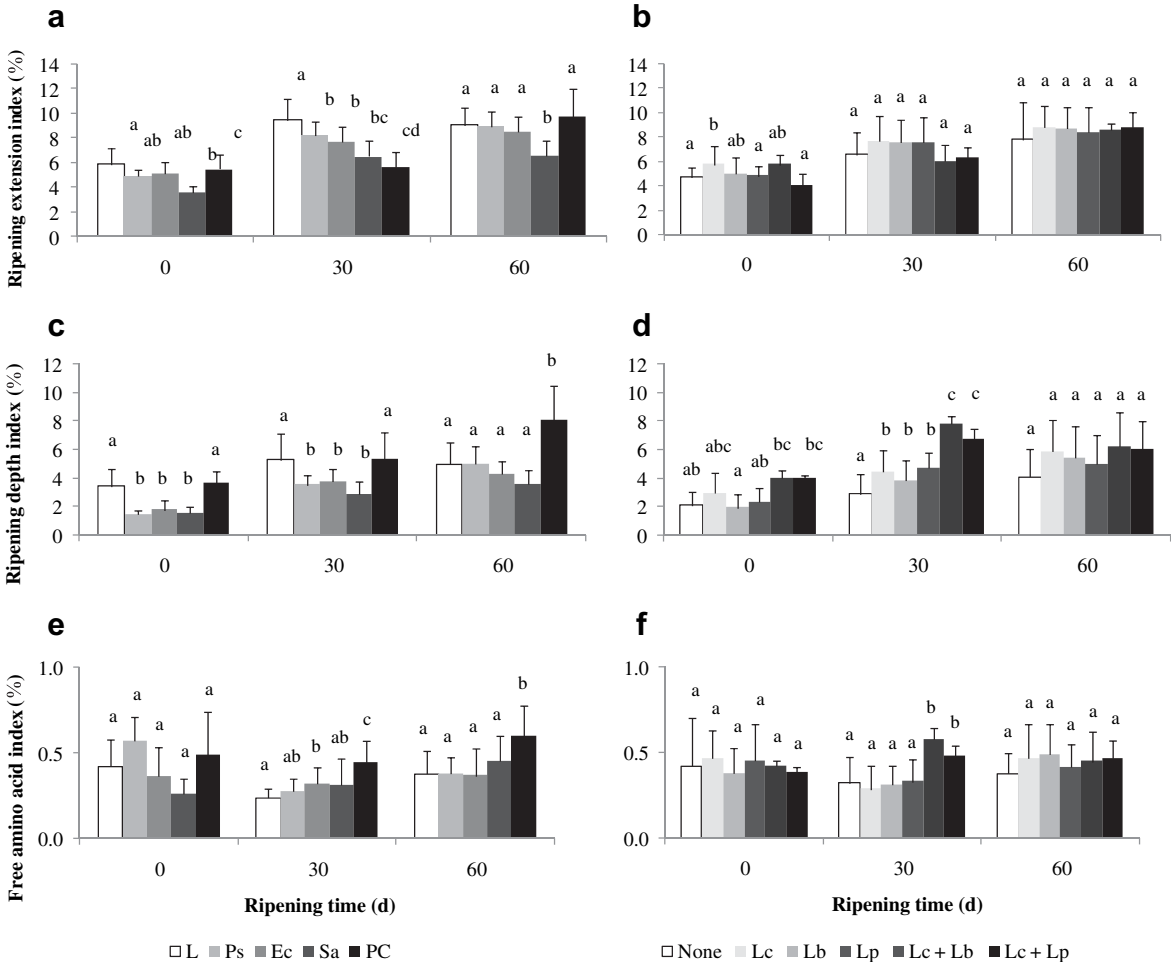

Fig. 3. Variation in the proteolysis indices of model cheeses throughout ripening: (a, b) ripening extension index – WSN/TN; (c, d) ripening depth index – TCASN/TN; and (e, f) free amino acid index – PTASN/TN; as a function of: (a, c, e) contaminant inoculum; and (b, d, f) LAB inoculum. L: *L. innocua*; Ps: *P. aeruginosa*; Ec: *E. coli*; Sa: *S. aureus*; PC: contaminant consortium; Lc: *L. lactis*; Lb: *L. brevis*; Lp: *L. plantarum*; Lc + Lb: *L. lactis* and *L. brevis*; and Lc + Lp: *L. lactis* and *L. plantarum*. Results are expressed as mean ± standard deviation. [a–d] Means within the same set of columns for each ripening time, without a common superscript, are significantly different ($P < 0.05$).

except for topical minor discrepancies among the various contaminant inocula. On the other hand, a few trends in the ripening depth index evolution (Fig. 3c and d) should be outlined: the faster action of LAB by 30 d, either as a plain or, more notoriously, as a mixture, and the synergistic effect by 60 d of the contaminant consortium. This could explain the similarities found between model cheeses not inoculated or inoculated with LAB, by the same ripening time. The release of free amino acids (Fig. 3e and f) followed a similar trend, except for the possible cooperative effect exerted by the LAB mixtures by 30 d.

