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The potential effect of FOS and inulin upon probiotic bacterium performance in curdled milk matrices

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ABSTRACT

Inulin and fructooligosaccharides were studied for their prebiotic effect upon growth/survival of probiotic bacteria and technological potential in probiotic food processing, via characterization of glycolysis, proteolysis and lipolysis in curdled milk matrices; the ultimate goal is the manufacture of synbiotic cheeses. Prebiotic compounds did not significantly affect growth/viability of all strains studied, except *Lactobacillus acidophilus* La-5. Proteolysis indices revealed considerable casein degradation in probiotic and synbiotic matrices inoculated with *Bifidobacterium lactis* B94 and *Lactobacillus casei*-01; lower values were achieved in those inoculated with *L. acidophilus* La-5, yet a synbiotic effect was apparent in NPN values. Lipolysis was not extensive over storage, irrespective of matrix type; however, interesting differences in terms of the qualitative free fatty acids profile were observed. CLA isomers, and α-linolenic and γ–linolenic acids were detected upon 15 d of ripening of all inoculated matrices. Principal component analysis was able to discriminate the various matrices according to degree of maturation, throughout the ripening period. Microbiological and biochemical parameters unfolded a very good technological potential, especially of *B. lactis* B94 and *L. casei*-01, to produce novel types of functional dairy matrices – although extrapolation to actual cheeses should still be done with care, because e.g. syneresis was not considered.

1. Introduction

Development of foods that promote general health and well-being has been a research priority for food industry; foods enriched with such healthy compounds as probiotics and prebiotics are indeed sought to further and further extents. The definition of probiotics has evolved over time, but one of the most commonly accepted one states that they are live microorganisms which, when administered in adequate amounts, confer a health benefit on the host (FAO/WHO, 2002; Sanders, 2003).

Health benefits claimed for such probiotic bacteria as *Lactobacillus acidophilus, Bifidobacterium* spp. and *Lactobacillus casei* have driven their tentative incorporation into dairy foods (Minelli et al., 2004); those benefits include active digestion capacity (thus contributing to improved digestibility and nutritional properties of the food), antagonistic activity against pathogens, modulation of gut beneficial microflora, improved colon integrity, down-regulated allergic response and immunomodulation (Gomes & Malcata, 1999; Sanders, 2003). Their viability in food matrices is of the utmost importance, since it is well known that food components can influence growth, viability and survival, as well as acid and bile tolerance, antimicrobial activity and functionality of probiotics – which will eventually determine their efficacy in the gastrointestinal tract after ingestion. Careful investigation on the interaction of existing probiotics with natural (or added) food components should thus be considered in attempts to formulate new functional foods.

Among dairy foods, cheese has been shown to be a promising alternative to yoghurt and fermented milk as a vehicle for probiotic delivery – due to its versatility, organoleptic appeal, nutritional value and suitability for all age groups (Gomes & Malcata, 1999). On the other hand, it has been shown that probiotic strain viability in a food matrix may be maintained (or even enhanced) by combination with prebiotic ingredients, which can contribute to their

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protection while in the food matrix and to their nutrition once in the intestinal tract (Capela, Hay, & Shah, 2006; Gibson, Probert, van Loo, Rastall, & Roberfroid, 2004). Recall that prebiotics are non-digestible dietary components that can reach the colon essentially intact, where they will stimulate proliferation and activity of desirable bacteria *in situ* (Mattila-Sandholm, Myllarinen, Crittenden, Mogensen, Fondén, & Saarela, 2002).

Synbiotic products – a combination of pre- and probiotics in a given food product (Holzapfel & Schillinger, 2002), are a more recent concept; they can enhance health promotion in a synergistic manner, over either probiotics or prebiotics alone, and are thus already marketed in Europe and Japan – but as fermented milk or yoghurt only (Maukonen, Matto, Kajander, Mattila-Sandholm, & Saarela, 2008; Roy, 2005). Fructooligosaccharides (FOS) and inulin are among the most famous prebiotic compounds (Buriti, Cardarelli, Filisetti, & Saad, 2007; Cardarelli, Saad, Gibson, & Vulevic, 2007; Gilliland, 2001; Roberfroid, 2005). It is important to emphasize that these compounds may be added to foods in attempts to increase fiber ingestion – to levels ranging between 3 and 6 g per portion, or simply because of their bifidogenic power – in which case 3–8 g per portion is usually considered appropriate (Coussement, 1999); however, they may also be added because of their intrinsic technological properties, e.g. low-calorie sweeteners or fat substitutes (Tungland & Meyer, 2002).

Therefore, the main objective of this research effort was to analyse the potential effect of prebiotic ingredients (as a 50:50 FOS:Inulin mix) upon growth and survival of commercial probiotic bacteria in curdled milk matrices. Further assessment of their metabolic activity was via monitoring of glycolysis, proteolysis and lipolysis in said probiotic or synbiotic curdled milk matrices – in attempts to characterize their technological potential towards manufacture of actual probiotic or synbiotic cheeses.

2. Materials and methods

2.1. Probiotic bacterium selection

Selection of probiotic bacteria for the preparation of curdled milk matrices was based on specific technological properties, viz. proteolytic and lipolytic activities assessed by agar diffusion tests (Freitas, Pintado, Pintado, & Malcata, 1999; Larsen & Jensen, 1999). Eight distinct strains of probiotic bacteria were thus tested on 10% (w/v) skim milk agar (Himedia, India) and 10%(w/v) tributyrin agar (Merck, Germany), with incubation at 37 °C for 72 h – under aerobic conditions for *L. casei* and *Lactobacillus paracasei* strains, and anaerobic conditions for the other strains. Inoculation of each strain was done in duplicate.

L. paracasei LAFTI® L26, *Lactobacillus acidophillus* LAFTI® L10 and *Bifidobacterium lactis* LAFTI® B94 were obtained as DELVO-PRO® freeze dried cultures from DSM (Australia); Nu-trish® probiotic cultures of *Bifidobacterium animalis* BB-12, *L. acidophillus* La-5 and *L. casei*-01 were obtained in freeze dried form from Chr. Hansen (Denmark); and *L. acidophilus* Ki and *B. lactis* Bo were obtained as ultra-concentrated frozen cultures from CSK (Netherlands). Cultures unable to decrease the turbid zone around the inoculum in said agars were considered negative for proteolytic or lipolytic activity (as appropriate), whereas those giving rise to a clear zone were taken as positive –using one of three levels: (+) for a small zone (<5 mm), (++) for an intermediate zone (5–10 mm) and (+++) for a large zone (>10 mm).

2.2. Probiotic bacterium inocula

To obtain each probiotic inoculum, freeze dried bacteria were inoculated twice in MRS broth (Biokar Diagnostics, France); 2% of a 24-h probiotic culture was then inoculated in MRS broth supplemented with 0.5 g L^{-1} L-cysteine-HCl (Panreac, Spain) in 50-mL flat-bottomed glass flasks, and incubated for another 24 h. This procedure was applied to *L. casei*-01 (Chr. Hansen), *L. acidophilus* La-5 (Chr. Hansen) and *B. lactis* B94 (DSM).

