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# Whey fermentation by thermophilic lactic acid bacteria: Evolution of carbohydrates and protein content

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**Abstract**

Whey, a by-product of the cheese industry usually disposed as waste, is a source of biological and functional valuable proteins. The aim of this work was to evaluate the potentiality of three lactic acid bacteria strains to design a starter culture for developing functional whey-based drinks. Fermentations were performed at 37 and 42 °C for 24 h in reconstituted whey powder (RW). Carbohydrates, organic acids and amino acids concentrations during fermentation were evaluated by RP-HPLC. Proteolytic activity was measured by the *o*-phthaldialdehyde test and hydrolysis of whey proteins was analyzed by Tricine SDS–PAGE. The studied strains grew well (2–3 log cfu/ml) independently of the temperature used. *Streptococcus thermophilus* CRL 804 consumed 12% of the initial lactose concentration and produced the highest amount of lactic acid (45 mmol/l) at 24 h. *Lactobacillus delbrueckii* subsp. *bulgaricus* CRL 454 was the most proteolytic (91 μg Leu/ml) strain and released the branched chain amino acids Leu and Val. In contrast, *Lactobacillus acidophilus* CRL 636 and *S. thermophilus* CRL 804 consumed most of the amino acids present in whey. The studied strains were able to degrade the major whey proteins, α-lactalbumin being degraded in a greater extent (2.2–3.4-fold) than β-lactoglobulin. Two starter cultures were evaluated for their metabolic and proteolytic activities in RW. Both cultures acidified and reduced the lactose content in whey in a greater extent than the strains alone. The amino acid release was higher (86 μg/ml) for the starter SLb (strains CRL 804 + CRL 454) than for SLa (strains CRL 804 + CRL 636, 37 μg/ml). Regarding α-lactalbumin and β-lactoglobulin degradation, no differences were observed as compared to the values obtained with the single cultures. The starter culture SLb showed high potential to be used for developing fermented whey-based beverages.


*Keywords:* Lactic acid bacteria; Whey; Fermentation; Proteolysis; β-Lactoglobulin

## 1. Introduction

Whey is a by-product of the cheese industry which was often disposed as a waste in the past, causing high environmental contamination. Considerable efforts have been made over the past years to find new outlets for whey utilization and reduce environmental pollution (González-Martínez et al., 2002). Nowadays technologies, such as ultra filtration and spray drying, are non-expensive and allow the separation of different fractions of whey (lactose, whey proteins as whey protein concentrate, lactalbumin, lactoglobulin, etc.) that are commercialized in the food and pharmaceutical industries. Whey and its protein concentrate are used as ingredients in the food industry mainly due to their foaming and emulsifying properties (Ji and Hauque, 2003). Furthermore, whey proteins provide an excellent way to fortify foods increasing the nutritional quality of cheese, dairy desserts, bakery products, etc. (Mistry et al., 1996; Kenny et al., 2001; Carunchia Whetstine et al., 2005).

Liquid whey is composed of lactose (5%), water (93%), proteins (0.85%), minerals (0.53%) and a minimum amount of fat (0.36%). The main whey proteins are β-lactoglobulin (BLG) (58%) and α-lactalbumin (ALA) (13%) while immunoglobulins, serum albumins and proteose peptones are present in lesser extent.

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The BLG is the most abundant whey protein secreted in ruminant milk and is highly resistant to gastric digestion. This protein is the major cause of milk intolerance and/or allergenicity in humans since it is absent in human milk (Høst and Halken, 1998). Industrial treatments such as sterilization, heating, or hydrostatic high pressure enhance the digestibility of whey proteins, especially BLG. Hydrolysis of BLG can potentially reduce the allergenic content and, moreover, improve its digestibility since the released peptides could be better absorbed in the intestinal tract than intact proteins (Peñas et al., 2006). Enzymes, acids and alkalis can carry out this degradation but the process tends to be difficult to control and yields products with reduced nutritional qualities. Moreover, chemical hydrolysis can form toxic substrates like lyso-alanine (Sinha et al., 2007).

Lactic acid bacteria (LAB) have been extensively used as starter cultures in the fermented food industry due to their metabolic activity on proteins, sugars and lipids, thus contributing to food digestibility and preservation as well as the improvement of texture and sensory profile of the end product (Wood, 1997). These microorganisms have complex nutritional requirements (Hebert et al., 2000; Morishita et al., 1981). The concentration of free amino acids in milk and whey are very limited, thus the sustained growth of LAB depends on the production of proteinases, peptidases and specific peptide and amino acid transport systems (Kunji et al., 1996; Mierau, et al., 1996).

Whey fermentation by LAB could decrease the high lactose content in whey, producing mainly lactic acid and other metabolites such as aroma compounds contributing to the flavor and texture and increasing carbohydrate solubility and sweetness of the end product (Mauriello et al., 2001). Proteolysis of BLG and ALA by LAB could increase their digestibility and hydrolyze allergenic peptides (Schlimme et al., 1990; Bertrand-Harb et al., 2003). A few LAB species such as *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* have been studied recently for their ability to degrade whey proteins in milk products (Morr and Foegeding, 1990; Bertrand-Harb et al., 2003; El-Zahar et al., 2003). Also, Prioult et al. (2005) suggested that *Bifidobacterium lactis* NCC362 could be a potential probiotic for preventing cow's milk allergy through degradation of the allergenic portion of BLG generated by trypsin/chymotrypsin hydrolysis. Furthermore, fermentation of milk products could increase the susceptibility of BLG towards pepsin digestion resulting in reduced stability of this allergenic protein, decreasing in consequence the immunoreactive BLG content of fermented products (Maier et al., 2006).