Rheology

Changes in the viscoelastic properties – G', or storage modulus, and G'', or loss modulus, as obtained by dynamic-mechanical analysis of the model cheeses throughout ripening, are plotted in Fig. 4. As can be inferred from these data, G' is higher than G'' in all cases, thus indicating a major contribution of the elastic, or solid character, component to the viscoelasticity of the model cheeses throughout ripening. Furthermore, the loss tangent, tan δ (G''/G'), exhibited values always below unity (data not shown), hence corroborating the dominant elastic nature of the model cheese matrices. Additionally, the presence of LAB mixtures – especially when *L. lactis* and *L. brevis* were employed, led to higher G' and G'', but tan δ was not affected (data not shown). The various contaminant inocula led to no significant differences, except for the outstandingly higher values of G' and G'' recorded in 60 d-model cheeses inoculated with the contaminant consortium.

Discriminant analysis

The 18 physicochemical quantitative parameters assayed for included bulk composition (viz. pH, moisture, SDM, FDM and PDM), organic acids (viz. glucose, lactose, and citric, acetic, formic, lactic and succinic acids), proteolysis (viz. WSN/TN, TCA/TN and PTA/TN) and rheology (viz. G', G'' and tan δ); these parameters were selected for various stepwise discriminant analyses, in attempts to establish a classification pattern of such model cheeses. However, depending on the distinct qualitative variables, ripening time, contaminant inoculum type or concentration, or LAB inoculum type, and according to their discriminant power or collinearity with other variables, different physicochemical parameters were included in each analysis. All such parameters were discriminated by 10, 14, 8 and 1 variables, and the total variances among discriminator variables were explained by 2, 4, 5 and 1 discriminant functions, respectively. The whole variation among the various grouping variables was thus accounted for by these discriminant functions.

The standardized discriminant function coefficients of the variables are listed in Tables 2–4. According to the classification results, model cheeses were better distinguished by ripening time