2.3. Curdled milk matrices

For each probiotic bacterium, 48 portions of 50-mL volumes of cow's milk previously sterilized (at 110–112 °C for 10 min) were prepared – and divided into 3 groups, so as to obtain control, probiotic and synbiotic curdled matrices. For the former, 6 mL L^{-1}_{milk} of three times diluted animal rennet (1:15 000) (Naturen®, from Chr. Hansen), 6 mL L^{-1}_{milk} of $CaCl_2$ (Panreac) and 10 g L^{-1}_{milk} of NaCl (Panreac) were added. Probiotic matrices shared the same composition, but were inoculated at 2% with *L. casei*-01, *L. acidophilus* La-5 or *B. lactis* B94, respectively. Besides the probiotic inoculum, the synbiotic matrices were supplemented with 20 g L^{-1}_{milk} of a 50:50 mix of FOS:Inulin (Beneo-Orafti, Belgium).

Milk coagulation was performed at 30 °C for 5 h. Upon coagulation, duplicates of each curdled milk matrix type were withdrawn, for microbiological and chemical analyses – and labeled as 0 d. The remaining matrices were incubated at 12 °C throughout a 60-d ripening period – and duplicate samples were as well withdrawn by 1, 5, 7, 15, 30, 45 and 60 d. These were true replicates, representative of parallel, independent runs – which were later subjected to replicated analyses as well. Although another negative control could have been provided – consisting of milk with rennet and prebiotic compounds, previous experience had indicated that it would be redundant as FOS or Inulin are not substrates for the rennet used.

2.4. Microbiological and chemical analyses

A 4-g aliquot of each curdled matrix was homogenized in 40 mL of sterile 2%(w/v) sodium citrate (Fisher Scientific, UK) for 3 min, in a Stomacher blender (Model 400 circulator, from Seward Laboratory Systems, UK). Sequential dilutions, were made, with sterile 0.1% (w/v) peptone water (Himedia), of curdled matrix homogenates – and 100-μL aliquots were plated, in duplicate, on Petri dishes with MRS agar or PCA (Biokar Diagnostics). Viable cells of probiotic bacteria were obtained in MRS agar containing 0.5 g L^{-1} L-cysteine-HCl, following incubation at 37 °C for 48 h, under aerobic conditions for *L. casei*-01, and under anaerobic conditions for *L. acidophilus* La-5 and *B. lactis* B94. Microbiological counts performed on PCA incubated aerobically at 37 °C for 48 h were used as check of contamination – which was found not to exist at all.

The pH of the curdled matrices was measured directly with a pH meter (Micro pH 2002, Crison, Spain), whereas titratable acidity was determined according to AOAC method (1990).

Duplicated samples of each curdled milk matrix were assessed for organic acids and sugars using an HPLC apparatus from Merck LaChrom (Fullerton CA, USA), in a single run, based on calibration curves previously prepared with appropriate chromatographic standards; an Aminex HPX-87X cation exchange column from BioRad (Richmond CA, USA) was used for separation; the eluant was pumped at 0.8 mL min^{-1}, and consisted of 13 mM H_2SO_4 (Merck); and detection was by refractive index at 65 °C for sugars, and UV absorbance at 220 nm for organic acids. Prior to analysis, all samples were pre-treated as follows: 5 mL was homogenized with 20 mL of 13 mM H_2SO_4 in an Ultra-Turrax (IKA®, Canada) at 4000 rpm, allowed to stand for 3 min in an ice-bath, centrifuged at 4937 × g for 10 min at 4 °C, and then filtered through a 0.22-μm membrane filter (Millipore, USA).

Total nitrogen (TN), water-soluble nitrogen (WSN) and non-protein nitrogen (NPN) were determined by Kjeldahl's method. WSN was obtained via fractionation with water, according to Kuchroo and Fox (1982a, 1982b). NPN – i.e. the nitrogen fraction soluble in 12%(w/v) trichloroacetic acid (TCA, from Panreac), was prepared via addition of 7.5 mL of an aqueous solution of 48%(w/v) TCA to 22.5 mL of water-soluble extract; the mixture was allowed to stand for 30 min at room temperature, and then filtered through No. 42 filter paper (Whatman, UK) prior to application of said Kjeldahl's method.

The methodology proposed by Alonso, Cuesta, and Gilliland (2003) was followed, with some modifications, in assaying for free fatty acids (FFA) and conjugated linoleic acids (CLA): a 6-mL sample was mixed with 60 μL of a 6.44 mg/mL hexane solution of heptadecanoic acid (Sigma) – which served as internal standard, and 12 mL of 2-propanol (Fluka, Germany), and then shaken vigorously; 9 mL of hexane (Panreac) was then added, and the mixture was shaken for an extra 3 min; finally, the sample was placed in an ultra-sound bath (Ultrasonik, Canada) for 30 min.

After settling, the upper layer was collected by aspiration and filtered through anhydrous sodium sulphate (Panreac); then, this sodium sulphate bed was washed with an additional 7 mL of hexane. The lipid fraction was collected in a 100-mL pear-shaped flask, and placed in a Rotavapor (Laborota 4000, from Heidolph, USA) kept at 40 °C until dryness. The dried sample was redissolved in 500 μL of hexane, and transferred to a microtube; 100 μL of a methanolic (Riedel-de Haën, Germany) solution of 1 N sodium hydroxide (Pronalab, Portugal) was added to the tube containing the lipid fraction, the mixture was vortexed for 1 min, and then held at 70 °C for 15 min in a sand bath. Methylation was carried out with 200 μL of 14%(w/v) boron trifluoride in methanol (Sigma), at room temperature for 30 min; then, 0.2 mL of hexane was added, and the samples were stored at −20 °C until analysis by GC–MS (QP5000, from Shimadzu, USA) was in order.

FFA and CLA were resolved by said GC–MS using a DB5 capillary column (0.25 μm film × 0.25 mm × 30 m), operated in SIM mode. Helium was used as carrier gas, at a linear velocity of 35 cm s^{-1}. The sample (1 μL) was injected under splitless mode, at 250 °C. The temperature program started at 80 °C, and increased until 300 °C at a rate of 8 °C min^{-1}. The MS detector temperature was kept also at 250 °C.

2.5. Statistical analyses

A three-way analysis of variance (ANOVA) was initially planned to assess whether probiotic bacteria (i.e. *L. casei*-01, *L. acidophilus* La-5 or *B. lactis* B94), curdled matrix (i.e. control, probiotic or synbiotic) or maturation time (0–60 d) were statistically significant sources of variation. However, ANOVA is strictly valid only if the experimental errors are independently and normally distributed, and if they possess a constant variance. Since these homoschedasticity requirements were not met, non-parametric tests had to be applied to our dataset – which yielded reasonable conclusions, so resorting to the Central Limit Theorem was not warranted at all.

Differences between probiotic bacteria or between matrix types were accordingly analyzed via Mann–Whitney tests – which assess whether two observations come from the same distribution. Putative differences between the three probiotic bacteria or the three matrices were investigated using Kruskal–Wallis tests, at each storage time. Since statistical differences were detected between values, the influence of storage time was assessed via Wilcoxon test; this test involves comparing differences between measurements, so it requires data to be measured at an intermediate level (storage time). All tests were performed to a 5% significance level, using SPSS software v. 15.0 (SPSS, USA).