The beverage market represents a large and growing industry. Recently, there has been a widespread increase on the consumption of lactic drinks of which whey-based beverages constitute an emerging segment of non-conventional dairy products that require sensory, physical and chemical characterization for quality control and product development (Gallardo-Escamilla et al., 2007). Manufacture of beverages through lactic fermentations that can provide desirable sensory profiles has already been considered an option to add value to whey (Salminen et al., 1991; Skudra et al., 1998). The production of a functional beverage produced upon whey fermentation by LAB could be an interesting alternative for whey utilization.

The aim of this work was to evaluate the technological potential of thermophilic LAB strains in reconstituted whey (RW) and their ability to hydrolyze the major whey protein BLG, to further develop a functional/healthy hydrolyzed whey-based beverage.

## 2. Methods

### 2.1. Microorganisms and media

The strains *Lactobacillus acidophilus* CRL 636, *L. delbrueckii* subsp. *bulgaricus* CRL 454 and *S. thermophilus* CRL 804 used in this work were obtained from the Culture Collection of the Centro de Referencia para Lactobacilos (CERELA), San Miguel de Tucumán, Argentina. Cultures were stored at −20 °C in 10% (w/v) sterile reconstituted skim milk containing 0.5% (w/v) yeast extract, 1.0% (w/v) glucose and 10% (v/v) glycerol.

Sweet cheese whey powder (kindly provided by DANONE S.A., Argentina) was obtained from the cheese manufacture by rennet addition; whey was then demineralized and crystallized before drying. Whey was reconstituted with distilled water to 10% (w/v) final concentration and the pH was adjusted to 6.7 with 2 M NaOH. The RW was heat treated at 80 °C for 30 min (Fialho et al., 1999), stored at 4 °C (no longer than 1 week) and used as fermentation medium. The composition (%, w/w) of the whey powder employed in this study was the following: fat, 2.0; protein, 10.0; moisture, 3.0; lactose, 76.5; and sodium salts, 0.4.

### 2.2. Fermentation conditions

Cultures were transferred twice in RW prior to experimental use; 16-h-old cultures were used as inoculums (2%, v/v) individually or combined in pairs at a 1:1 (v/v) ratio. Fermentations were performed in sealed bottles containing 200 ml of RW and incubated at 37 or 42 °C for 24 h. Samples were aseptically withdrawn every hour during 12 h and at 24 h of incubation and mixed uniformly with a vortex before diluting. Cell viability was determined by plating appropriate dilutions of the cultures in MRS agar (MRS Britania, Buenos Aires, Argentina, plus 15 g/l agar), Plates were incubated at 37 °C for 48 h and the colony-forming units (cfu)/ml were determined. Results were expressed as log cfu/ml. pH measurements were determined with a digital pH meter (Altronix TPX 1, New York, USA).

### 2.3. Analysis of metabolites

Sugar content (lactose, galactose and glucose) and organic (lactic, acetic, formic) acids production were analyzed during fermentation by high performance liquid chromatography (HPLC). HPLC was performed using a Knauer Smartline System HPLC (Berlin, Germany) with a Knauer Smartline 2600 UV detector (Berlin, Germany) fitted with a Biorad Aminex HPX-87H column (300 × 7.8 mm, Hercules, CA, USA). The operating conditions were the following: 5 mM $H_2SO_4$ was used as eluent at a flow rate of 0.6 ml/min and an internal temperature of 41 °C.

Citric acid was measured using the enzymatic Bio Analysis/Food Analysis Kit (Boerhinger Mannhein/R Biopharm AG, Darmastadt, Germany). Fermented RW samples were placed in a water bath at 80 °C for 15 min, centrifuged at 13,000 rpm for 10 min and the supernatants used for citric acid determination. Samples were treated according to the manufacturer's indications.

### 2.4. Proteolysis assessment

Proteolytic activity by LAB was measured every hour during 12 h and at 24 h incubation by the *o*-phthaldialdehyde (OPA) test (Church et al., 1983). The increase in optical density at 340 nm ($OD_{340}$) relative to the control was determined using a VERSAmax$^{TM}$ Tunable Microplate reader (Sunnyvale, CA, USA). The OPA solution contained: 2.5 ml of 20% (w/v) SDS, 25 ml of 100 mM sodium tetraborate (Sigma Chemical Co., St Louis, MO, USA), 40 mg of OPA (Sigma Chemical Co.) (previously dissolved in 1 ml methanol), 100 μl of 2-mercaptoethanol (Merck, Darmstadt, Germany) and distilled water up to a 50 ml final volume. Fermented samples were incubated with 0.75 M trichloroacetic acid (Sigma Chemical Co.) at a sample: TCA ratio = 1:3 at 4 °C for 30 min and centrifuged (5000 rpm, 10 min). A 10 μl supernatant aliquot of this mixture was added to 0.2 ml of OPA reagent and incubated at room temperature for 20 min until the $OD_{340}$ was read in the microplate spectrophotometer. Proteolytic activity was arbitrarily expressed as μg leucine (Leu) released/ml using a standard curve of L-leucine (BDH Chemicals Ltd., Poole, UK).

### 2.5. Free amino acids determination

The free amino acid content of RW and the fermented RW samples (37 °C) was determined. The amino acids were extracted as described by Jones et al. (1981). The reaction was prepared by mixing 200 μl of RW with 2% (w/v) of SDS (dissolved in 0.4 M sodium borate buffer, pH 9.5) and 200 μl of the OPA methalonic solution. The mixture was shaken, incubated for 1 min and the reaction was stopped by adding 400 μl of 0.1 M sodium phosphate buffer (pH 4.0) and filtered through 0.2 μm nylon membrane (Alltech Associates Inc., Deerfield, IL, USA). The amino acids used as standards (Sigma Chemical Co.) were treated in the same way as the fermented samples.