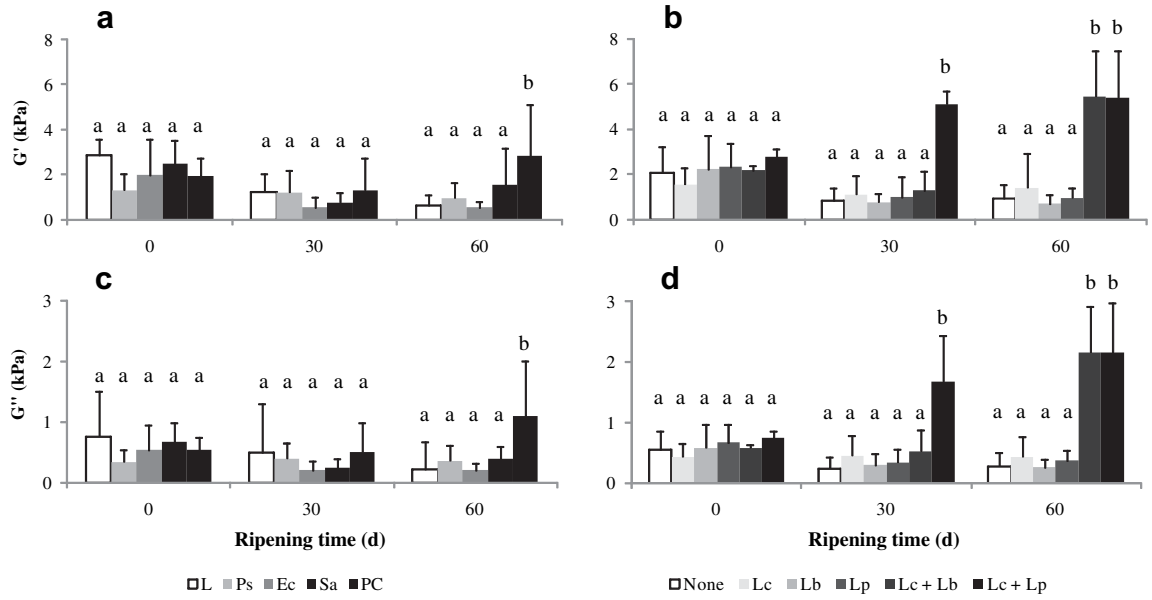

Fig. 4. Variation in the viscoelastic properties of model cheeses throughout ripening: (a, b) elastic modulus – G'; and (c, d) viscous modulus – G'', as a function of: (a, c, e) contaminant inoculum; and (b, d, f) LAB inoculum. L: *L. innocua*; Ps: *P. aeruginosa*; Ec: *E. coli*; Sa: *S. aureus*; PC: contaminant consortium; Lc: *L. lactis*; Lb: *L. brevis*; Lp: *L. plantarum*; Lc + Lb: *L. lactis* and *L. brevis*; and Lc + Lp: *L. lactis* and *L. plantarum*. Results are expressed as mean ± standard deviation. [a–c] Means within the same set of columns for each ripening time, without a common superscript, are significantly different ($P < 0.05$).

as 89.3% of the original grouped cases were classified correctly, followed by contaminant inoculum type (78.5%), LAB inoculum type (59.8%) and contaminant inoculum concentration (56.2%). All cheeses were also correctly discriminated for ripening time. Function 1 indeed determined the separation between unripened, or a positive value, and ripened, or a negative value, model cheeses (Fig. 5a). The variable contributing the most to separation of fresh model cheeses was lactose content (Table 2). Function 2 permitted separation of 30 d-ripened model cheeses, with a negative value, from 60 d-ripened model cheeses, with a positive value; sugar contents contributed negatively, and lactic acid positively.

Concerning the contaminant and LAB inoculum type (see Tables 3 and 4, respectively), most variance was explained by the first two discriminant functions, i.e. 92.4% and 82.3%, respectively; therefore, only these were chosen and plotted for each grouping variable, as done in Fig. 5b and c. The remaining functions accounted for only a marginal fraction of the whole variance, so they have not been considered afterwards. Function 1 separated better model cheeses inoculated with *E. coli* alone, and was associated specifically with moisture and glucose contents, from those inoculated with the consortium, which were characterized by specific pH and total protein and TCA-soluble peptides contents. On the other hand, both functions determined the classification of model cheeses inoculated with *S. aureus*, *P. aeruginosa* and *L. innocua*. Variables exhibiting a higher weight for this function were lactose, moisture and salt contents (Table 3). Model cheeses were only partially classified by LAB inoculum type (Fig. 5c), whereas the lack of inoculum, associated with a positive contribution of pH, and the mixed inocula *L. lactis* with *L. brevis*, or *L. lactis* with *L. plantarum*, associated with a negative contribution of citric acid, were separated by function 1

Table 2
Standardized canonical discriminant function coefficients, for the discriminator variables associated with ripening time of model cheeses.

Variables	Function 1	Function 2
WSN/TN[a]	−0.405	0.087
PTASN/TN[b]	0.148	0.208
G'[c]	0.253	0.099
Lactose	0.773	−0.349
Glucose	−0.225	−0.242
Lactic acid	0.227	0.659
SDM[d]	0.193	0.292
FDM[e]	−0.020	0.488
PDM[f]	−0.300	−0.802
pH	0.286	0.719
Variance explained (%)	81.6*	18.4*

*$P < 0.05$ (Wilks' Λ-test).
[a] WSN/TN – Ripening extension index.
[b] PTASN/TN – Free amino acid index.
[c] G' – Elastic modulus.
[d] SDM – Salt per dry matter.
[e] FDM – Fat per dry matter.
[f] PDM – Protein per dry matter.

Table 3
Standardized canonical discriminant function coefficients, for the discriminator variables associated with contaminant inoculum of model cheeses.

Variables	Function 1	Function 2
WSN/TN[a]	−0.361	0.961
TCASN/TN[b]	0.614	−0.598
PTASN/TN[c]	0.166	−0.064
Citric acid	0.341	0.347
Lactose	0.207	1.183
Glucose	−0.898	0.064
Lactic acid	0.393	0.211
Formic acid	−0.011	−0.396
Acetic acid	−0.078	0.159
Moisture	−0.659	1.245
SDM[d]	0.166	−1.883
FDM[e]	0.287	0.042
PDM[f]	0.659	−0.038
pH	0.639	−0.130
Variance explained (%)	76.3*	16.1*

*$P < 0.05$ (Wilks' Λ-test).
[a] WSN/TN – Ripening extension index.
[b] TCASN/TN – Ripening depth index.
[c] PTASN/TN – Free amino acid index.
[d] SDM – Salt per dry matter.
[e] FDM – Fat per dry matter.
[f] PDM – Protein per dry matter.