Additionally, principal component analysis (PCA) was performed to ascertain tentative relationships among the various samples of probiotic or synbiotic curdled milk matrices, and their corresponding storage times – based on the microbiological and chemical parameters assayed for. For the multivariate analysis, mean values of said microbiological and chemical parameters – assessed for each sample of probiotic or synbiotic curdled milk matrix, were used to build a standardized data matrix.

3. Results and discussion

Evidence for proteolytic and lipolytic activities brought about by each probiotic strain selected, on 10% milk agar and on 10% tributyrin agar, is tabulated in Table 1. Hydrolysis of casein – a phenomenon requiring availability of proteinases, was brought about chiefly by *L. paracasei* L26, *L. casei*-01 and *B. lactis* B94, and by *L. acidophilus* La-5, *L. acidophillus* Ki and *B. lactis* Bo to a much lesser extent. Lipolytic activity was apparent in the case of *L. acidophilus* La-5 and *B. lactis* B94, and much less for *B. animalis* BB-12 and *L. acidophilus* Ki. Based on the combined proteolytic and lipolytic capacities, *L. casei*-01, *L. acidophilus* La-5 and *B. lactis* B94 were selected for further inoculation in curdled milk matrices.

3.1. Viability of microorganisms and glycolysis

B. lactis B94 and *L. casei*-01, in both curdled matrices, attained maximum viable cell numbers of 10^9–10^{10} cfu g^{-1} between 5 and 30 d of storage; a statistically significant decreasing tendency in cell numbers ($p < 0.05$) was detected upon 30 d of storage (Fig. 1a,b) – and although higher values were generally observed in synbiotic matrices, they were not statistically significant ($p > 0.05$). On the other hand, *L. acidophilus* La-5 did not exhibit a significant growth in the curdled milk matrix; however, addition of FOS:Inulin contributed favorably to a continuous increase of viable cells until 30 d of ripening (Fig. 1c). Statistically significant differences were observed only between inoculated matrices and the corresponding control matrices ($p < 0.05$).

Despite the decrease in viable cells observed in the last 15 d of ripening – especially of *B. lactis* B94 and *L. casei*-01, the levels of each probiotic bacterium in curdled matrices were ca. 10^8 cfu g^{-1}. Although it is not possible to hypothesize the minimum dose of probiotics needed for a beneficial effect in the gut be observed, Douglas and Sanders (2008) concluded that studies showing positive effects at levels below 10^8 cfu d^{-1} are quite uncommon. Considering this heuristic guideline, our probiotic and synbiotic curdled milk

Table 1
Proteolytic and lipolytic activities of the various *Lactobacillus* (5) and *Bifidobacterium* (3) strains tested with.

Probiotic strain	Origin	Incubation conditions	
		10% Skim milk agar	10% Tributyrin agar
		72 h/37 °C	72 h/37 °C
L. paracasei L26	LAFTI®, DSM (Australia)	+ +	–
L. casei-01	Nu-trish®, Chr. Hansen (Denmark)	+ + +	–
L. acidophilus L10	LAFTI®, DSM (Australia)	–	–
L. acidophilus La-5	Nu-trish®, Chr. Hansen (Denmark)	+	+ +
B. animalis BB-12	Nu-trish®, Chr. Hansen (Denmark)	–	+
B. lactis B94	LAFTI®, DSM (Australia)	+ + +	+ +
L. acidophilus Ki	CSK (Netherlands)	+	+
B. lactis Bo	CSK (Netherlands)	+	–

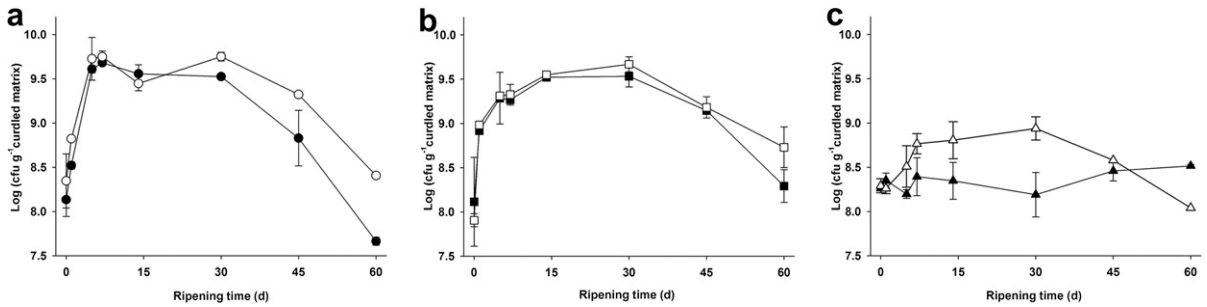

Fig. 1. Variation of viable cell numbers, throughout ripening, in curdled milk matrices inoculated with: *B. lactis* B94 (a), in probiotic matrix (●) and synbiotic matrix (○); *L. casei*-01 (b), in probiotic matrix (■) and synbiotic matrix (□); and *L. acidophilus* La-5 (c), in probiotic matrix (▲) and synbiotic matrix (△).

matrices proved in principle adequate food vectors during a shelf-life of 60 d, if delivery of sufficient concentrations of viable probiotic microorganisms able to promote potential health benefits is sought; however, this statement will need tests to be run under (at least) simulated gastrointestinal conditions for backup, especially because our matrices have a higher a_w than regular cheeses.

Cheese may be a potential synbiotic vector, in particular for the *L. acidophilus* strains under consideration here. According to Cardarelli et al. (2007), combination of probiotics and prebiotics resulted in a rather promising functional *petit-suisse* cheese, whereas presence of inulin had no implications upon growth and viability of *L. paracasei* in a synbiotic fresh cream cheese (Buriti et al., 2007).

Growth and survival behaviors of probiotics in the various curdled milk matrices assessed (i.e. control, probiotic and synbiotic) entertained significant correlations with the acidification profiles obtained throughout storage (Fig. 2); as expected, negative and positive correlations were found between log (viable cells) and pH ($r = -0.782$, $p < 0.05$) and titratable acidity ($r = 0.698$, $p < 0.05$). A lower acidification rate was observed in samples inoculated with *L. acidophilus* La-5 than with *L. casei*-01 or *B. lactis* B94; the latter were both independent of whether prebiotic supplementation existed. Such an observation is consistent with the lower production and utilization rates of lactic acid and lactose, respectively – as will be discussed below.

In agreement with the trends entertained by microbiological viability, no statistically significant differences were found for pH and titratable acidity among the three probiotic bacteria considered, or among probiotic and synbiotic matrices ($p > 0.05$) at each storage time. Statistically significant differences were observed only between inoculated matrices and the corresponding control matrices ($p < 0.05$). As expected, storage time was statistically significant ($p < 0.05$) in terms of acidification parameters.

