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Bioactive action of β-glucosidase enzyme of *Bifidobacterium longum* upon isoflavone glucosides present in soymilk

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Summary Bioconversion of isoflavone glucosides and antioxidant activity by probiotic strain (*Bifidobacterium longum*) during soymilk fermentation was investigated, as well as partial characterisation of the produced enzyme β-glucosidase. The enzyme has higher affinity for genistin than for other substrates assayed. Maximum activity occurred at 42 °C and at pH 6.0; keeping 70–80% of activity for 60 days stored at low temperatures. *Bifidobacterium longum* grew well in soymilk (8.26 log CFU mL^{-1} and pH of 3.9 at 24 h) and were produced in good quantities of organic acids. High hydrolysis degree of isoflavone glucosides (81.2%) was observed at 24 h. Enhancements in bioactivity were assessed in fermented soymilk by monitoring the radical-scavenging activity, antioxidant activity and DNA protective action. The use of probiotic Bifidobacterium strain as β-glucosidase producer increased bioactive isoflavone content and demonstrated that this enzyme plays a key role in the bioavailability of soymilk isoflavones, reducing the bioconversion time compared to other studies.

Keywords Antioxidant activity, *Bifidobacterium*, isoflavone, soymilk fermentation, β-glucosidase.

Introduction

Isoflavones are nonsteroidal compounds that have structural/functional similarity to human oestrogens (Tsangalis *et al*., 2002) and therefore are considered to play an important role in the prevention of cancers (Adlercreutz, 2002), heart disease (De Kleijin *et al*., 2002), menopausal symptoms (Messina, 2000) and osteoporosis (Arjmandi *et al*., 1998). Some of these diseases have been correlated with oxidative damage (Kehrer, 1993). Besides, it is known that regular ingestion of antioxidative supplements, or foods containing antioxidants, may reduce the oxidative damage on the human body (Thomas, 2000). Isoflavones are scarcely distributed in nature; they are mainly found in soybean and unfermented soybean products as conjugated glucosides. Genistin and daidzin correspond to 80–95% of total isoflavones, and provide the basic chemical structures of their aglycones (genistein and daidzein) (Wang & Murphy, 1994). Unfortunately, only isoflavone aglycones exhibit biological activity (i.e. oestrogenic action, antioxidant capacity). For this reason, considerable efforts have been carried out to develop processing techniques such as basic, acid and thermal hydrolysis as well as enzymatic treatment to modify the isoflavone content as well as their composition (Delmonte *et al*., 2006; Mathias *et al*., 2006). Isoflavone conjugates can be transformed into their aglycones during soybean processing by β-glucosidase (EC 3.2.1.21) enzyme (King & Bignell, 2000). Bifidobacteria and some *Lactobacillus*, microorganisms usually present in the gut, produce β-glucosidase enzyme capable to hydrolyse the glucose moiety of isoflavones conjugates releasing the aglycones with biological activity. This transformation could occur at the gut during soymilk consumption or its fermentation (Chun *et al*., 2007; Wei *et al*., 2007; Pham & Shah, 2008; Marazza *et al*., 2009). Bifidobacteria are widely used in food industry because they constitute one of the main bacterial groups of the gut microflora in mammalians, and they are the first colonisers of the sterile gastrointestinal tract of newborns (Tannock *et al*., 1990). Besides, these microorganisms are considered probiotics due to their health beneficial effects such as reduction in serum cholesterol, immune system activation and growth inhibition of potential pathogens that may cause infectious disease in the host (Lomax & Calder, 2009).

Several reports indicated the ability of bifidobacteria to hydrolyse isoflavone β-glucosides during soymilk

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fermentation (Otieno *et al*., 2006; Otieno & Shah, 2007a,b). Tsangalis *et al*. (2002) found that *Bifidobacterium (B.) animalis* produced a significant increase in isoflavone aglycone concentration higher than that of *B. pseudolongum* and *B. longum* reached after 48 h of soymilk incubation. Extensive times affect the productivity of the process.