Table 4
Standardized canonical discriminant function coefficients, for the discriminator variables associated with lactic acid bacteria inoculum of model cheeses.

Variables	Function 1	Function 2
WSN/TN[a]	0.547	0.036
G''[b]	−0.517	−0.335
Citric acid	−0.815	0.403
Glucose	0.180	0.386
Succinic acid	0.344	0.007
Lactic acid	−0.371	0.670
Acetic acid	−0.014	−0.949
pH	0.990	0.063
Variance explained (%)	50.2*	32.1*

*$P < 0.05$ (Wilk's Λ-test).
[a] WSN/TN − Ripening extension index.
[b] G'' − Viscous modulus.

(Table 4). Function 2 determined separation between *L. lactis* and the lactobacilli, with the higher contributions arising from lactic and acetic acids, respectively (Table 4). Finally, the two initial concentrations of contaminants, viz. 10^3–10^4 and 10^7–10^8 cfu/g, were only differentiated by one function, with acetic acid acting as the single discriminator factor (statistical results not shown).

Discussion

The use of model systems allows one to overcome a number of extrinsic factors that would otherwise have masked the conclusions drawn. In this way, the restriction applied to the initial microbial load and diversity, as well as the strict control of manufacture parameters afterwards have permitted independent assessment of the phenomena that take place in actual cheeses, while allowing one to ascertain the effect of a few technological factors upon ripening (Pereira et al., 2008a,b). Note that the lack of control has often been claimed to lead to unexplained and unpredictable heterogeneity of the final product.

In order to attain our primary goal, the control of the microflora was essential; hence, our experimental cheeses were manufactured from milk previously subjected to thermal treatment. It is well documented that heating induces several physicochemical changes in milk – e.g. partial denaturation of whey proteins (Grappin and Beuvier, 1997), followed by interaction with caseins and eventual aggregation, which in turn increase the water-holding capacity of the proteinaceous gel matrix (Hinrichs, 2001). However, trends and comparisons within our experimental results could still be drawn, relating to the various factors considered, as also done elsewhere using similar experimental conditions (Pereira et al., 2008a,b).

Concerning the various physicochemical parameters assayed for pertaining to bulk composition, organic compounds, proteolysis and rheology, a major outcome is the high weight of the initial composition of milk and of the organic acid metabolic pathway, and consequent acidification, upon discrimination between model cheeses. In fact, the discriminant analysis pointed at these as the main vectors responsible for differentiation between the experimental conditions tested. Although proteolysis and evolution of viscoelastic properties are intrinsic processes in cheese ripening, they appeared to be relatively insensitive to variations in the starting wild microflora.

The evolution of the ripening extension index accounts for the release of large, water-soluble peptides directly from casein. This phenomenon is caused mainly by the residual rennet retained in the curd following manufacture, so bacteria are not expected to be relevant in this event. The evolution in the ripening depth index corresponds, in turn, to release of medium- and small-sized peptides. This is the result of the combined action of both rennet and microbial enzymes upon the aforementioned large peptides, previously released by rennet enzymes. On the other hand, fractionation with 5% PTA is able to extract only low molecular weight peptides and free amino acids. Bacterial enzymes are relevant actors during these two latter stages of proteolysis. However, the relatively slow appearance of free amino acids may also be due to their substantial uptake as nitrogen source, during exponential growth of the viable bacteria upon inoculation (data not shown).

Parameter G' is used to measure the energy stored in, or the elastic properties of the cheeses; conversely, G'' measures the energy lost by, or the flow properties thereof (Park, 2007). The evolution of tan δ reveals, in turn, changes in flexibility, and eventual weakening of the casein structure (van Vliet et al., 1991) as a result of proteolysis. The values of tan δ obtained were always below 1 (data not shown), thus indicating that the elastic nature of the cheese matrix dominates over its viscous counterpart. A gradual transition from an initial network dominated by denaturated whey proteins attached to the surface of micelles, to a network dominated by casein–casein interactions at lower pH (Haque et al., 2001), as a result of LAB metabolism, probably accounts for our observations. Furthermore, the breakdown of citrate by LAB leads to the production of carbon dioxide, which may as well influence the texture of certain fermented dairy products (Fox et al., 1993).

