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Functional fermented whey-based beverage using lactic acid bacteria

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ABSTRACT

Whey protein concentrate (WPC) is employed as functional food ingredient because of its nutritional value and emulsifying properties. However, the major whey protein β-lactoglobulin (BLG) is the main cause of milk allergy. The aim of this study was to formulate a fermented whey beverage using selected lactic acid bacteria and WPC35 (WPC containing 35% of proteins) to obtain a fermented product with low lactose and BLG contents and high essential amino acid concentration. Cell viability, lactose consumption, lactic acid production, proteolytic activity, amino acid release and BLG degradation by the selected strains *Lactobacillus acidophilus* CRL 636, *Lactobacillus delbrueckii* subsp. *bulgaricus* CRL 656 and *Streptococcus thermophilus* CRL 804, as single or mixed (SLaB) cultures were evaluated in WPC35 (10%, w/v) incubated at 37 °C for 24 h. Then, the fermented WPC35 was mixed with peach juice and calcium lactate (2%, w/v) and stored at 10 °C for 28 days. During fermentation, single cultures grew 1.7–3.1 log CFU/ml and produced 25.1–95.0 mmol/l of lactic acid as consequence of lactose consumption (14.0–41.8 mmol/l) after 12 h fermentation. *L. delbrueckii* subsp. *bulgaricus* CRL 656 was the most proteolytic strain (626 μg/ml Leu) and released the branched-chain essential amino acids Leu (16 μg/ml), Ile (27 μg/ml) and Val (43 μg/ml). All strains were able to degrade BLG in a range of 41–85% after 12 h incubation. The starter culture SLaB grew 3.0 log CFU/ml, showed marked pH reduction, produced 122.0 mmol/l of lactic acid, displayed high proteolytic activity (484 μg/ml Leu) releasing Leu (13 μg/ml), Ile (18 μg/ml) and Val (35 μg/ml), and hydrolyzed 92% of BLG. The addition of calcium lactate to WPC35 maintained the drink pH stable during shelf life; no contamination was detected during this period. After 28 days, a decrease in cell viability of all strains was observed being more pronounced for *L. delbrueckii* subsp. *bulgaricus* CRL 656 and *L. acidophilus* CRL 636 (2.3 and 1.9 log CFU/ml, respectively). The results showed that WPC fermentation by rationally selected lactic acid bacteria might be used for developing functional beverages with improved characteristics such as reduced BLG content and increased branched-chain essential amino acids.

1. Introduction

Over the years numerous efforts have been made to transform large volumes of whey generated as sub-product of the cheese industry into a suitable product for food use (Djurić et al., 2004). Whey constitutes about 85–90% of the milk volume used for transformation into ripened cheese, and it retains about 55% of the milk nutrients. Liquid whey is composed of lactose (5%), water (93%), proteins (0.85%), minerals (0.53%) and a minimum amount of fat (0.36%). Whey proteins have high biological value superior to other proteins such as those of egg, soy and caseins of milk (Smithers, 2008) mainly due to the high content of branched-chain essential amino acids (isoleucine, leucine and valine). These amino acids stimulate specific intracellular pathways associated with muscle protein synthesis (Katsanos et al., 2006) and may play a role in the hormonal response to feeding as stimulate insulin secretion (Calbet and MacLean, 2002).

Whey proteins are recovered commercially by ultrafiltration (UF) and because of their size, they are separated from lactose and ash, which pass through the membrane into the permeate. The retentate stream is fed into spray dryers to produce powdered whey protein concentrate (WPC) (Yee et al., 2007), in which the protein concentration is within a range of 35–80%.

Despite the fact that whey proteins have multiple qualities which are considered to be healthy, one of its main proteins β-lactoglobulin (BLG) is the major allergen of milk. Lactic acid bacteria (LAB), microorganisms extensively used in the elaboration of dairy fermented products, can hydrolyze milk proteins and moreover, some of them can degrade BLG during growth in whey and milk (Bertrand-Harb et al., 2003; Pescuma et al., 2008). More interestingly, strains of *Lactobacilus acidophilus*, *L. paracasei*, and *Bifidobacterium* have been reported to breakdown the BLG allergenic epitopes *in vitro* (Pescuma et al., 2007, 2009; Prioult et al., 2003). Other studies showed that certain probiotic strains of LAB and bifidobacteria may induce oral

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tolerance to BLG, restore aberrant protein transport and have specific effect on protein degradation in the intestinal mucosa (Mizumachi and Kurisaki, 2002; Pessi et al., 1998; Prioult et al., 2003). Kirjavainen et al. (2003) demonstrated that supplementation of infant formula containing viable *Lactobacillus* GG could prevent cow's milk allergy.

As metabolically active products, fermented milks show modifications throughout their shelf life, such as post-acidification and loss of starter viability, impairing the quality of the product. *Lactobacillus delbrueckii* subsp. *bulgaricus* produces lactic acid during storage, known as post-acidification, which is claimed to affect the viability of probiotic bacteria (Dave and Shah, 1997). The use of WPC may improve culture viability due to its protein and phosphate contents, thus enhancing the buffering capacity of the yogurt (Kailasapathy and Supriadi, 1996).

The aim of this study was to formulate a novel functional fermented whey beverage using WPC35 and selected LAB strains able to lower the lactose and BLG contents and to release essential amino acids. LAB viability during storage of this fermented beverage was also assessed.

2. Materials and methods

2.1. Microorganisms and media

The strains *L. acidophilus* CRL 636, *L. delbrueckii* subsp. *bulgaricus* CRL 656 and *Streptococcus thermophilus* CRL 804 used in this work were obtained from the Culture Collection of 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.

Whey protein concentrate 35%, w/w protein (WPC35), powder (kindly provided by MILKAUT S.A., Argentina) was reconstituted with distilled water to 10% (w/v) and the pH was adjusted to 8.0 with 2 mol/l NaOH. The reconstituted WPC35 was heat treated at 116 °C for 20 min, stored at 4 °C until use (no longer than one week) and used as fermentation medium.