Bifidobacterium (B.) longum CRL849 is a strain extensively studied in our laboratory, which shows technologically important traits and health-promoting properties. In addition, *B. longum* CRL849 possess a high α-galactosidase (EC 3.2.1.22) activity (Garro *et al*., 1998, 1999, 2004; LeBlanc *et al*., 2004a,b,c) and metabolises soy-oligosaccharides, such as α-D-galactose-oligosaccharides (raffinose, stachyose), considered bifidogenic factors. α-Galactosidase activity in *B. longum* allows it scavenging a wide variety of oligosaccharides commonly found in the human gastrointestinal tract contributing towards its competitive advantage over other bacteria. Additionally, this *B. longum* strain exhibits high β-glucosidase activities on *p*-nitrophenyl-β-D-glucopyranoside (*p*-NPGlu) (Garro *et al*., 2006) displaying great potential to breakdown β-glucoside linkages of soymilk isoflavones yielding biologically active aglycones.

This study was undertaken to evaluate the use of β-glucosidase-producing *B. longum* CRL849 as functional starter culture to increase bioactive isoflavone levels in fermented soymilk and to determine the antioxidant action of the fermented soymilk. A partial biochemical characterisation of this β-glucosidase enzyme was also conducted.

Materials and methods

Microorganism and growth conditions

Bifidobacterium longum CRL849 belonging to the culture collection (CRL) of the Centro de Referencia para Lactobacilos (CERELA-CONICET, Tucumán, Argentina) was used in this study. Before experimental use, culture was propagated (2%, v/v) twice in sterile modified MRS medium incubated at 37 °C for 18 h without agitation in microaerophilic conditions. Modified MRS medium consisted of MRS (De Man *et al*., 1960) broth with 1% (w/v) sucrose, supplemented with 0.05% (w/v) L-cysteine hydrochloride, 0.0005% (w/v) hemin and 0.00005% (w/v) vitamin K. All aggregates were sterilised separately (0.22 μm filtration), and then added to the MRS broth.

Cell-free extracts preparation

Cells at the end of growth exponential phase were collected by centrifugation (10 000 ***g***, 10 min, 4 °C), washed twice with 100 mM McIlvaine buffer (Na_2HPO_4-citric acid, pH 5.8) (McIlvaine, 1921) and resuspended in the same buffer to give a final concentration of 30 % wet weight (w/v). The cell suspensions were disintegrated in a French press (French Pressure cell, Thermo Spectronic, Rochester, NY, USA) at a constant pressure of 12000 psi. Cell debris was removed by centrifuging at 30 000 ***g*** for 10 min at 4 °C. The supernatant fluid was used as a crude enzyme extract cell-free extract (CFE). In order to study the enzyme localisation, the CFE was ultra centrifuged at 207 900 ***g*** (1 h, 4 °C) (optima L-90K ultracentrifuge; Beckman Coulter, Fullerton, CA, USA). The fractions of membrane-wall (FMW) and cytoplasmatic fraction (CP) were used for β-glucosidase assay.

β-glucosidase activity assay

Enzyme activity was measured as previously described (Garro *et al*., 2006) by following the release of *p*-nitrophenol (pNP) from *p*-nitrophenyl-β–D-glucopyranoside (*p*NPGlu, Sigma-Aldrich, Buenos Aires, Argentina). One unit of enzyme (UE) was defined as the amount of enzyme required to liberate 1.0 μmol of pNP per ml per min under the assay conditions. Specific activity was expressed as UE per mg of protein. Protein concentration was determined by using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA) based on the method of Bradford (1976) using bovine serum albumin as a standard. The enzymatic activity was determined in culture supernatant, in CFE, FMW and CP.

β-glucosidase enzyme characterisation

The effect of pH on β-glucosidase activity was determined by incubating of mixture at 37 °C for 15 min in 100 mM McIlvaine buffer with pH ranging from 3.0 to 7.6.

The influence of the temperature on enzymatic activity was determined by incubating the assay mixture in 100 mM McIlvaine buffer pH 5.8 for 15 min at temperatures from 10 to 55 °C.

Storage stability of β-glucosidase was evaluated using suspensions from the microorganism stored at 4 and −20 °C during 2 months. The residual enzyme activity was determined throughout this period.