The amino acid content of the samples was analyzed by reverse phase-high performance liquid chromatography (RP-HPLC) with an ISCO model 2360 (ISCO, Inc., Lincoln, NE, USA) fitted with an Ultrasphere ODS $C_{18}$ column (4.6 × 25 mm, particle size 5 μm, Beckman Instruments Inc., Fullerton, CA, USA). The equipment was coupled with an ISCO model 2350 pump (ISCO Inc.) and an ISCO FL-2 fluorescence detector (ISCO Inc.). The operating conditions were the following: flow rate, 1.7 ml/min; solvent A, tetrahydrofurane:methanol:sodium acetate (1:19:80, v/v/v) 0.05 M pH 5.9 (Sigma Chemical Co.) in ultra pure water; solvent B, methanol:sodium acetate 0.05 M pH 5.9 (80:20, v/v) (Sigma Chemical Co.). Elution was performed by applying a linear gradient of 100% solvent A over 1 min, then 0–50% solvent B over the following 20 min, and 50–100% solvent B over the last 20 min. Absorbance was recorded at 305–395 nm and 430–460 nm excitation and emission wavelengths, respectively. The injection volume of derivatized amino acids was 10 μl. The HPLC was coupled with the software Chem Research 150 Data System 3.0.2 (1994, ISCO Inc.). All the amino acids, except proline, cystein and methionine, were determined under the assayed conditions. Amino acids concentration was expressed in μg/ml.

### 2.6. Hydrolysis of whey proteins

Whey proteins degradation was measured in 12 and 24-h-incubation samples by Tricine SDS–PAGE electrophoresis (Schagger and von Jagow, 1987) and performed as follows: fermented whey samples (8 μl) were suspended in 5 μl of sample buffer (6.5 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS and 5% β-mercaptoethanol) and heated at 100 °C for 5 min. The corresponding controls (non-fermented, heated RW), and the molecular weight (MW) marker (Protein marker, Broad Range, 2–212 kDa, New England Biolabs Inc., Ipswich, MA, USA) were loaded separately. Tricine SDS–PAGE were carried out on 17% (w/v) polyacrylamide gels on vertical slab electrophoresis cells (BIO RAD Mini PROTEAN® 3 System, Hercules, CA, USA) for 4 h at 60 V. Coomassie brilliant blue R250 was used for staining the gels. The degradation of the whey proteins was evaluated by densitometric analysis of gels by using the QuantiScan software (BIOSOFT 1.5, USA).

### 2.7. Strains compatibility

Strains compatibility was evaluated by the plate diffusion assay (Parente et al., 1995). Briefly, overnight cultures grown in MRS were washed twice with saline solution and resuspended at the same volume. The plates were prepared by pouring 15 ml of MRS soft agar (MRS plus 7 g/l agar) containing 60 μl of the cell suspension on the same agar.

After overlay solidification, 5 mm diameter wells were made by using sterilized plastic cylinders. Wells were inoculated with 60 µl of culture supernatants from the other strains. After incubation at 37 °C for 16 h, the appearance of inhibition zones were observed.

### 2.8. Statistical analysis

All assays were carried out in duplicate and results were expressed as means. Variations in all assays were less than 10%.

## 3. Results

### 3.1. Growth and metabolites production by LAB in RW

*L. delbrueckii* subsp. *bulgaricus* CRL 454, *S. thermophilus* CRL 804 and *L. acidophilus* CRL 636 were able to grow in RW either at 37 or 42 °C, but populations remained under $10^9$ cfu/ml (Fig. 1a–c). The growth temperature had no effect on the growth rate of *L. acidophilus* CRL 636 ($\mu_{max}$: 0.23 and 0.21 $h^{-1}$ at 37 and 42 °C, respectively) and *L. delbrueckii* subsp. *bulgaricus* CRL 454 (0.51 and 0.45 $h^{-1}$ at 37 and 42 °C, respectively) while, *S. thermophilus* CRL 804 grew better at 37 °C ($\mu_{max}$: 0.31 $h^{-1}$) than at 42 °C (0.13 $h^{-1}$). At 24 h incubation, cell growth (Δlog cfu/ml) of *S. thermophilus* CRL 804 and *L. acidophilus* CRL 636 was 0.6- and 0.4-fold higher at 37 °C than at 42 °C, respectively. No differences were observed at both temperatures for *L. delbrueckii* subsp. *bulgaricus* CRL 454. RW fermented by *S. thermophilus* CRL 804 showed a constant pH reduction since the beginning of the fermentation (Fig. 1a) whilst pH started to decrease after 7 h for the *L. delbrueckii* subsp. *bulgaricus* CRL 454 and *L. acidophilus* CRL 636 cultures. *L. delbrueckii* subsp. *bulgaricus* CRL 454 displayed the lowest pH values (~4.5) at 24 h. In general, the pH decrease was similar at both temperatures. Only lactic acid was produced by all the strains reaching values of 4–45 mmol/l (24 h), the highest concentration being produced by *S. thermophilus* CRL 804. Neither acetic acid nor formic acid was detected, as expected for homofermentative LAB strains. The citric acid concentration present in RW (9 mmol/l) started to decrease (6–24%) after 12 h incubation, no significative differences were observed on citrate consumption either at 37 or 42 °C, except for *S. thermophilus* CRL 804 which showed a marked reduction (40%) at 37 °C at 24 h (Fig. 1a). Lactose consumption by LAB was within a range of 1.8–11.6% of original values at 24 h incubation, *S. thermophilus* CRL 804 being the strain which consumed the highest lactose amount. An accumulation of galactose (3–23 mmol/l) in the cultures of *L. acidophilus* CRL 636 and *S. thermophilus* CRL 804 was detected in 24 h cultures while the glucose moiety was completely metabolized for the strains' growth since it was not detectable throughout the fermentation.