Lactic acid was significantly produced in both probiotic and synbiotic matrices, when inoculated with either *L. casei*-01 or *B. lactis* B94 – and correlated well with lactose degradation, and frutose consumption over the initial 7-d period in the latter case (Table 2). In fact, *L. casei*-01 exhibited a more constant (and higher) rate of lactose utilization rate than *B. lactis* B94 throughout this period, irrespective of being in a probiotic or synbiotic matrix. On the other hand, *B. lactis* B94 showed a higher frutose utilization rate than *L. casei*-01 in the synbiotic matrices, coupled with a lower lactose utilization rate than in their probiotic counterparts; such observations reflect the metabolic preference of *B. lactis* B94 for the prebiotic compounds FOS and Inulin.

According to Su, Henriksson, and Mitchell (2007), both *L. casei* and *B. lactis* are able to grow in basal medium supplemented with FOS or Inulin. As previously realized with the pH-lowering effect (Fig. 2), the presence of prebiotic compounds contributes to a higher lactic acid production; a similar accelerating effect of Inulin – i.e. a reduction by ca. 10% of the fermentation time of different binary co-cultures, was reported by Oliveira, Perego, Converti, and de Oliveira (2009).

Acetic acid content increased in probiotic curdled matrices inoculated with *B. lactis* B94 – and to a greater extent than its *L. casei*-01 counterpart, likely as a consequence of the inherent metabolic activities of those microorganisms. Interestingly, acetic acid concentrations in the synbiotic curdled matrix containing *B. lactis* B94 were one half of their its probiotic counterpart, thus reflect a slight inhibition of the heterofermentative features of *B. lactis* B94; this – such may contribute to a better stability and organoleptic quality of the final functional cheeses. *L. acidophilus* La-5 revealed, among all strains tested, the mildest acidification capacity within the same period – both in terms of lactose utilization rate and lactic acid production rates; it was also the strain least affected by the presence of prebiotic compounds.

Despite the putative differences detected between probiotic and synbiotic curdled matrices, the results of Mann–Whitney tests did not indicate any statistically significant variation in lactose consumption and lactic acid production - hence suggesting that supplementation with FOS and Inulin does not affect overall post-acidification ($p > 0.05$).

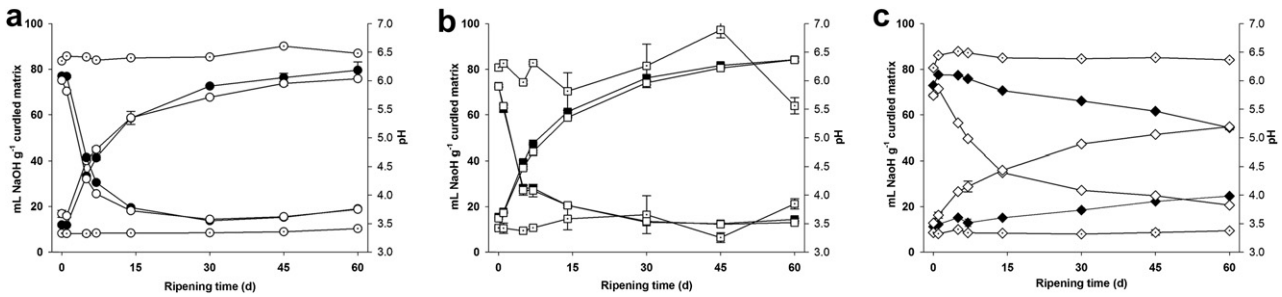

Fig. 2. Variation of titratable acidity and pH, throughout ripening, in curdled milk matrices inoculated with: *B. lactis* B94 (a), in control matrix (⊙), probiotic matrix (●) and synbiotic matrix (○); *L. casei*-01 (b), in control matrix (▣), probiotic matrix (■) and synbiotic matrix (□); and *L. acidophilus* La-5 (c), in control matrix (◈), probiotic matrix (◆) and synbiotic matrix (◇).

Table 2
Variation of concentrations of organics acids (lactic, acetic, formic and citric acids) and sugars (lactose, fructose and glucose), throughout ripening, in curdled milk matrices inoculated with each probiotic strain.

Probiotic bacterium	Curdled matrix	Ripening time (*d*)	(mg g^{-1})						
			Lactic acid	Acetic acid	Formic Acid	Citric Acid	Lactose	Fructose	Glucose
B. lactis B94	Probiotic	0	1.0 ± 0.0	0.23 ± 0.01	0.8 ± 0.0	1.76 ± 0.01	42.0 ± 0.5	<0.049[c]	1.51 ± 0.02
		1	0.7 ± 0.0	0.2 ± 0.0	1.1 ± 0.4	1.83 ± 0.03	42.2 ± 0.0	<0.049	1.6 ± 0.0
		5	3.49 ± 0.01	0.5 ± 0.0	0.4 ± 0.0	1.37 ± 0.02	39.0 ± 0.2	<0.049	1.62 ± 0.01
		7	5.1 ± 0.2	0.48 ± 0.02	0.29 ± 0.03	1.33 ± 0.04	36.9 ± 0.3	<0.049	1.60 ± 0.01
	Synbiotic	0	1.0 ± 0.0	<0.19[a]	0.71 ± 0.03	1.82 ± 0.04	41.7 ± 0.9	3.4 ± 0.1	0.98 ± 0.05
		1	0.8 ± 0.0	<0.19	0.79 ± 0.02	1.90 ± 0.02	41.9 ± 0.6	2.96 ± 0.01	1.02 ± 0.03
		5	4.6 ± 0.0	0.2 ± 0.0	0.34 ± 0.04	1.8 ± 0.2	42.1 ± 0.5	2.8 ± 0.2	1.4 ± 0.1
		7	5.66 ± 0.05	0.24 ± 0.01	0.26 ± 0.03	1.78 ± 0.03	41.0 ± 0.9	1.8 ± 0.1	1.51 ± 0.04
L. casei-01	Probiotic	0	1.33 ± 0.02	<0.19	<0.20[b]	1.79 ± 0.01	42.9 ± 0.1	<0.049	1.10 ± 0.02
		1	1.07 ± 0.06	<0.19	0.86 ± 0.01	1.93 ± 0.01	41.6 ± 0.7	<0.049	1.2 ± 0.3
		5	4.9 ± 0.6	0.23 ± 0.01	1.4 ± 0.2	1.78 ± 0.05	37.6 ± 0.4	<0.049	1.60 ± 0.03
		7	5.1 ± 0.8	0.24 ± 0.02	0.39 ± 0.01	1.6 ± 0.2	32.9 ± 4.3	<0.049	1.4 ± 0.2
	Synbiotic	0	1.6 ± 0.3	<0.19	<0.20	2.1 ± 0.3	46.1 ± 7.0	3.0 ± 0.5	1.2 ± 0.2
		1	1.2 ± 0.2	<0.19	0.8 ± 0.0	1.91 ± 0.07	40.8 ± 1.5	1.98 ± 0.00	1.2 ± 0.3
		5	4.7 ± 0.5	0.2 ± 0.0	1.1 ± 0.3	1.73 ± 0.03	36.2 ± 0.7	1.85 ± 0.03	1.01 ± 0.05
		7	5.2 ± 0.1	0.26 ± 0.01	0.4 ± 0.0	1.71 ± 0.03	35.7 ± 1.0	1.95 ± 0.08	1.02 ± 0.05
L. acidophilus La-5	Probiotic	0	1.27 ± 0.03	<0.19	<0.20	1.82 ± 0.01	42.7 ± 0.1	<0.049	1.34 ± 0.01
		1	1.15 ± 0.02	<0.19	<0.20	1.71 ± 0.05	39.6 ± 1.4	<0.049	1.25 ± 0.02
		5	1.20 ± 0.09	<0.19	<0.20	1.8 ± 0.1	43.7 ± 3.0	<0.049	1.33 ± 0.07
		7	0.92 ± 0.07	<0.19	<0.20	1.71 ± 0.02	39.7 ± 0.3	<0.049	1.25 ± 0.04
	Synbiotic	0	1.06 ± 0.04	<0.19	<0.20	1.79 ± 0.01	41.5 ± 0.1	3.0 ± 0.2	0.71 ± 0.02
		1	0.97 ± 0.01	<0.19	<0.20	1.8 ± 0.0	41.7 ± 0.5	2.7 ± 0.2	0.76 ± 0.02
		5	1.48 ± 0.01	<0.19	0.46 ± 0.01	1.81 ± 0.01	41.1 ± 0.7	2.5 ± 0.8	0.85 ± 0.00
		7	1.95 ± 0.06	<0.19	0.4 ± 0.0	1.81 ± 0.04	40.9 ± 0.6	1.84 ± 0.02	0.91 ± 0.01