The presence of deteriorating microorganisms was assessed by plating pure or diluted (ten times) beverage samples in Baird Parker agar supplemented with egg yolk and tellurite (for *Staphylococcus aureus*), violet red bile lactose agar (VRBA, for total coliforms), plate count agar (PCA, for mesophilic microorganisms), and potato dextrose agar (PDA, for fungi and yeasts). All media were purchased from Britania S.A (Buenos Aires, Argentina). Plates were incubated according to the manufacturer's indications.

2.2. Fermentation conditions

Cultures were transferred twice in WPC35 prior to experimental use; 16 h old cultures (2% v/v) were used as inocula individually, or combined as follows: *L. delbrueckii* subsp. *bulgaricus* CRL 656: *S. thermophilus* CRL 804: *L. acidophilus* CRL 636 at a 1:1.5:6.4 CFU/ml ratio. Fermentations were performed statically in sealed bottles containing 300 ml of WPC35 and incubated at 37 °C for 24 h. Samples were aseptically withdrawn every 2 h during 12 h and at 24 h of incubation. Cell viability was determined by plating appropriate dilutions of the cultures in MRS agar (MRS Britania, Buenos Aires, Argentina, plus 15 g/l agar). To determine the viable cell count of the *L. acidophilus* strain in the mixed culture, 1.5% (w/v) bile salt (Sigma Chemical CO, St. Louis, USA) was added to MRS agar (Vinderola and Reinheimer, 2000). The strains *L. delbrueckii* subsp. *bulgaricus* CRL 656 and *S. thermophilus* CRL 804 were selectively counted by means of their shape in the mixed culture by plating the fermented WPC35 in MRS agar (aerobic conditions) as recommended by the International Dairy Federation for the selective count of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* in yogurt. (Vinderola and Reinheimer, 1999). Plates were incubated at 37 °C for 48 h and colony-forming units (CFU)/ml were determined. Cells of *L. delbrueckii* subsp. *bulgaricus* CRL 656 appeared as irregular white large colonies while those of *S. themophilus* CRL 804 as small round white colonies. To confirm the identity of the colonies, cell morphology was observed with an Olympus Vanex microscope (Tokyo, Japan). Cell count was expressed as log CFU/ml. Decrease in pH was followed with a digital pH meter (Altronix TPX 1) every 2 h during the first 12 h and after 24 h incubation.

2.3. Beverage formulation

WPC35 was allowed to ferment for 12 h, cooled down in ice and diluted 1:3 with peach juice (ZUCO, Corandes S.A., Argentina), previously dissolved in sterile water or calcium lactate 2% (w/v). Calcium lactate was added as acidity regulator following the indications of the Codex Alimentarius (CODEX STAN, 192-1995). The resulting beverages were distributed in sterile plastic bottles in triplicates and stored at 10 °C for 28 days. Viable cell count, pH, sugar and lactic acid concentrations, proteolytic activity, free amino acid content and whey protein degradation were determined after 0, 7, 14, 21 and 28 days of storage.

2.4. Analysis of metabolites

Sugar content (lactose, galactose and glucose) and organic acids (lactic, acetic, and formic) 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 RI detector fitted with a Biorad Aminex HPX-87H column (300×7,8 mm, Hercules, CA, USA). The operating conditions were the following: 5 mmol/l H_2SO_4 was used as eluent at a flow rate of 0.6 ml/min during 30 min and an internal temperature of 45 °C. For the quantification of sugars and organic acids, calibration curves for each compound were performed using pure standards at different concentrations.

2.5. Proteolysis assessment

The proteolytic activity of LAB was measured in samples of fermented WPC35 (every 2 h during 12 h and at 24 h) and of the beverage during storage (0 and 28 days) by using 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™ Tunable Microplate reader (Sunnyvale, CA, USA). The OPA solution contained: 2.5 ml of 20% (w/v) SDS, 25 ml of 100 mmol/l sodium tetraborate (Sigma Chemical Co), 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 mol/l trichloroacetic acid (Sigma Chemical Co) at a sample: trichloroacetic acid ratio = 1:3 at 4 °C for 30 min and centrifuged (5000 rpm 10 min). Ten microliters of the supernatant of this mixture was added to 0.2 ml of OPA reagent and then incubated at room temperature for 5 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, England).

2.6. Free amino acid determination

The free amino acid content of non-fermented and fermented WPC35 as well as the stored beverage was determined. Samples were treated to eliminate proteins and the amino acids were extracted as described by Jones et al. (1981). The reaction was prepared by mixing 200 µl of WPC35 with 2% (w/v) of SDS (dissolved in 0.4 mol/l 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 mol/l 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 above 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 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 mol/l pH 5.9 (Sigma Chemical Co.) in ultra pure water; solvent B, methanol:sodium acetate 0.05 mol/l 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 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 acid concentration was expressed in µg/ml.

2.7. Hydrolysis of β-lactoglobulin in WPC35

Degradation of β-lactoglobulin was monitored by RP-HPLC using a Knauer Smartline System (Manager 5000, pump 1000) with a UV detector (2000) fitted with a C18 column (Pursuit 4.6×250 mm, 300 A, 5 µm, Varian, Lexington, USA). The method used included buffer A: water/acetonitrile/trifluoroacetic acid (90/10/0.1, v/v/v), and buffer B acetonitrile/trifluoroacetic acid (100/0.1, v/v) with a flow rate of 1 ml/min. The gradient used was 100% buffer A up to 10 min and 10 to 60% buffer B in a linear fashion between 10 and 60 min. Eluted peaks in the chromatograms were detected at 214 nm. Samples for HPLC were prepared as follows: WPC35 samples (fermented and non-fermented) were mixed 1:1 with reduction buffer containing urea and 20 mmol/l dithiothreitol (DTT) and incubated for 60 min at 30 °C. Prior to injection in the column, the reduced sample was diluted 5-fold in buffer A containing 6.0 mol/l urea. BLG hydrolysis was expressed as percentage and was calculated by measuring its relative peak area with respect to the control (non-fermented sample).

