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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.

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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

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