### 3.2. Proteolysis by LAB in RW

The proteolytic activity of the studied strains was not affected by the temperatures (37 or 42 °C) used. *L. delbrueckii* subsp. *bulgaricus* CRL 454 showed higher proteolytic activity (90 µg Leu/ml) than *S. thermophilus* CRL 804 (30 µg Leu/ml) and *L. acidophilus* CRL 636 (36 µg Leu/ml) after 24 h of incubation. The amino acid analysis of non-fermented whey revealed only the presence of glutamic acid (Glu), glycine (Gly), threonine–arginine (Thr–Arg) and alanine (Ala). The strains displayed a different behavior regarding the consumption and release of amino acids (Fig. 2a–c). *S. thermophilus* CRL 804 and *L. acidophilus* CRL 636 consumed most of the amino acids originally present in whey although in a different extent. Thr–Arg was consumed (27–77%) by all the strains after 24 h incubation. *L. delbrueckii* subsp. *bulgaricus* CRL 454 (Fig. 2c) released Glu (4.5 µg/ml), Ser–His (17 µg/ml), Gly (33 µg/ml), valine (Val; 9.2 µg/ml) and Leu (4.6 µg/ml) while *L. acidophilus* CRL 636 released only small amounts (8.2 µg/ml) of Gly (Fig. 2b).

### 3.3. Hydrolysis of whey proteins

Hydrolysis of whey proteins by the LAB strains was further analyzed by Tricine SDS–PAGE gels (Schagger and von Jagow, 1987) (Fig. 3a–c). All the studied microorganisms degraded whey proteins but in a different extent. Concerning the major proteins ALA (14 kDa) and BLG (18.6 kDa), *L. delbrueckii* subsp. *bulgaricus* CRL 454 showed the greatest ALA hydrolysis (24–27% at 37 and 42 °C, respectively) while *L. acidophilus* CRL 636 displayed the lowest degradation (11–10% at 37 and 42 °C, respectively). In general, BLG was hydrolyzed in a lesser extent than ALA; the degree of degradation being similar for all the strains assayed (7–10%). Regarding the high MW proteins, for instance bovine serum albumin and immunoglobulins, the 69 kDa fraction was 1.1–3.0-fold more susceptible to hydrolysis than ALA by all the strains.

### 3.4. Whey fermentation by starter cultures

The diffusion plate assay indicated that *L. delbrueckii* subsp. *bulgaricus* CRL 454 and *L. acidophilus* CRL 636 were not compatible strains, therefore, starters combining *S. thermophilus* CRL 804 and *L. acidophilus* CRL 636 (named SLa) or *S. thermophilus* CRL 804 and *L. delbrueckii* subsp. *bulgaricus* CRL 454 (named SLb; yogurt-type) were used. Due to the better behavior of the strains grown in RW at 37 °C than at 42 °C, the former temperature was chosen for the fermentations' assays. The starter culture SLb displayed a higher cell growth ($\mu_{max}$: 0.31 $h^{-1}$) than the starter SLa ($\mu_{max}$: 0.22 $h^{-1}$) while no differences in cell viability were detected at 24 h (Fig. 4a). In general, both starter cultures showed higher acidification rates (0.42–0.44 units/h for SLa and SLb, respectively) than the strains grown separately (0.28–0.27–0.09 units/h for