[a] Detection limit of acetic acid.
[b] Detection limit of formic acid.
[c] Detection limit of fructose.

3.2. Proteolysis

WSN and NPN fractions increased throughout time, when compared with control matrices; this supports the actual role of probiotic enzymes in this proteolytic endeavor, besides that of animal rennet ones (Fig. 3). Statistically significant differences were indeed detected between inoculated matrices and the corresponding control matrices ($p < 0.05$).

L. casei-01 accounted for slightly higher values of the aforementioned proteolytic parameters in both curdled matrix types, up to 15 d at 12 °C. Probiotic and synbiotic matrices inoculated with *B. lactis* B94 also presented a significant level of proteolysis – especially

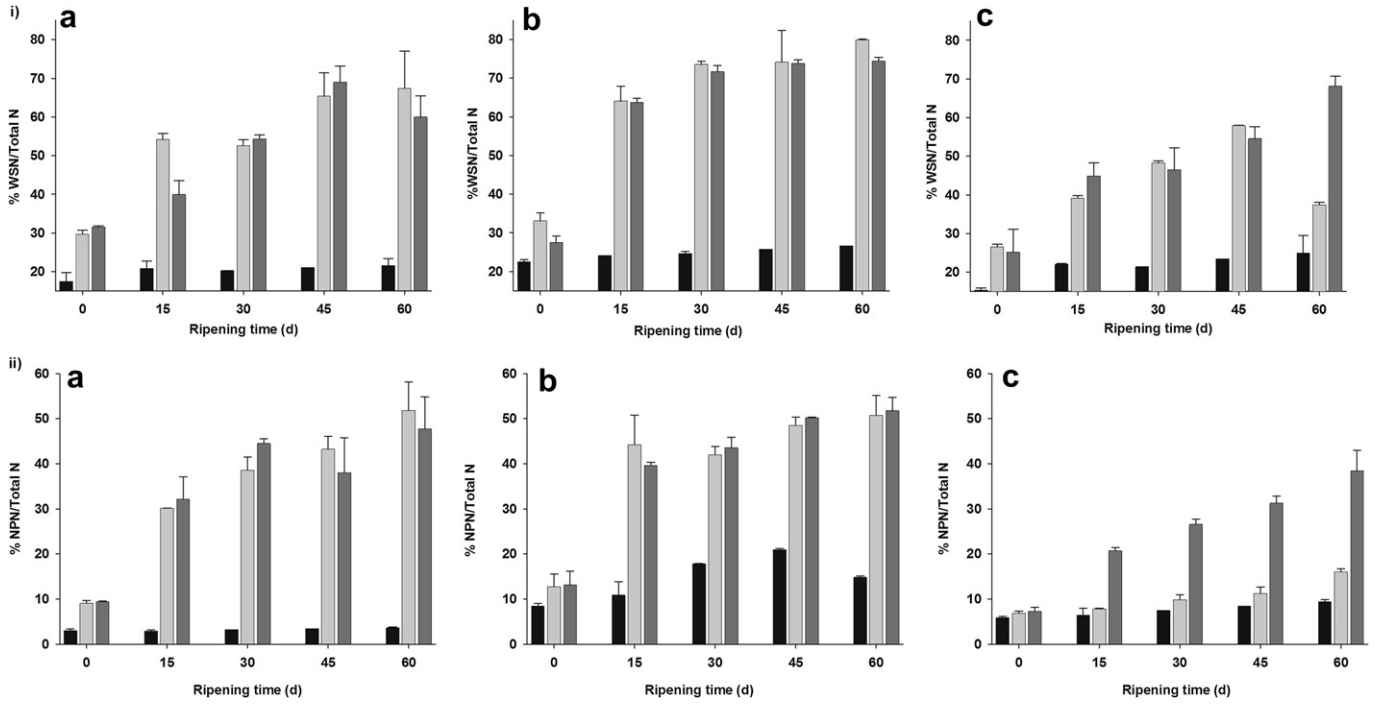

Fig. 3. Variation of WSN (i) and NPN (ii), throughout ripening, in control (■), probiotic (□) or synbiotic (▩) curdled milk matrices inoculated with: *B. lactis* B94 (a), *L. casei*-01 (b) or *L. acidophilus* La-5 (c).

by 60 d at 12 °C. However, compared with those inoculated with *L. casei*-01, this phenomenon was more gradual in the first 30 d of ripening. These results apparently corroborate those by Ong, Henriksson, and Shah (2007) – who reported higher degrees of casein hydrolysis in Cheddar cheese inoculated with *L. casei* or *L. paracasei*, as well as higher concentrations of free amino acids in all probiotic cheeses. However, they disagree with regard to the levels of WSN in probiotic and synbiotic matrices inoculated with *B. lactis* B94 – as Ong et al. (2007) claimed that *Bifidobacterium* sp. was a weak proteolytic bacterium, when compared with strains of *Lactobacillus*.

The lowest values of WSN and NPN were found in both types of matrix inoculated with *L. acidophilus* La-5; once again, this points at the role of microbial enzymes – recall that the viable counts of *L. acidophilus* La-5 were lower than those of *L. casei*-01 or *B. lactis* B94, which produced a lower microbial enzymatic activity on milk proteins or peptides. Although at a lower order of magnitude, it is still interesting to realize that a synbiotic effect was apparent upon NPN in curdled matrices inoculated with *L. acidophilus* La-5, thus leading to a gradual increase of NPN throughout time. According to Mann–Whitney test, significant differences of NPN values were observed between matrices inoculated with *L. casei*-01 and *L. acidophilus* La-5, at all sampling times ($p < 0.05$) except for 60 d. In terms of ripening evolution – and as far as both nitrogen fractions are concerned, Wilcoxon test demonstrated significant differences between 0 and 45 d of storage ($p < 0.05$), but not between 45 and 60 d ($p > 0.05$).