2.8. Strains compatibility

Strains compatibility was evaluated by the plate diffusion assay (Parente and Zottola, 1991). Briefly, overnight cultures grown in MRS were washed twice with saline solution and suspended at the initial volume. Plates were prepared by pouring 15 ml of MRS soft agar (MRS plus 0.7%, w/v, agar) containing 60 µl of the cell suspension on the agar. After overlay solidification, 5 mm diameter wells made with sterilized plastic straws were inoculated with 60 µl of culture supernatants from the other strains. After incubation at 37 °C for 16 h, appearance of inhibition zones were observed.

2.9. Statistical analysis

All assays were carried out in triplicate, and results were expressed as mean values with standard deviations. Statistical analyses were performed using MINITAB 14 software (State College, PA, USA). Comparisons were accomplished by ANOVA general linear model followed by Tukey's post-hoc test, and $p<0.05$ was considered significant.

3. Results

3.1. Growth and metabolite production by LAB in WPC35

The assayed strains *L. delbrueckii* subsp. *bulgaricus* CRL 656, *L. acidophilus* CRL 636 and *S. thermophilus* CRL 804 grew between 1.7 and 3.1 log CFU/ml after 12 h of incubation in WPC35 at 37 °C, the highest cell count values being observed for *L. delbrueckii* subsp. *bulgaricus* CRL 656 (Fig. 1a–c). After this period, cell viability started to decline specially for the *L. delbrueckii* subsp. *bulgaricus* strain. *S. thermophilus* CRL 804 and *L. delbrueckii* subsp. *bulgaricus* CRL 656 showed a constant drop of pH (reaching about 4.9–4.5, respectively) during the first 8 h of incubation showing a major consumption of lactose during this period (32.5–40.0 mmol/l). On the contrary, *L. acidophilus* CRL 636 showed a slight decrease in pH, and lactose reduction was only detected at 12 h. As expected, the assayed strains released only lactic acid (25.1–95.0 mmol/l, at 12 h) from lactose fermentation, *L. delbrueckii* subsp.*bulgaricus* CRL 656 being the strain which produced the highest amount. An accumulation of galactose (4.3–46.2 mmol/l, 12 h) during LAB growth in WPC35 was found while glucose was entirely consumed by all the assayed strains.

3.2. Proteolytic activity and amino acid release by LAB in WPC35

The proteolytic activity of the studied LAB during WPC35 fermentation was strain dependent (Fig. 2). *L. delbrueckii* subsp. *bulgaricus* CRL 656 was the most proteolytic strain (626 µg Leu/ml) while no differences in the amino group concentration were observed for *S. thermophilus* CRL 804 during the incubation period. Non-fermented WPC35 showed low OPA value (82.3 µg/ml) and no amino acids were detected by RP-HPLC (data not shown). *L. delbrueckii* subsp. *bulgaricus* CRL 656 released 14 amino acids (Table 1) and displayed the highest amino acid concentration (428 µg/ml, Fig. 2) at 12 h incubation. The most abundant amino acids released by this strain were Ser-His (114 µg/ml), Gly (62 µg/ml) and Glu (53 µg/ml) and also the branched-chain amino acids Leu (16 µg/ml), Ile (27 µg/ml) and Val (43 µg/ml). *L. acidophilus* CRL 636 released mainly the amino acids Lys (21 µg/ml), Thr-Arg (18 µg/ml) and Glu (16 µg/ml) while *S. thermophilus* CRL 804 released only Thr-Arg (6 µg/ml) after 12 h incubation (Table 1).

3.3. β-lactoglobulin degradation in WPC35

All LAB strains were able to degrade BLG (Fig. 3c–h) in a range of 41–85% after 12 h incubation releasing mainly hydrophilic peptides (2–24 min elution); however, some small peaks corresponding to more hydrophobic peptides (30–38 min) were also observed for *L. delbrueckii* subsp. *bulgaricus* CRL 656, the most proteolytic strain (85% at 12 h incubation) (Fig. 3e). Although certain peaks (denoted as 1–4 in Fig. 3c–h) were found in all hydrolysates, distinct peptides were observed for each sample (marked with arrows).

3.4. WPC35 fermentation by a starter culture

The three studied strains were compatible as observed by the diffusion plate assay; thus, a starter culture named SLaB was formulated combining the strains *S. thermophilus* CRL 804, *L. delbrueckii* subsp. *bulgaricus* CRL 656 and *L. acidophilus* CRL 636 in a CFU/ml ratio of 1:1.5:6.4.

S. thermophilus CRL 804 showed the highest cell growth (1.9 log CFU/ml) during the first 4 h of incubation in WPC35 as compared with the other strains (0.59–1.02 log CFU/ml); however, similar cell count values were attained by all the strains after 6 h (Fig. 4a) indicating that *L. acidophilus* CRL 636 displayed a lower growth rate since it was inoculated in a higher ratio than the other strains.

The SLaB culture showed a fast drop in pH reaching 4.5 after 8 h and remained almost constant until 12 h fermentation; this behavior was similar to that found for the *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* strains when grown separately in WPC35 during the same incubation period. Lactose consumption was approximately 78.1 mmol/l releasing 122 mmol/l of lactic acid after 12 h of fermentation; an increase in galactose concentration was also observed (56.4 mmol/l).

SLaB showed high proteolytic activity reaching a maximum value of 648.6 μg/ml Leu at 24 h incubation (Fig. 2); however, this value was lower than that found for the *L. delbrueckii* subsp. *bulgaricus* CRL 656 culture (721.0 μg/ml, Fig. 2) after the same incubation period. The free amino acid concentration of the SLaB culture was also lower to that of the CRL 656 strain due to lower concentrations of the Glu (24%), Ser-His (26%), Gln (45%), Gly (39%) and Ile (33%) amino acids. In contrast, a higher amount (46%) of the amino acids Thr-Arg was observed with respect to the *L. delbrueckii* subsp. *bulgaricus* culture (Table 1).

The starter SLaB was able to hydrolyze BLG (92%) after 12 h incubation releasing mainly hydrophilic peptides and three peptides eluting between 15 and 20 min (Fig. 4b).