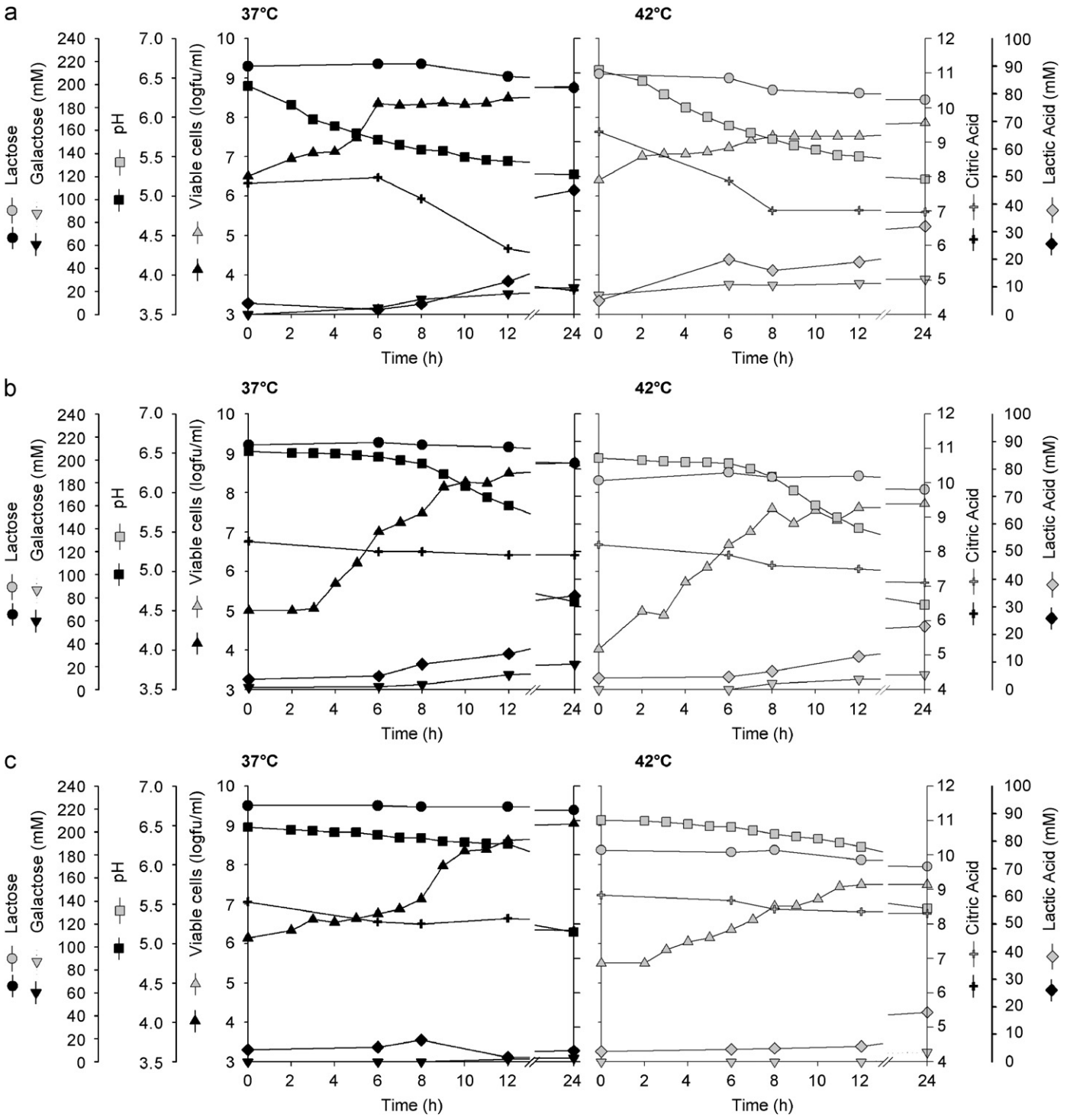


Fig. 1. Cell viability, pH decrease, lactic acid production and sugar and citric acid concentration during growth of (a) *S. thermophilus* CRL 804, (b) *L. delbrueckii* subsp. *bulgaricus* CRL 454 and (c) *L. acidophilus* CRL 636 in RW at 37 and 42 °C.

*S. thermophilus* CRL 804, *L. delbrueckii* subsp. *bulgaricus* CRL 454 and *L. acidophilus* CRL 636, respectively). The pH profile showed a marked decrease from the beginning of the fermentations. Among the organic acids analyzed, only lactic acid was produced, the values found being 2.0-fold higher (82 and 92 mmol/l for SLa and SLb, respectively) respect to those obtained with the strains alone (Fig. 1a–c). In accordance to the growth behavior, the amount of lactose consumed was higher (20–22% of original content) than that obtained when using single cultures. Regarding citrate concentration, a decrease was observed at the end of the incubation period. The starter SLb was more proteolytic than SLa (Fig. 4b); the concentration of amino acids (OPA) increased rapidly for

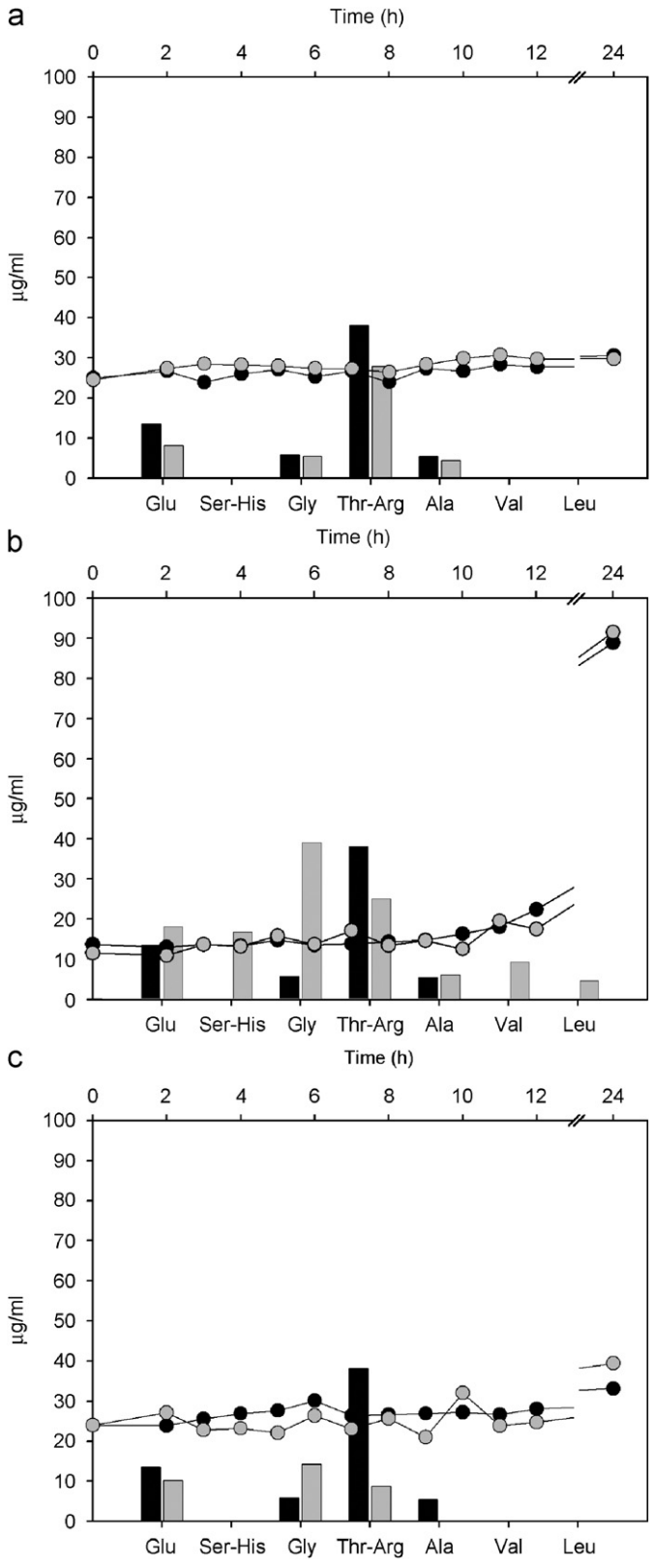


Fig. 2. Proteolytic activity (OPA) at 37 °C (●) and 42 °C (◎) and amino acid release at 37 °C for (a) *S. thermophilus* CRL 804, (b) *L. delbrueckii* subsp. *bulgaricus* CRL 454 and (c) *L. acidophilus* CRL 636. Non-inoculated (▮) and 24-h-fermented (▯) RW.