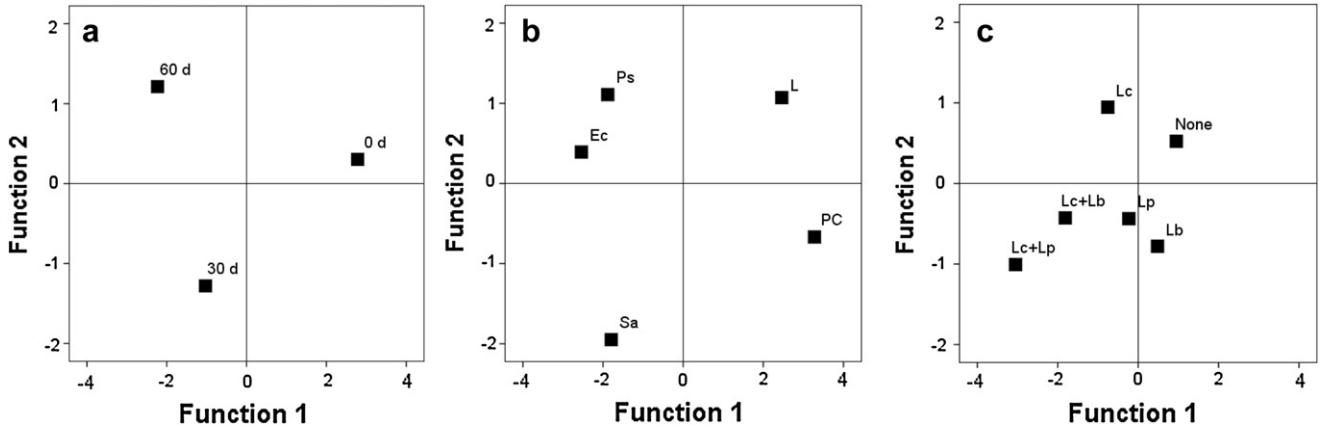

Fig. 5. Canonical biplots, encompassing all model cheeses. Symbols indicate group centroids for (a) ripening time, (b) contaminant inoculum and (c) LAB inoculum. L: *L. innocua*; Ps: *P. aeruginosa*; Ec: *E. coli*; Sa: *S. aureus*; PC: pathogen consortium; Lc: *L. lactis*; Lb: *L. brevis*; Lp: *L. plantarum*; Lc + Lb: *L. lactis* and *L. brevis*; and Lc + Lp: *L. lactis* and *L. plantarum*.

Our experimental results proved that organic compounds were a direct consequence of the action of the starting wild microflora (Akalin et al. 2002). Therefore, those compounds can be used as an indicator of qualitative changes during cheese ripening (Lombardi et al., 1994; González de Llano et al., 1996; Ballesteros et al., 2004). Production of flavour compounds, or precursors thereof, relies on the milk-degrading enzymes synthesized by each strain; it also hinges upon the complementary metabolic routes of the various strains present, which may enhance the level and quality of flavours (Irlinger and Mounier, 2009). As expected, the production of lactic acid was attributed to the presence of LAB, and it was relevant when also exposed to the contaminant consortium; this was so likely because of the occurrence of *L. innocua* (Kelly and Patchett, 1996), since *P. aeruginosa* and *E. coli* were not implicated in lactose or glucose depletion. Furthermore, the homo- or heterofermentative character of the LAB used determined a higher production of acetic acid by lactobacilli, especially in the presence of *S. aureus*, as well as an accumulation of citric and succinic acids by *L. lactis*, especially in the presence of the contaminant consortium. Cheese composition, chiefly protein and moisture contents and pH, were also parameters associated with continuing presence of certain LAB or contaminants.

One should realize that cheese constitutes a heterogeneous environment, in terms of physical structure and chemical composition. Consequently, it is anticipated that microbial growth will be spatially heterogeneous as well. In fact, the functional diversity that is closely related to the complexity of the cheese microbiota plays a crucial role in the multiplicity of flavour compounds produced during ripening (Irlinger and Mounier, 2009). Finally, the alteration of the physicochemical composition can be regarded as a stress response to physiologically adverse environmental conditions, which may severely affect sensory and other acceptability criteria (Fleet, 1999).

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