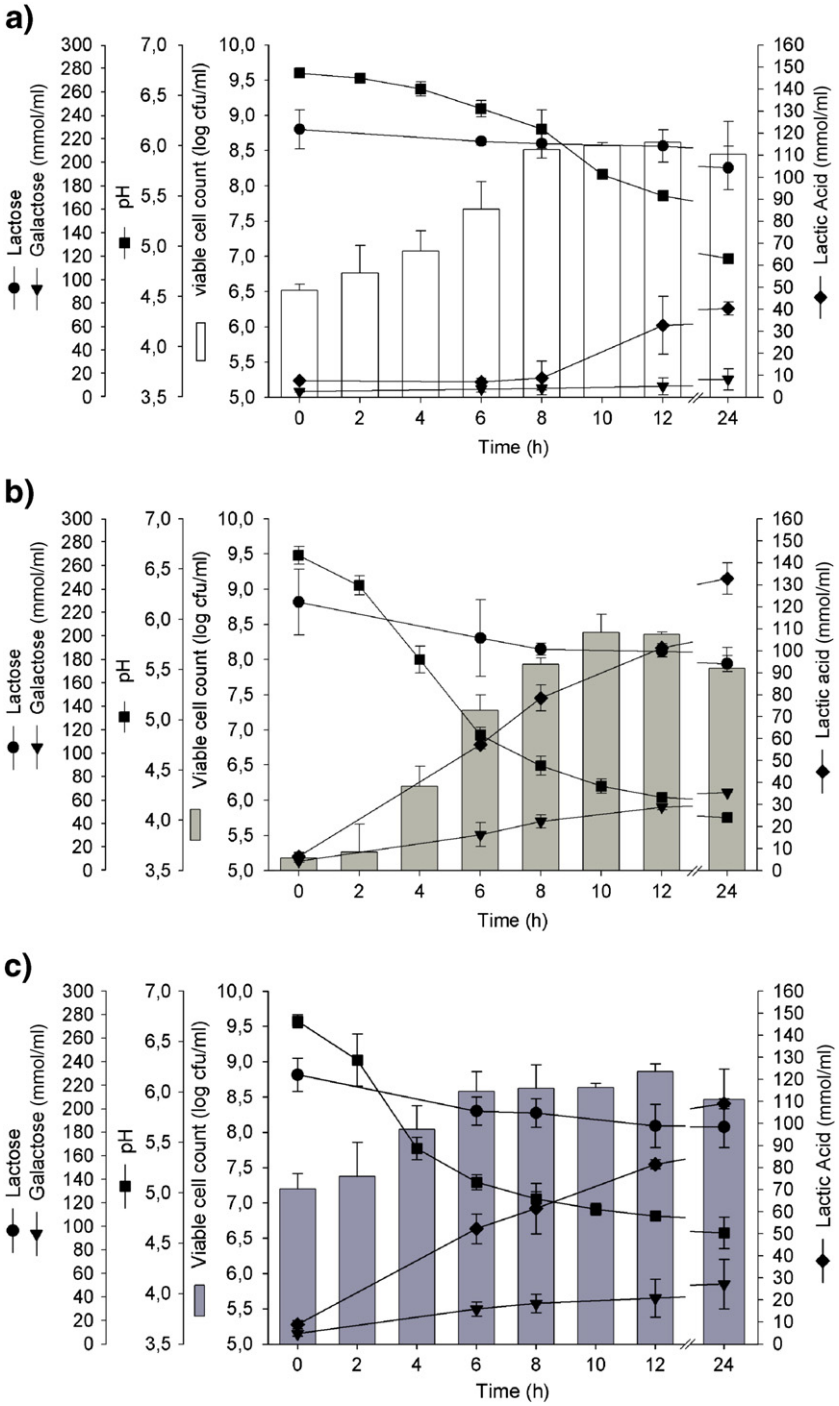

Fig. 1. Viable cell count, pH, sugar concentration and lactic acid release by a) *L. acidophilus* CRL 636, b) *L. delbrueckii* subsp. *bulgaricus* CRL 656 and c) *S. thermophilus* CRL 804 incubated in WPC35 at 37 °C for 24 h.

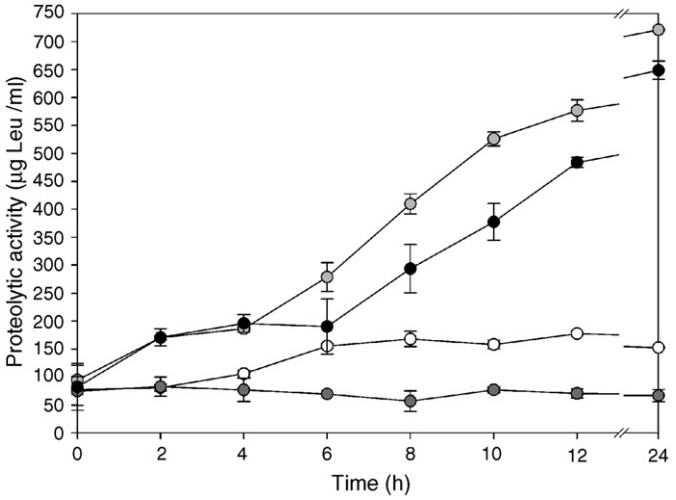

Fig. 2. Proteolytic activity (µg Leu/ml) of *L. acidophilus* CRL 636 (○), b) *L. delbrueckii* subsp. *bulgaricus* CRL 656 (◐), c) *S. thermophilus* CRL 804 (◑) and the starter culture SLaB (●) in WPC35 fermented at 37 °C for 24 h.

3.5. Fermented WPC35-based drink

A WPC35-based drink was formulated by mixing fermented WPC35 and distilled water (W), peach juice (P) or 2% (v/v) calcium lactate in peach juice (PL) at a 1:3 (v/v) ratio. Non-inoculated WPC35 but incubated for 12 h at 37 °C was used as control (C). Peach juice was used to mask the bitter flavor of whey. The drinks were kept at a storage temperature of 8–10 °C.