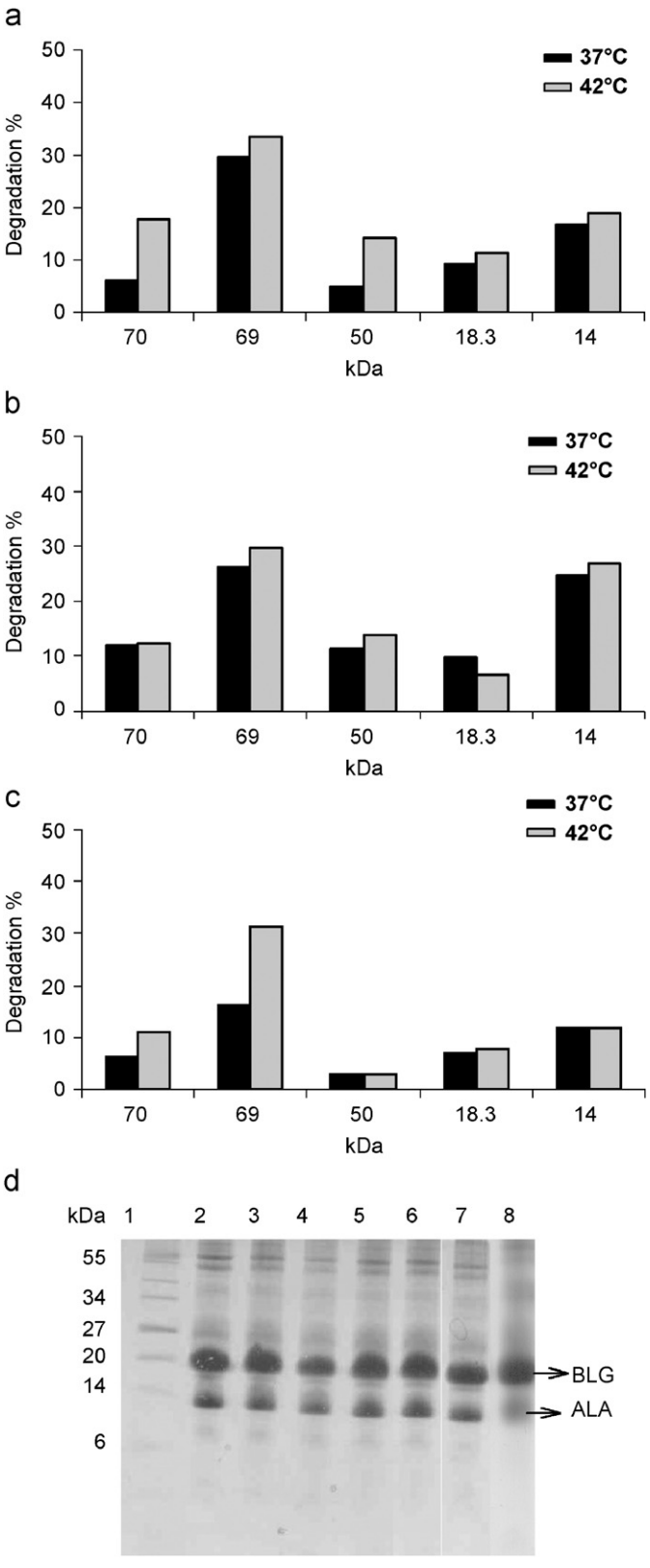


Fig. 3. Degradation of whey proteins (70, 69, 50, 18.3 and 14 kDa) by (a) *S. thermophilus* CRL 804, (b) *L. delbrueckii* subsp. *bulgaricus* CRL 454, (c) *L. acidophilus* CRL 636 grown in RW at 37 and 42 °C after 24 h of incubation and (d) Tricine SDS–PAGE analysis of fermented RW, lanes: 1, broad range weight protein marker (2–212 kDa); 2, non-fermented RW ($t = 0$, control); 3 and 4, *S. thermophilus* CRL 804 incubated at 37 and 42 °C for 24 h, respectively; 5 and 6, *L. acidophilus* CRL 636 incubated at 37 and 42 °C for 24 h, respectively; and 7 and 8, *L. delbrueckii* subsp. *bulgaricus* CRL 454 incubated at 37 and 42 °C for 24 h.