Several authors (e.g. Cruz, Buriti, Souza, Faria, & Saad, 2009; Gomes, Malcata, Klaver, & Grande, 1995; Gomes, Vieira, & Malcata, 1998) have shown that incorporation of probiotic bacteria in cheese does not generally affect primary proteolysis – which is brought about by residual coagulant or even milk plasmin; yet it affects secondary proteolysis, via increasing the total free amino acid content – which may indirectly contribute to cheese flavor and/or aroma (Cruz et al., 2009).

3.3. Lipolysis

Lipolysis was not a major event throughout storage, in all matrices – as can be observed in Fig. 4. The total free fatty acids (FFA) content increased from 4.41 to 5.15 mg g^{-1} in probiotic or synbiotic matrices over the 60-d ripening period, which represents an increase of 17%; from a statistical point of view, it was significant relative to the corresponding controls ($p < 0.05$). Ripened probiotic cheeses have been manufactured elsewhere, in which marginal changes in the extent of lipolysis have contributed to a positive overall quality of the final cheese (Gomes et al., 1998). In our case, although no statistically significant differences were unfolded between total FFA content in probiotic or synbiotic matrices, *L. casei*-01 was responsible for slightly higher contents.

Besides the differences in total FFA relative to the corresponding control matrices, the probiotic inoculated ones also unfolded significant differences in terms of qualitative FFA profile (see Fig. 4 and Table 3) – unlike the claim by some authors (Corbo, Albenzio, de Angelis, Sevi, & Gobetti, 2001; Gomes et al., 1998) that addition of probiotic cultures does not affect the FFA profile of cheese. The possible formation of conjugated linoleic acid (CLA) isomers in all probiotic and synbiotic matrices throughout ripening would be welcome in this study – as CLA (which comprises a mixture of positional and geometric isomers of octadecadienoic acid) entails two major isomers (i.e. cis-9:trans-11 and trans-10:cis-12) responsible for biological properties. Dietary CLA has in fact been reported to hold anticarcinogenic and antiatherogenic effects in animal models, as well as immunomodulating and fat reduction features (Bassaganya-Riera, Hontecillas, & Wannemuehler, 2002; Benjamin & Spener, 2009).

Microbial enzymes are known to be major contributors to CLA production: linoleic acid (LA) isomerase activity can convert LA to CLA, and is responsible for the significant differences in CLA content found between inoculated matrices and their control counterparts. The use of probiotic bacteria able to synthesize free CLA in cultured dairy products brings about additional health and nutritional benefits; studies focusing on CLA production during fermentation of milk by *Lactobacillus, Lactococcus, Streptococcus* and *Bifidobacterium* spp. have indeed shown this potential (Coakley et al., 2003; Jiang, Bjorck, & Fonden, 1998; Lin, Lin, & Lee, 1999).

In this study, CLA isomers – c_9t_{11}, $t_{10}c_{12}$ and t_9t_{12}, as well as α-linolenic and γ-linolenic acids, were detected by 15 d of ripening in both probiotic and synbiotic matrices. These results somehow contrast with those by Prandini, Sigolo, Tansini, Brogna, and Piva (2007) – who reported that the only CLA isomer detected in yoghurts, fermented milks and cheeses was c_9t_{11}. In any case, our study revealed a predominant production of c_9t_{11} by the three bacteria at stake – which underwent 2- to 4-fold increases between 15 and 60 d of storage: a level of 465 mg g^{-1} was attained by c_9t_{11} in the *B. lactis* B94 probiotic matrix by 60 d. In agreement with the total FFA results, storage time was a significant factor toward CLA production ($p < 0.05$). Finally, no significant differences were found between probiotic and synbiotic curdled matrices.

3.4. Principal component analysis

To aid in unfolding relationships among the data pertaining to the various probiotic or synbiotic curdled matrices (including storage time), based on microbiological (i.e. viable numbers) and

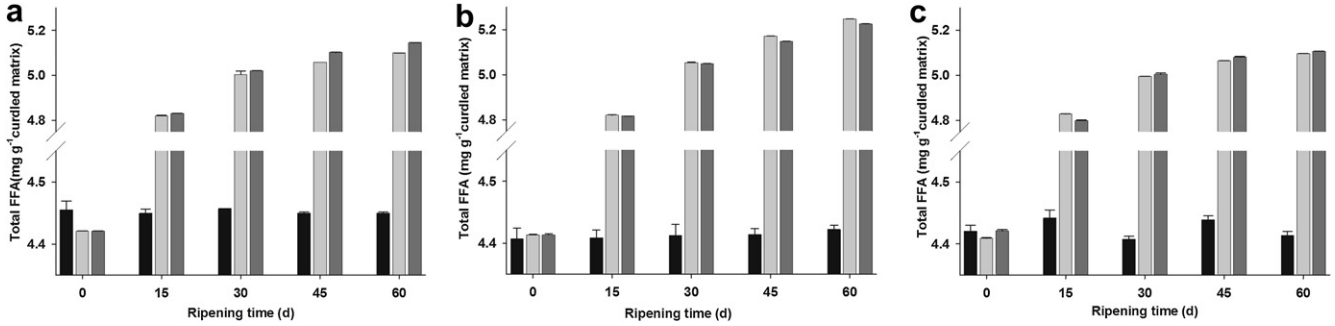

Fig. 4. Variation of total free fatty acids, throughout ripening, in control (■), probiotic (□) or synbiotic (▒) curdled milk matrices inoculated with: *B. lactis* B94 (a), *L. casei*-01 (b) or *L. acidophilus* La-5 (c).

Table 3
Variation of concentrations of long-chain, unsaturated fatty acids, throughout ripening, in curdled milk matrices inoculated with each probiotic strain.