Soymilk preparation and fermentation

Whole soybeans were washed and soaked overnight in distilled water. The swollen soybeans were manually dehulled and then ground with water. The ratio of dry soybeans to water used for grinding was 1:5 (w/v). The slurry was cooked at 80 °C for 15 min, and then, filtered through a double layered cheese cloth to separate insoluble residues. The liquid was transferred into

glass bottles and sterilised by autoclaving at 121 °C for 15 min. The resulting soymilk was cooled; sucrose (previously sterilised by filtration) was then added to a final concentration of 1% (w/v) and stored at 4 °C before use. Five hundred millilitres of soymilk were inoculated with 4% (v/v) of an active culture of *B. longum* CRL849, previously transferred in soymilk, and allowed to ferment at 37 °C for 24 h. Samples were aseptically withdrawn at 0, 3, 6, 9, 12 and 24 h and immediately cooled on ice to determine cell viability, pH, titratable acidity, organic acids, isoflavone content and β-glucosidase activity. Noninoculated soymilk incubated under the same experimental conditions was used as a control.

Microbiological and chemical analysis

Cell viability in fermented soymilk was determined by the plate dilution method using modified MRS agar that consisted in MRS agar with 1% (w/v) sucrose and 0.05% (w/v) L-cysteine hydrochloride. Serial dilutions of each fermented soymilk sample were plated in duplicate, and plates were incubated at 37 °C for 48 h in anaerobiosis. Results were expressed as colony-forming units per ml (CFU mL^{-1}).

The pH of the samples was measured with a pH metre (Portable Meter PT-10, Sartorius AG, Goettingen, Germany).

Titratable acidity (%TA) was measured by titration with 0.11N NaOH and expressed as percent of lactic acid.

For evaluation of organic acids, samples were centrifuged at 10 000 ***g*** for 10 min at 4 °C, and supernatants were stored at −20 °C until analysis. Lactic and acetic acids were determined by HPLC (Knauer Smartline, Berlin, Germany) using an Aminex HPX-87H column 300 × 7.8 mm (Bio-Rad Laboratories) at 41 °C. As mobile phase, a 5 mM H_2SO_4 solution was used; the flow rate was 0.6 mL min^{-1}. Detection and data acquisition were performed with a differential refractometer (Knauer K-2301, Berlin, Germany) and Eurochrom® software. Pure samples of lactic and acetic acids obtained from Sigma were used as standards for calibration purposes.

β-glucosidase assay in fermented soymilk

Samples (1.5 mL) of soymilk and fermented soymilk were centrifuged at 10 000 ***g*** for 10 min at 4 °C, and the cell pellet was washed once and resuspended with 100 mM McIlvaine buffer (pH 5.8). This suspension was permeabilised by adding 50 μL of toluene–acetone (1:9) per ml of cell suspension. The mixture was stirred for 5 min. Permeabilised samples were kept at 4–6 °C before enzymatic analysis. β-glucosidase activity was measured as described by Garro *et al.* (2006) and expressed as the amount of enzyme required to liberate 1.0 μmol of pNP from the substrate (*p*NPGlu, Sigma) per min under the assay conditions. The results are expressed as UE per ml.

Isoflavone assays

For determining isoflavone content, samples were freeze-dried and stored at −20 °C until used. Isoflavone extraction including β-glucosides and aglycones from fermented and nonfermented soymilk was carried out according to the modified method of Lee *et al.* (2004). Briefly, 250 mg of each freeze-dried samples was extracted with 100 mM HCl (1 mL), acetonitrile (3.5 mL) and bidistilled water (1.5 mL) with shaking at room temperature for 2 h. The insoluble residue was separated by centrifugation (10 000 ***g***, 5 min, 4 °C), and the supernatant was then filtered with a syringe filter (0.45 μm PVDF membrane, Millipore®, Bedford, MA, USA). The supernatants (isoflavone extracts) were stored at −20 °C for quantification by HPLC and bioactivity assays (antioxidant effects).