Cell viability of the starter SLaB during storage was analyzed (Fig. 5a–c). A decrease in cell count was observed for all strains after 28 days. The viable cell count of *L. acidophilus* CRL 636 was higher in P and PL (6.5 and 6.3 log CFU/ml, respectively, at 28 days) than in W (4 log CFU/ml). On the contrary, the decrease in *S. thermophilus* CRL 804 and *L. delbrueckii* subp. *bulgaricus* CRL 656 was more pronounced in P (1.0 and 2.5 log CFU/ml) than in the other beverage formulations after 28 day incubation.

The pH of the W beverage decreased only 0.2 U after the 28 day storage, which was consistent with an increase of lactic acid concentration of 8.5 mmol/l. In contrast, no differences in the pH values were observed for the beverage containing peach juice (P and PL) while an increase of 11 mmol/l in lactic acid concentration was observed for PL (Fig. 5a–c). No changes in pH (Fig. 5d) were observed for the control (C). Growth of deteriorating microorganisms in the beverages was not detected during the 28 day storage as assessed in the different selective growth media.

Table 1
Free amino acid concentration of fermented WPC35 after 12 h incubation at 37 °C.

Amino acids	Concentration of free amino acids (µg/ml)			
	L. acidophilus CRL 636	*L. delbrueckii* subsp. *bulgaricus* CRL 656	*S. thermophilus* CRL 804	Starter culture SLaB
Asp	ND	7.73 ± 0.26[a]	ND	7.06 ± 0.14
Glu	16.20 ± 0.80	53.20 ± 1.00	ND	40.40 ± 1.50
Asn	ND	1.38 ± 0.01	ND	1.35 ± 0.04
Ser-His	ND	114.00 ± 1.00	ND	84.70 ± 3.60
Gln	ND	40.00 ± 0.70	ND	21.70 ± 1.10
Gly	1.38 ± 0.20	61.60 ± 4.00	ND	37.80 ± 10.20
Thr-Arg	18.00 ± 1.00	24.10 ± 1.60	6.24 ± 1.09	51.60 ± 1.00
Ala	3.09 ± 0.08	12.10 ± 0.00	ND	12.70 ± 0.30
Val	1.90 ± 0.03	43.30 ± 1.00	ND	35.10 ± 0.60
Ile	8.40 ± 0.17	26.70 ± 0.50	ND	18.50 ± 0.70
Leu	8.22 ± 0.04	16.50 ± 0.30	ND	13.50 ± 0.50
Lys	21.2 ± 1.5	27.20 ± 0.80	ND	21.00 ± 0.20

ND, not detected.
[a] Mean value ± standard deviation.

No significant ($p>0.05$) differences in the proteolytic activity of the strains (OPA values) were observed during drink storage (data not shown); however, the amino acid concentration decreased between 19 and 29% after 28 days (Fig. 6a–c). The drink containing peach juice but not calcium lactate (P) showed lower amino acid concentration, probably due to the reduction of Gly (50%), Gln (62–100%) and Asn (25–100%) content at 28 day storage; however, the Ala concentration increased five times with respect to the control (W). BLG was not further hydrolyzed in any of the assayed drinks during the 28 days (data not shown).

4. Discussion

Fermentation of WPC by LAB could be an interesting alternative for the production of dairy functional foods with high nutritional content since whey proteins are known to have a high biological value (Smithers, 2008). Most of the research made regarding fermented whey drink formulations was based on the use of deproteinized whey losing the most valuable component of this sub-product (Dragone et al., 2009; Kar and Misra, 1999; Maity et al., 2008). This fact is due to the difficulties of heating whey avoiding protein precipitation. A few studies have been done on probiotic growth and survival (Dralić et al., 2005) fermentation with yogurt starters (Almeida et al., 2009; Gallardo-Escamilla et al., 2007) or kefir grains in reconstituted whey or whey and milk mixtures (Athanasiadis et al., 2004). However, no information was given on the proteolytic activity or amino acid release during bacterial growth. In this work, we show the results obtained during lactic fermentation of WPC35 a substrate that has similar protein concentration to that found in milk.

The thermophilic LAB strains assayed in this work were able to grow well in WPC35 after 12 h. In previous studies (Pescuma et al., 2008) we showed that LAB could grow and degrade whey proteins when incubated in reconstituted whey (RW) for 24 h. The results obtained here indicated that the use of WPC35 improved cell growth (1.5–1.9-fold) and the proteolytic activity of the lactobacilli strains (5 and 8 times for *L. delbrueckii* subsp. *bulgaricus* CRL 656 and *L. acidophilus* CRL 636, respectively) with respect to RW.

No free amino acids from unfermented WPC35 were detected as formation of glycosil-amino acids during heat treatment (116 °C, 20 min) could have taken place. These glycosil-amino acids are modified to Amadori compounds that cannot be determined using the regular amino acid standards for RP-HPLC (Brands and van Boekel, 2001). Glycated amino acids are poorly absorbed and after ingestion are excreted by the urine so that their impact in human health is limited (Meltretter and Pischetsrieder, 2008). However, LAB are able to consume Amadori compounds during growth (Sgarbieri et al., 1973) hence; the release of essential amino acids from whey protein hydrolysis by the assayed LAB strains can enhance the nutritional quality of the end product. *L. delbrueckii* subsp. *bulgaricus* CRL 656 released the essential amino acids Leu, Val, Ile, Lys and Thr in higher concentrations comparing to the values reported by Simova et al. (2006) for strains of *L. delbrueckii* subsp. *bulgaricus* isolated from kefir after 16 h incubation in milk. Leu, Ile and Val are branched-chain amino acids, which provide metabolic energy to the muscles and promote the synthesis of Ala and Glu during stress (i.e. traumatism, infection, intense exercise, etc.). The amino acids Leu, Lys, Trp, Ile and Thr are also known to play a role as metabolic regulators of glucose and protein metabolism, and as such, they are important for weight control (Zemel, 2004).

When using the formulated starter culture SLaB the lactose consumption and the acidification rate were higher (57% and 0.076/h, respectively) than those of the strains incubated separately (6–19% and 0.016–0.067/h, respectively). The amount of remaining lactose (4 g/l) was similar to that found in commercial yogurts, which are known to alleviate lactose maldigestion (Onwulata et al., 1989; Vesa et al., 2000).