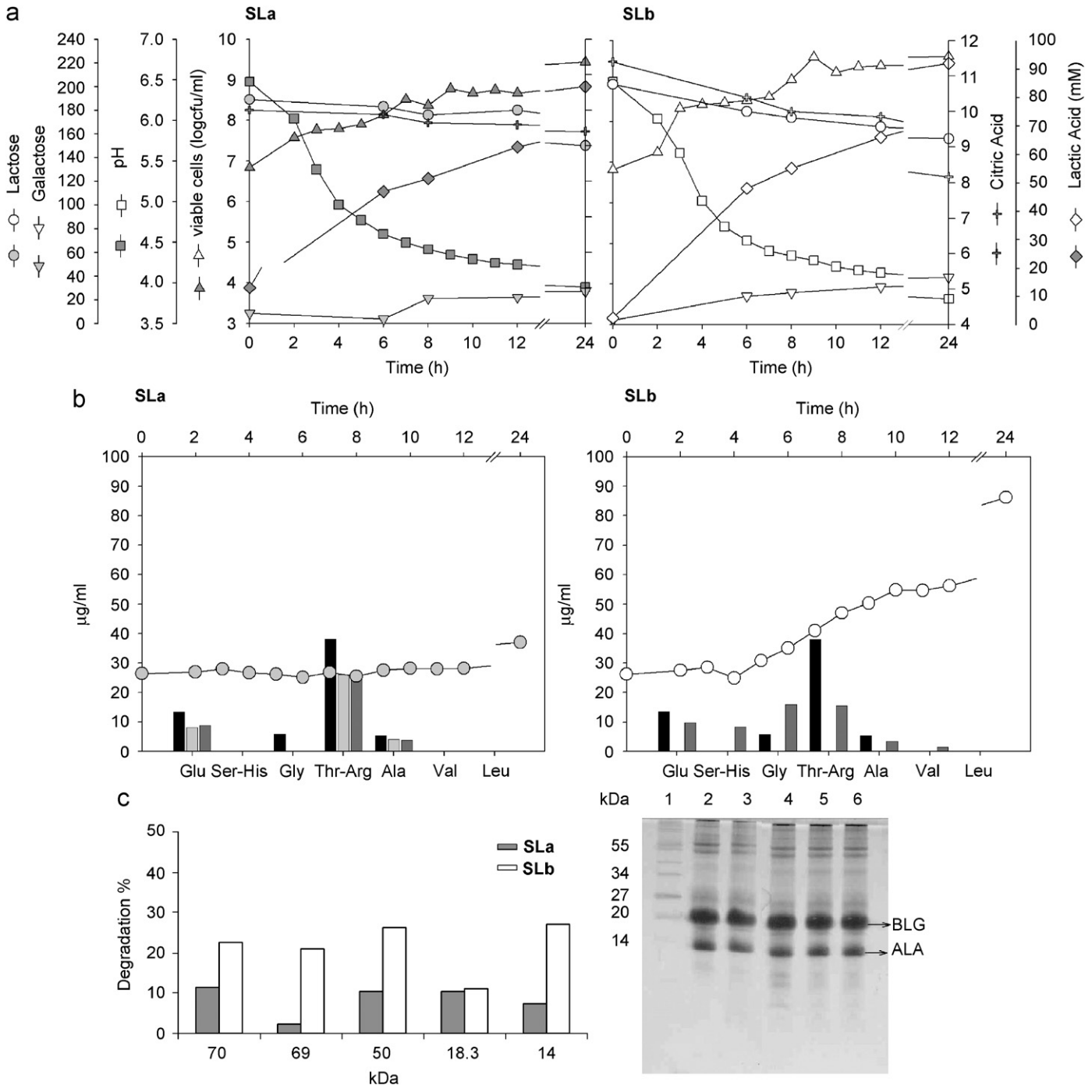


Fig. 4. (a) Growth of starter cultures SLa and SLb, pH decrease, lactic acid production and sugar and citric acid concentration in RW at 37 °C. (b) Proteolytic activity (OPA) of SLa (●) and SLb (○) and amino acid release in non-inoculated (■), 8 h (▯) and 24-h-fermented (▮) RW. (c) Degradation of whey proteins after 24 h of incubation and Tricine SDS–PAGE analysis of fermented RW, lanes: 1, broad range weight protein marker (2–212 kDa); 2, non-fermented RW ($t = 0$, control); 3 and 4, SLb and SLa incubated at 37 °C for 24 h, respectively; 5 and 6, non-fermented RW incubated at 37 and 42 °C for 24 h.

the starter SLb (41.0 µg Leu/ml, at 7 h) reaching maximum values of 86.0 µg Leu/ml at 24 h. Regarding the amino acids profile for these cultures, Glu, Ala and Thr–Arg were consumed (51–54, 25–100 and 31–54%, respectively) while the Gly concentration increased (30%) at 8 h fermentation. An increase in the concentration of Glu, Gly and Ala as well as the release of Val (1.4 µg/ml) and Ser–His (8.4 µg/ml) were observed only at 24 h incubation respect to 8 h profile. The starter SLa showed a similar behavior as compared to the strains grown separately: low proteolytic activity during the first hours of incubation and maximum values of 37.0 µg Leu/ml at 24 h. The amino acids present in whey were consumed during the first 8 h of incubation and only an increase of Glu was observed at 24 h respect to the results obtained at 8 h incubation.

Hydrolysis of BLG (9%) and ALA (8%) by SLa was observed at 24 h incubation, no degradation of these proteins was detected in earlier incubation periods (12 h). These results were consistent with the OPA values which remained constant along the first 12 h. Despite the fact that SLb showed an increasing amino acid concentration since the first 6 h incubation, BLG and ALA degradation (7% and 27%, respectively) was detectable only at 24 h incubation. Moreover, BLG and ALA hydrolysis was not superior to that found with the strains alone. Hydrolysis of the immunoglobulins fractions of 50 and 69 kDa was higher (2.0-fold) when using the starter SLb than SLa.

## 4. Discussion

Whey fermentation by LAB can be an interesting alternative to improve proper disposal of whey and to provide it an extra nutritional value. This study shows the potentiality of three industrially important LAB strains to grow in RW and to degrade the major whey proteins BLG and ALA. The studied strains had been selected from previous studies for their proteolytic activity on whey proteins (Pescuma et al., 2007).