Probiotic bacterium	Curdled matrix	Ripening time (*d*)	(μg g^{-1})							
			C16:1	C18:1	C18:2	CLA[a]			ALA[b]	GLA[c]
						c_9t_{11}	$t_{10}c_{12}$	t_9t_{12}		
B. lactis B94	Control	0	1063.3 ± 7.7	826.7 ± 2.1	88.7 ± 0.1	<0.048[d]	<0.059[e]	<0.050[f]	<0.077[g]	<0.066[h]
		60	1055.1 ± 1.7	824.1 ± 0.0	88.3 ± 0.2	<0.048	<0.059	<0.050	<0.077	<0.066
	Probiotic	0	1053.6 ± 0.0	820.7 ± 0.1	88.7 ± 0.1	<0.048	<0.059	<0.050	<<0.077	<0.066
		15	1058.6 ± 0.2	815.1 ± 0.1	87.4 ± 3.1	88.2 ± 0.3	66.1 ± 0.1	88.8 ± 0.2	61.8 ± 0.2	66.1 ± 0.2
		30	1059.6 ± 0.1	813.3 ± 0.2	89.3 ± 2.1	244.5 ± 0.2	74.6 ± 0.1	95.4 ± 0.2	64.1 ± 0.1	70.3 ± 0.1
		45	1060.2 ± 0.0	808.0 ± 0.0	91.7 ± 0.0	263.8 ± 0.0	82.7 ± 0.0	97.6 ± 0.0	64.9 ± 0.0	72.0 ± 0.0
		60	1062.7 ± 0.1	804.7 ± 0.3	91.6 ± 0.2	285.1 ± 0.1	85.2 ± 0.1	99.7 ± 0.0	65.7 ± 0.1	73.1 ± 0.2
	Synbiotic	0	1057.4 ± 0.1	824.3 ± 0.2	87.8 ± 0.1	<0.048	<0.059	<0.050	<0.077	<0.066
		15	1061.2 ± 0.1	822.3 ± 0.1	89.9 ± 0.1	90.6 ± 0.1	67.5 ± 0.1	90.7 ± 0.1	61.4 ± 0.2	67.9 ± 0.2
		30	1062.6 ± 0.1	819.4 ± 0.1	91.9 ± 0.1	247.4 ± 0.1	74.9 ± 0.1	96.5 ± 0.1	62.4 ± 0.1	71.1 ± 0.1
		45	1063.5 ± 0.1	816.6 ± 0.1	93.3 ± 0.1	291.4 ± 0.5	84.9 ± 0.2	99.2 ± 0.3	64.7 ± 0.1	73.0 ± 0.1
		60	1066.5 ± 0.1	813.6 ± 0.1	94.5 ± 0.1	308.4 ± 0.1	90.0 ± 0.1	100.4 ± 0.3	67.0 ± 0.1	74.0 ± 0.1
L. casei-01	Control	0	1043.7 ± 8.8	822.6 ± 1.1	86.4 ± 0.2	<0.048	<0.059	<0.050	<0.077	<0.066
		60	1058.7 ± 0.1	823.1 ± 1.6	86.5 ± 0.2	<0.048	<0.059	<0.050	<0.077	<0.066
	Probiotic	0	1045.5 ± 0.2	821.3 ± 0.2	84.5 ± 0.2	<0.048	<0.059	<0.050	<0.077	<0.066
		15	1051.7 ± 0.4	787.2 ± 0.1	88.4 ± 0.1	88.5 ± 0.2	66.4 ± 0.1	89.8 ± 0.2	59.3 ± 0.1	66.9 ± 0.2
		30	1061.4 ± 0.2	757.1 ± 0.3	96.9 ± 0.3	247.2 ± 0.2	76.3 ± 0.2	95.7 ± 0.1	65.0 ± 0.1	73.6 ± 0.1
		45	1064.2 ± 0.2	736.6 ± 0.5	104.7 ± 0.2	302.5 ± 0.2	83.1 ± 0.3	100.7 ± 0.1	70.3 ± 0.1	78.5 ± 0.2
		60	1069.3 ± 0.2	729.6 ± 0.3	111.4 ± 0.3	320.9 ± 0.1	87.8 ± 0.3	106.0 ± 0.4	75.5 ± 0.0	81.3 ± 0.2
	Synbiotic	0	1046.6 ± 0.6	821.4 ± 0.1	84.0 ± 0.1	<0.048	<0.059	<0.050	<0.077	<0.066
		15	1050.9 ± 0.1	786.5 ± 0.1	88.0 ± 0.0	88.1 ± 0.1	66.1 ± 0.1	89.4 ± 0.1	59.5 ± 0.0	66.6 ± 0.2
		30	1058.4 ± 0.3	757.9 ± 0.1	96.9 ± 0.3	247.4 ± 0.2	76.1 ± 0.0	95.7 ± 0.2	64.9 ± 0.0	73.5 ± 0.2
		45	1062.8 ± 0.1	735.0 ± 0.2	105.7 ± 0.3	297.1 ± 0.1	81.2 ± 0.1	100.9 ± 0.0	70.2 ± 0.0	76.7 ± 0.2
		60	1066.2 ± 0.2	720.7 ± 0.2	112.4 ± 0.1	318.3 ± 0.2	87.2 ± 0.1	105.1 ± 0.1	74.9 ± 0.2	80.9 ± 0.1
L. acidophilus La-5	Control	0	1057.9 ± 5.8	821.3 ± 1.0	87.8 ± 0.1	<0.048	<0.059	<0.050	<0.077	<0.066
		60	1053.8 ± 9.2	823.1 ± 1.0	87.3 ± 0.3	<0.048	<0.059	<0.050	<0.077	<0.066
	Probiotic	0	1052.1 ± 2.1	821.1 ± 0.2	87.5 ± 0.3	<0.048	<0.059	<0.050	<0.077	<0.066
		15	1062.6 ± 0.1	820.2 ± 0.1	89.3 ± 0.1	92.0 ± 0.3	69.3 ± 0.1	93.4 ± 0.3	61.3 ± 0.1	69.1 ± 0.0
		30	1066.6 ± 0.8	817.6 ± 0.1	89.8 ± 0.1	240.9 ± 0.3	73.9 ± 0.3	94.0 ± 0.2	63.5 ± 0.1	69.6 ± 0.1
		45	1069.9 ± 0.1	814.1 ± 0.1	90.4 ± 0.0	283.2 ± 0.1	81.6 ± 0.1	96.2 ± 0.1	64.8 ± 0.1	71.1 ± 0.1
		60	1073.8 ± 0.1	813.0 ± 0.1	92.6 ± 0.2	284.2 ± 0.1	84.2 ± 0.1	96.5 ± 0.1	66.4 ± 0.1	71.9 ± 0.2
	Synbiotic	0	1057.9 ± 0.1	822.5 ± 0.6	87.0 ± 0.1	<0.048	<0.059	<0.050	<0.077	<0.066
		15	1059.6 ± 0.6	820.5 ± 0.1	88.7 ± 0.1	87.5 ± 0.1	65.3 ± 0.1	87.6 ± 0.1	57.7 ± 0.1	67.0 ± 0.1
		30	1062.2 ± 3.0	817.5 ± 0.1	90.8 ± 0.1	249.0 ± 0.1	73.2 ± 0.1	94.9 ± 0.0	62.6 ± 0.2	70.1 ± 0.2
		45	1073.6 ± 2.3	815.1 ± 0.2	91.5 ± 0.1	285.5 ± 0.1	81.8 ± 0.1	97.1 ± 0.2	65.4 ± 0.1	71.8 ± 0.1
		60	1079.7 ± 0.2	813.0 ± 0.1	92.4 ± 0.2	288.6 ± 0.1	83.2 ± 0.1	96.1 ± 0.2	66.0 ± 0.1	72.7 ± 0.0

[a] Conjugated linoleic acid isomers.
[b] α-linolenic acid.
[c] γ-linolenic acid.
[d] Detection limit of CLA c_9t_{11}.
[e] Detection limit of CLA $t_{10}c_{12}$.
[f] Detection limit of CLA t_9t_{12}.
[g] Detection limit of ALA.
[h] Detection limit of GLA.

chemical parameters (i.e. pH, titratable acidity, WSN, NPN, total FFA and total CLA), a principal component analysis was performed. As observed in the resulting PCA biplot (Fig. 5), four main groups can be pinpointed along the first two components – which explained 91.3% of the total variation.