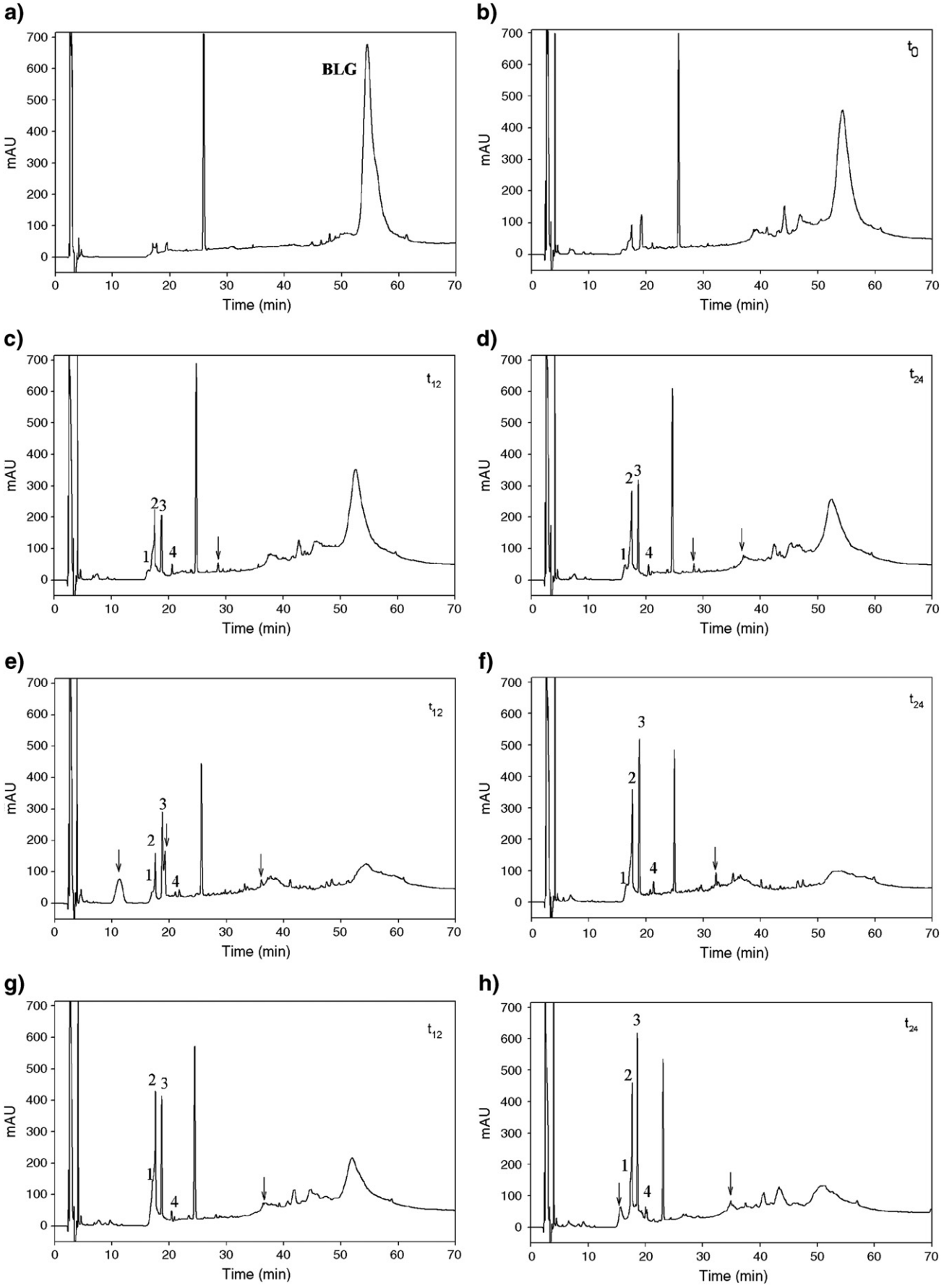

Fig. 3. RP-HPLC profiles of whey protein degradation from WPC35 fermented at 37 °C for 24 h. a) BLG (control), b) non-inoculated WPC35 incubated at 37 °C for 24 h, c) *L. acidophilus* CRL 636, 12 h and d) 24 h incubation; e) *L. delbrueckii* subsp. *bulgaricus* CRL 656, 12 h and f) 24 h incubation; g) *S. thermophilus* CRL 804, 12 h and h) 24 incubation.