The LAB strains used in this work grew well in whey and showed different acidification rates. Drgalić et al. (2005) reported the growth of three probiotic LAB strains (*L. acidophilus* La-5, *Bifidobacterium bifidum* Bb-12 and *Lactobacillus casei* Lc-01) in RW and observed similar cell viable counts to those found in our work but lower pH values (lower than 4.5) after 24 h incubation at 37 °C. Vinderola and Reinheimer (2000) showed that viable cell counts of Argentinean fermented dairy products were 8 log cfu/ml for *S. thermophilus* while 2–8 log cfu/ml for *L. delbrueckii* subsp. *bulgaricus* and *L. acidophilus*. Data from this study are also in line with those of Champagne et al. (1996) with yoghurt cultures in unsupplemented whey. However, this team also reported counts of $6.4 \times 10^9$ cfu/ml in whey supplemented with protein hydrolysates when the fermentation was carried out under pH control. Therefore, data by Champagne et al. (1996) suggest that if our strains are indeed active in whey protein proteolysis, much higher cell counts and protein hydrolysis could occur if the fermentation were carried out under pH control. This, however, remains to be determined.

The analysis of free amino acids present in the whey used in our study revealed only the presence of Glu, Gly, Thr–Arg and Ala. As compared to milk, whey contains less free amino acids. In addition, Letort et al. (2002) found in milk asparagine (Asn), aspartic acid, Ser, Leu, isoleucine (Ile), Val, tyrosine (Tyr), His and lysine (Lys). In our study, we showed that all the strains analyzed consumed Glu. This amino acid is required by most species of *Lactobacillus*, *Streptococcus*, *Pediococcus* and *Leuconostoc*, being this characteristic related to extensive genetic lesions encoding for this amino acid biosynthesis (Morishita et al., 1981; Hebert et al., 2004). *L. delbrueckii* subsp. *bulgaricus* CRL 454 released the essential branched chain amino acids Leu and Val. This property has been considered as a relevant issue since whey-based products are an important source of branched chain amino acids, commonly used in sports nutrition to improve muscle mass and physical performance (Pasin and Miller, 2000). *L. delbrueckii* subsp. *bulgaricus* CRL 454 released also the essential amino acid His (16.8 μg/ml), enhancing the nutritional value of whey. On the contrary, *S. thermophilus* CRL 804 consumed all the amino acids originally present in whey. Letort et al. (2002) reported the consumption (310 μM) of these amino acids as well as Asn, glutamine, Ser, Leu, Ile, Val, Lys and Arg to sustain the growth of a cell wall-associated proteinase positive *S. thermophilus* strain in milk at 7 h incubation. *S. thermophilus* strains have complex amino acid requirements and their growth is influenced by the availability and active transport/exchange of free amino acids from the growth medium (Desmazeaud and de Roissart, 1994). The assayed strains were able to degrade the whey proteins although the achieved BLG hydrolysis was not higher than 10%. In general, ALA (non-allergenic and with better digestibility than BLG) was more susceptible to hydrolysis than BLG. Similar results were found by Bertrand-Harb et al. (2003) who showed that ALA was more susceptible to proteolysis especially by the *S. thermophilus* strain when grown in MRS supplemented with whey proteins. In addition, El-Zahar et al. (2003) reported that ALA was hydrolyzed to a slightly greater extent than BLG during yogurt production using ovine milk.

The starter cultures made by combining the selected strains (a yogurt-type mixture and another including strains of *S. thermophilus* and *L. acidophilus*) displayed higher acidification rates and lactose consumption than the strains grown alone. Banina et al. (1998) reported that the growth and lactose consumption ability of *L. acidophilus* strains in milk were enhanced when co-cultivating them with other LAB species such as *S. thermophilus*. The proteolytic activity (OPA) was improved when using SLb as compared with single cultures, releasing amino acids in shorter incubation periods. However, this starter culture released only the branched chain amino acid Val and lower concentrations of Ser–His than the single culture of *L. delbrueckii* subsp. *bulgaricus* CRL 454. *S. thermophilus* CRL 804, present in SLb, could be responsible for the consumption of these amino acids and Leu, allowing a better growth of this strain and resulting in a faster acidification of whey. The importance of the proteolytic activity of *L. delbrueckii* subsp. *bulgaricus* with respect to its synergistic relationship with *S. thermophilus* in mixed starter cultures is well documented (Rajagopal and Sandine, 1990). When SLa was used, the amino acid release was not improved and only Glu was liberated at 24 h, all the amino acids being consumed at 8 h probably to support bacterial growth. Despite the faster amino acid release observed for SLb, BLG and ALA were not degraded in a shorter period than 24 h incubation. Bertrand-Harb et al. (2003) reported that strains used for

yogurt production (as the case of the starter SLb) did not cleave detectable amounts of BLG and ALA during yogurt manufacture and found that BLG proteolysis required a longer time (24–48 h) than that for elaboration of yogurt. Kleber et al. (2005) reported that LAB could reduce the antigenic response of BLG in whey and that a synergic effect was observed regarding this reduction when 1:1 mixtures of *L. acidophilus* and *S. thermophilus* or *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* were assayed, while no information on the amino acid release nor the percentage of BLG degradation was given. Prioult et al. (2004, 2005) reported that *B. lactis* NCC362 and *Lactobacillus paracasei* NCC2461 hydrolyzed BLG generating peptides that could promote derivation from a pro-allergy phenotype *in vitro*.

The fermentation of whey could lead to a more stable product with enhanced flavor and nutritional characteristics. The potential technological application of the SLb starter culture for the development of healthy fermented whey-based drinks was shown in this study. SLb was able to reduce the lactose concentration in whey and more interestingly, to degrade the major whey proteins. An increase in the free amino acid content, a relevant feature in nutrition, was also achieved. Finally, BLG was partially hydrolyzed increasing its digestibility and potentially decreasing its allergenicity. To our knowledge, this is the first detailed study on amino acid profiles during whey fermentation by LAB.

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