The first group (I) – located in the lower right quadrant, included only samples of probiotic and synbiotic matrices by 0 d of storage, as well as the synbiotic matrix inoculated with *L. acidophillus* La-5 by 15 d. These samples did not correlate with any parameter in particular, besides higher values of pH. Group (II), located in the upper right quadrant, included only samples of probiotic curdled matrices inoculated with *L. acidophillus* La-5 between 15 and 60 d of storage. This group encompasses the lower microbiological counts observed in these probiotic matrices – and consequently the decreased enzymatic activity in terms of glycolysis, proteolysis and lipolysis. The third group (III), displayed in the upper left quadrant, is constituted by all 60-day probiotic and synbiotic curdled matrices, as well as the 45-d synbiotic curdled matrices inoculated with *B. lactis* B94 or *L. acidophilus* La-5. These samples are characterized by higher values of WSN, and total FFA or CLA. Such a graphical distribution is consistent with influence of the FOS:Inulin mix – which justifies the differences in terms of proteolysis and lipolysis levels among the curdled matrices, especially those inoculated with *L. acidophilus* La-5. Finally, the fourth group (IV) – distributed all over the lower left quadrant, includes mainly data pertaining to 15–45-d probiotic or synbiotic curdled matrices, inoculated with *L. casei*-01 or *B. lactis* B94. These 45-d samples are apparently better correlated with NPN fraction and titratable acidity, whereas those by 15 or 30 d of storage are correlated chiefly with viable counts of probiotic bacteria.

According to the data depicted in Table 4 and Fig. 5, all variables contributed negatively to the first component (*x*-axis), but pH. Furthermore, all variables possessed approximately the same weight along this component, except the logarithm of viable cell counts of probiotic bacteria. With regard to the second component (*y*-axis), the highest weight was entertained by the microbiological parameter, together with titratable acidity and NPN – which also

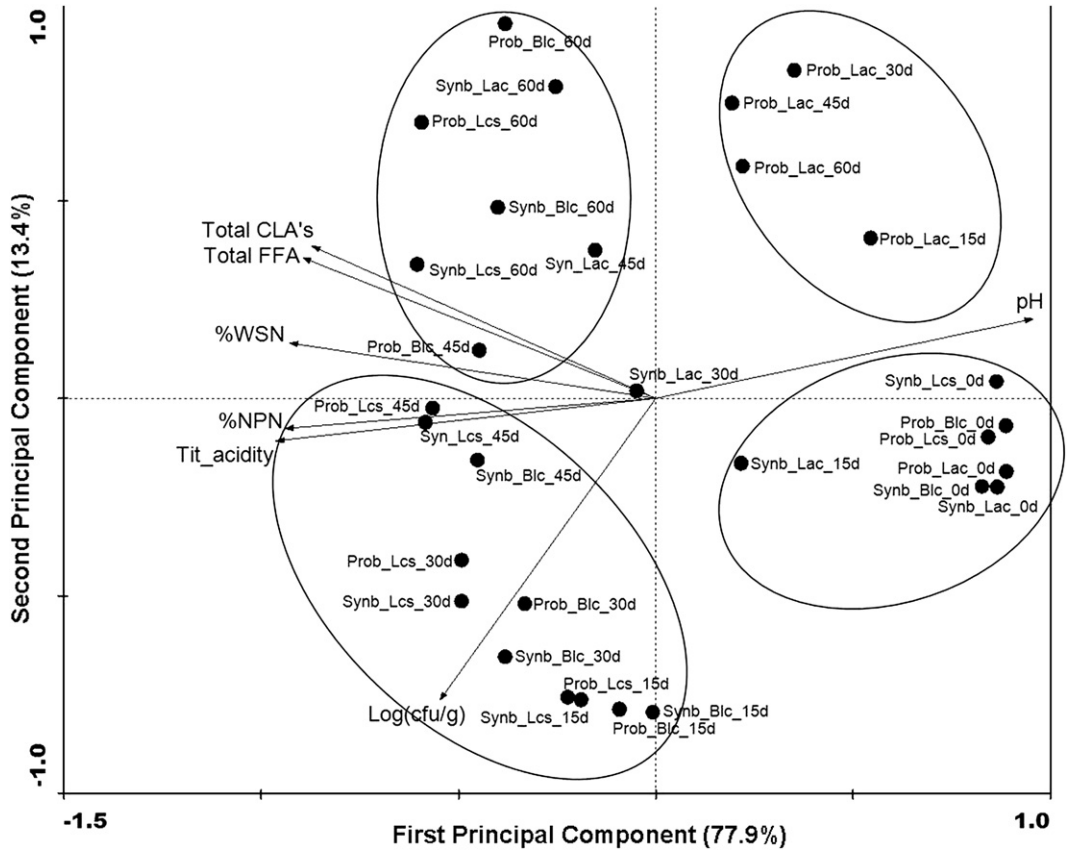

Fig. 5. Results of principal component analysis: biplot including samples of curdled milk probiotic or synbiotic matrices throughout ripening (e.g. Prob_Blc_60d: probiotic matrix inoculated with *B. lactis* B94 by 60 d of ripening; or Synb_Lcs_15d: synbiotic matrix inoculated with *L. casei*-01 by 15 d of ripening), and associated parameter values.

Table 4
Coefficients of the two principal components, accounting for 91.3% of total variation.

Variable	Component 1	Component 2
Log (viable numbers)	−0.235	**−0.789**
pH	**0.410**[a]	0.208
Titratable acidity	**−0.412**	−0.111
WSN	**−0.398**	0.145
NPN	**−0.402**	−0.078
Total FFA	**−0.384**	**0.368**
Total CLA	**−0.374**	**0.399**

[a] Significant coefficients are denoted in bold.

contributed negatively along this axis. Total FFA and CLA were the parameters with dominant positive contribution toward this component. The PCA was also able to discriminate the various curdled milk matrices according to degree of maturation.

4. Conclusions

The viable cell numbers of the three probiotic bacteria under assessment, in both probiotic and synbiotic curdled matrices, attained in general values between 10^8 and 10^{10} cfu g^{-1} throughout the 60-d ripening period; this was a clue to robustness with regard to survival, so a great potential exists for their eventual inclusion as part of a starter culture (rather than a plain additive) for inoculation in a probiotic or synbiotic cheese matrix. Despite the differences observed between probiotic and synbiotic curdled matrices, in terms of lactose consumption or lactic acid production, supplementation with FOS:Inulin did not significantly affect overall post-acidification; technological benefits were gained from their addition, as far as growth and survival of *L. acidophilus* La-5 is concerned.

Furthermore, the proteolysis and lipolysis indices – in particular the presence of CLA isomers, as well as α-linolenic and γ-linolenic acid after 15 d of ripening, in both probiotic and synbiotic curdled matrices, unfolds a great technological promise especially in the case of *B. lactis* B94 and *L. casei*-01, toward novel cheese products. Complementary studies are thus warranted to assess the effect of increasing prebiotic concentration upon growth, metabolic activity and survival of probiotic strains, besides nutritional convenience to the host himself (of at least a 3-g content per portion); however, organoleptic assessment will also be required to anticipate eventual consumer acceptance prior to commercial launching of any new food product based thereon.

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[1] *The chosen references (as key references) represent some recent and fundamental information on research about synbiotic potential (Buriti et al., 2007), probiotic cheese and its interest as a functional food (Cruz et al., 2009; Grattepanche et al., 2008; Ong et al., 2007) and CLA in dairy products (Prandini et al., 2007).