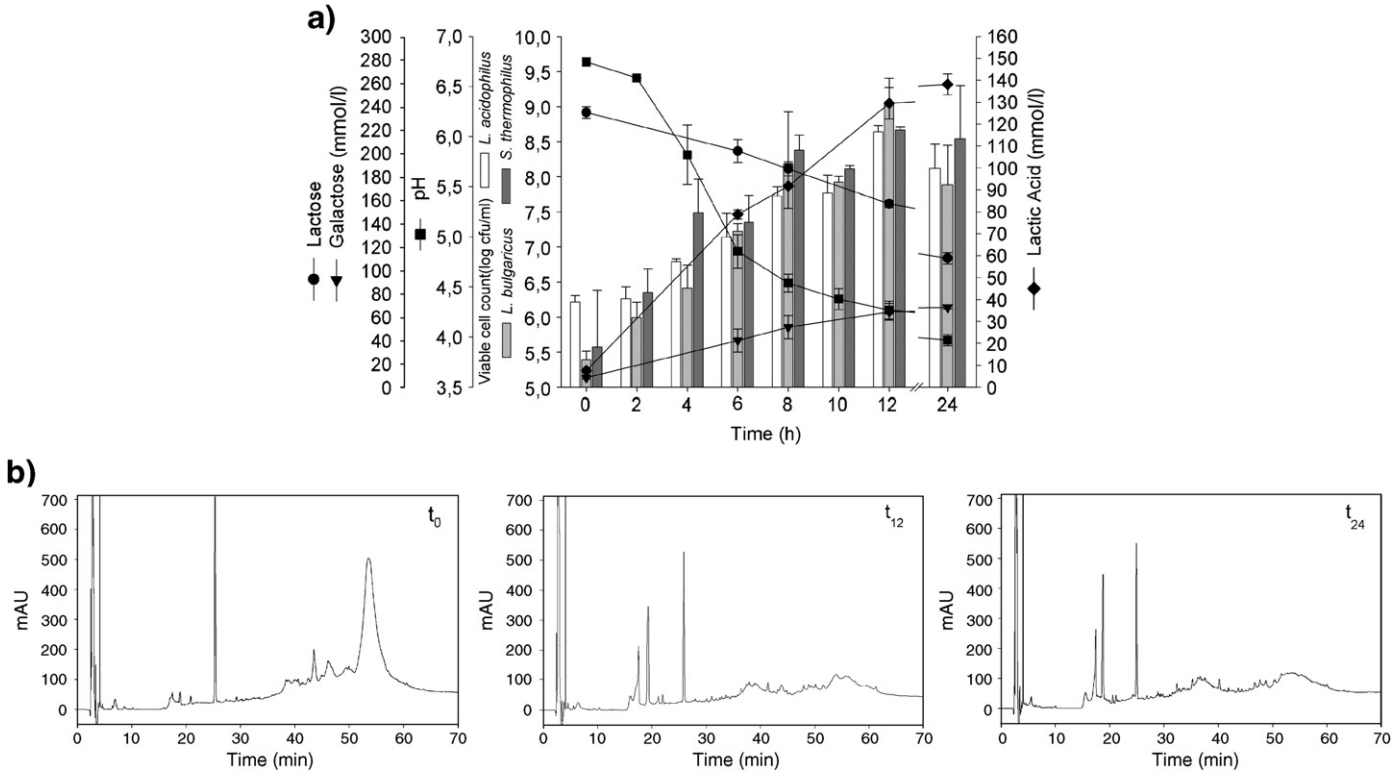

Fig. 4. Behavior of the starter culture SLaB in WPC35, a) Differential enumeration of each strain in the starter, pH, sugar concentration and lactic acid release, and b) RP-HPLC profiles of whey protein degradation.

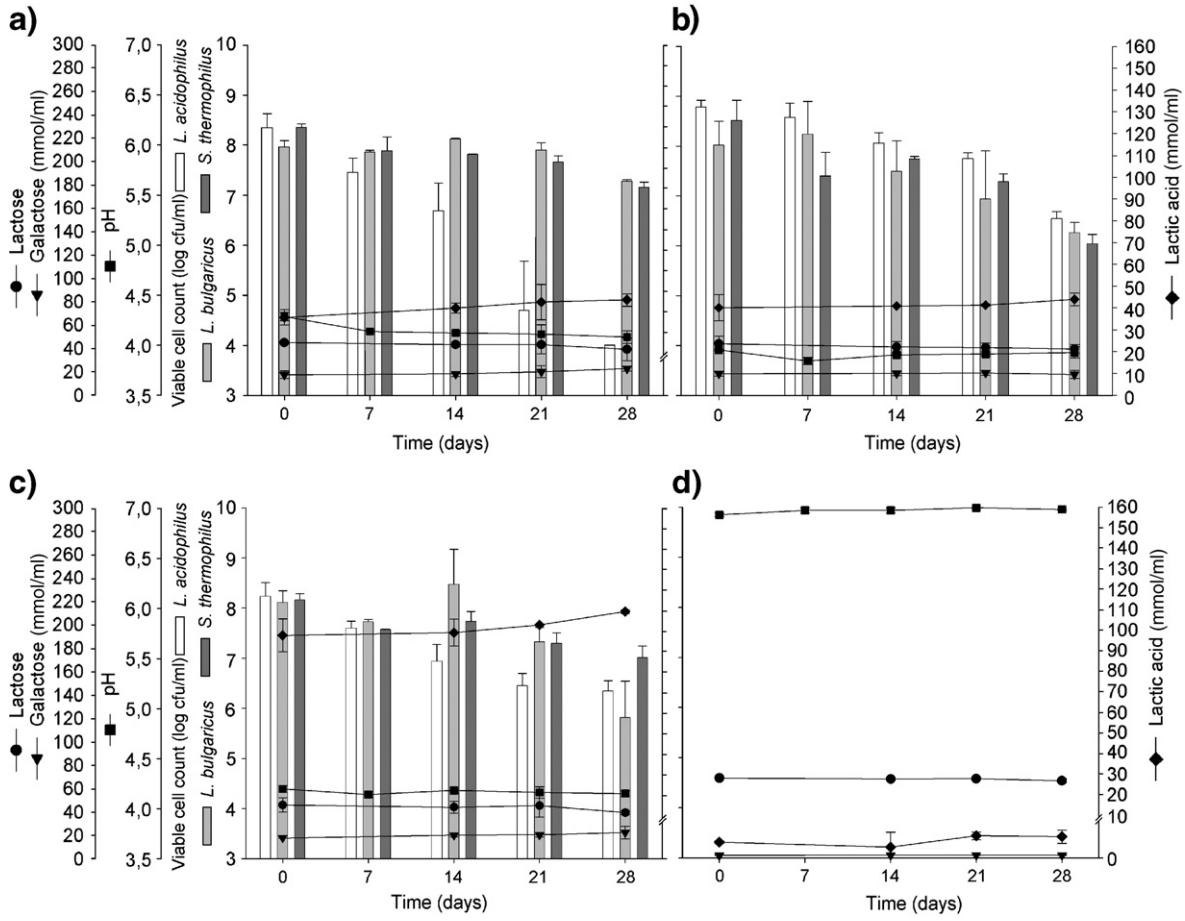

Fig. 5. Stability (cell viability, pH, sugar concentration and lactic acid release) of different beverage formulations during storage. a) W (fermented WPC35 plus water), b) P (fermented WPC35 plus peach juice), c) PL (fermented WPC35 plus peach juice and calcium lactate) and d) C (control, non-fermented WPC35).

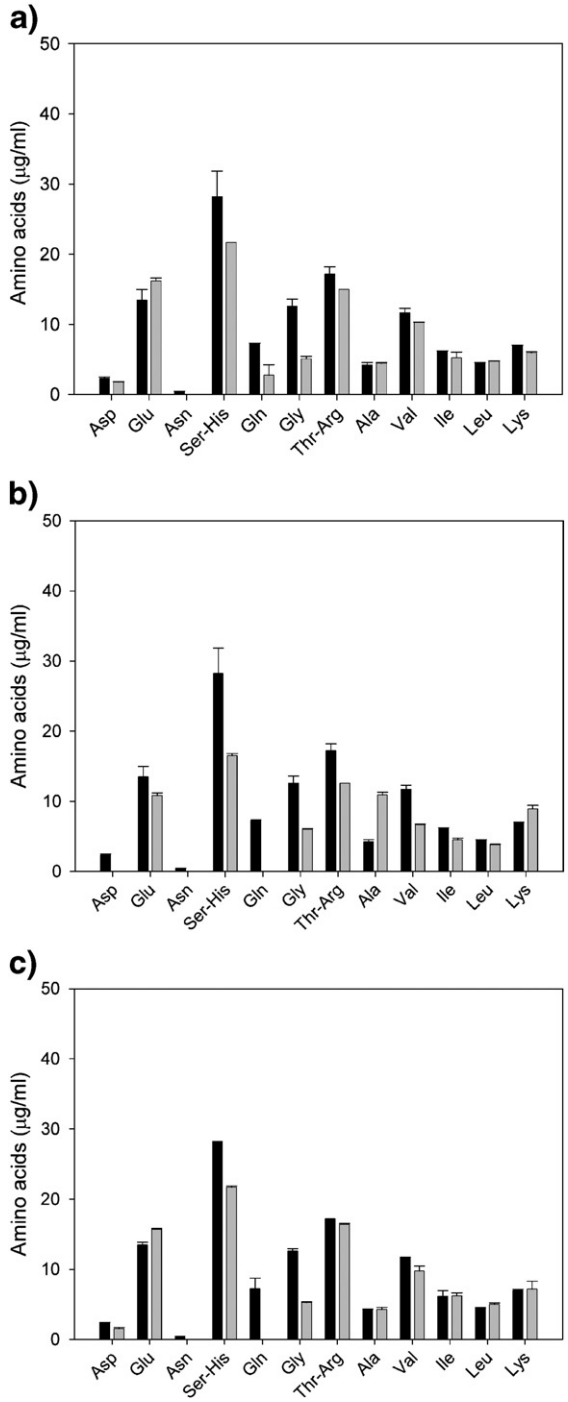

Fig. 6. Free amino acid concentration of different beverage formulations during storage. a) W (fermented WPC35 plus water), b) P (fermented WPC35 plus peach juice), and c) PL (fermented WPC35 plus peach juice and calcium lactate). Black bars: amino acid content of beverages (day 0); grey bars: amino acid content at day 28 of storage.

The SLaB culture liberated lower amount of free amino acids such as Ser-His (26%), Gln (45%) and Gly (39%) than the strain *L. delbrueckii* subsp. *bulgaricus* CRL 656 solely; this fact could be due to the consumption of the released amino acids by the *S. thermophilus* strain. Also, Letort et al. (2002) showed that when a *S. thermophilus* strain was grown in milk the concentration of the free amino acids Glu, Gln, Thr, Ser, Gly, Ala, Leu and Ile was diminished during the first hours of incubation.

Interestingly, BLG hydrolysis by SLaB was achieved at 12 h incubation at 37 °C with WPC35 instead of the 24 h needed using RW as fermentation medium; thus WPC35 being a more suitable substrate for industrial production of fermented beverages.

The addition of peach juice to fermented WPC35, which was used to mask the unpleasant flavor of whey (Djurić et al., 2004; Tranjan et al., 2009), decreased the pH from 4.3 to 3.9; this pH decrease was avoided by adding calcium lactate. After seven-day storage, post-acidification was observed in the drink diluted with distilled water while the addition of calcium lactate maintained the pH values invariable despite of the increase of lactic acid. In this respect, Antunes et al. (2005) showed that strains of *L. delbrueckii* subsp. *bulgaricus* were responsible for producing post-acidification in fermented milks and yogurts during storage.

The benefits of probiotic bacteria for consumers' health are widely recognized. Probiotic bacteria must be viable and present in high numbers at the time of consumption to perform their claimed effects (Gomes and Malcata, 1998). Furthermore, viability should be high in the final part of the gastrointestinal tract. We evaluated the survival of the LAB strains assayed during the beverage storage. *L. acidophilus* CRL 636 viability decreased markedly; however, the cell count (10^6 CFU/ml after 28 day storage) in PL was higher than that reported by Barreto et al. (2003) for 15 commercial Brazilian fermented milks ($<10^5$ CFU/ml). Akalin et al. (2007) showed that the supplementation of fermented milks with WPC had a positive effect on *L. acidophilus* and *Bifidobacterium* survival during storage probably due to the higher buffering capacity of whey proteins with respect to caseins thus preventing the post-acidification during this period. Also, sulfur amino acid release during heat treatment of whey could lower the redox potential leading to a beneficial effect on *L. acidophilus* survival.

Several studies showed that probiotic strains could degrade dietary antigens in the intestine and alleviate allergy symptoms (Bernasconi et al., 2006; Pessi et al., 1998; Schouten et al., 2009). *L. acidophilus* CRL 636 can hydrolyze the main BLG epitopes using a non proliferating cell system (Pescuma et al., 2009); however, it remains to be analyzed *in vivo* if this strain can continue BLG hydrolysis in the intestine and thus prevent allergy symptoms when given in this beverage formulation.

In this work the fermentation conditions of WPC35 with respect to RW were improved mainly due to the higher protein content of the former substrate, which made possible to reach the desired functional properties such as lower BLG and lactose content and higher essential amino acid concentration in a shorter period (12 h). The addition of peach juice and calcium lactate improved the beverage appearance without drastically decreasing the cell viability. Nevertheless, sensory analyses of the product remain to be evaluated.

The results obtained here evidence that fermentation of WPC35 by selected LAB strains might be used for developing novel functional fermented beverages with specific properties.

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