Reversed-phase high-performance liquid chromatography (RP-HPLC) analysis was performed with KNAUER Smartline System, using Pursuit XRs C18 column (150 × 4.6 mm, Varian Inc., Lake Forest, CA USA) and Smartline multiwavelength UV detector (Marazza *et al.*, 2009). The mobile phase was composed of 0.1% acetic acid in water (solvent A) and 0.1% acetic acid in acetonitrile (solvent B). The solvents flow rate was 0.8 mL min^{-1}, using a gradient of 80% A (20% B) at 0 min, steady for 2.5 min 80% A, decreasing to 70% A for 7.5 min, 65% A for 10 min, 60% A for 5 min, steady at 60% A for 12 min, decreasing to 15% A for 7 min, steady at 15% A for 3 min and then increasing to 80% A for 2 min and steady at 80% for A until completing the gradient programme of 60 min. Samples were monitored from 254 to 262 nm, and area responses were integrated. Glicitein (16 mg l^{-1}) was used as internal standard (the soymilk used in this study does not contain this glucoside). Isoflavones were identified by retention time, and multiwavelength UV spectrums were compared with those of standards. Standards of glucosides (daidzin and genistin) and aglycones (daidzein and genistein) were obtained from Sigma. Results are expressed as mg of flavonoid 100 g^{-1}dry soymilk to correct from concentration variations due to water evaporation.

Determination of antioxidant bioactivities

Antiradical activity determination

Free radical-scavenging ability was determined in the samples (isoflavone extracts) using a synthetic 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical as previously described (Wu *et al.*, 2003) with slight

modification (Marazza *et al*., 2012). DPPH radical was dissolved in methanol to obtain an absorbance equal to 1.00 at 517 nm. All the solutions were daily prepared before use. Aliquots of 300 μL of every isoflavone extracts were added to 3 mL of DPPH solution. The absorbance decrease at 517 nm was monitored spectrophotometrically in cycles of 30 s for 10 min. The antiradical activity percentage (%ARA) of the samples was calculated according to the equation described by Burda & Oleszek (2001). The absorbance of the system in a steady state was estimated by the mathematical fitting of kinetic curves performed with Origin 7.0 software. All determinations were performed in triplicate.

β-carotene bleaching assay

This assay was carried out according to the method described by Chaillou & Nazareno (2006) with some modifications. Briefly, an aliquot of a stock solution of β-carotene in chloroform was mixed with Tween 20. The mixture was evaporated using nitrogen and borate buffer pH 9 was added. For the control assay, 2.0 mL of the β-carotene solution and 200 μL of linoleic acid solution in Tween 20 were mixed in a 3-mL cuvette. Finally, 200 μL of LOX solution was added to initiate the reaction, and the absorbance was monitored at 460 nm in cycles of 30 s for 10 min. For the antioxidant activity determination, the previous procedure was performed by adding an aliquot of 300 μL of every isoflavone extract. All assays were carried out in triplicate at room temperature. Antioxidant activity (% AOA) was calculated as suggested by Burda & Oleszek (2001) as the percentage of inhibition of the β-carotene bleaching of the samples compared to that of the control.

Protective ability against oxidative DNA breakdown

Induction of DNA scission by Fenton's reagent was carried out using a small plasmid DNA (p29Kat 232), which was isolated by fast plasmid kit (AccuPred® Plasmid Mini Extraction Kit, Bionner, Alameda, CA, USA), according to the method described by Lee *et al*. (2002) with some modification. Briefly, 10 μL of each isoflavone extract and 0.5 μL of plasmid DNA in 1 × TE buffer (10 mM Tris-Cl and 1 mM EDTA) were mixed followed by the addition of 20 μL Fenton's reagent (100 mM H_2O_2, 0.1 mM acetic acid and 1.6 mM $FeCl_3$). The reaction mixture was incubated at 30 °C for 1 h. Plasmid and plasmid treated with Fenton's reagent were used as plasmid control and oxidation reaction control, respectively. After incubation, the samples were analysed by electrophoresis on 1% agarose gel containing GelRed nucleic acid gel stain at 65 V per 20 mA for 45 min. After electrophoresis, the gel was exposed under UV light, and the intensity of bands was analysed using a Quanti Scan densitometer (Biosoft) to evaluate the DNA protective action of isoflavone extracts.

Statistical analysis

All results presented in this paper were the average of three independent assays and reported as means ± standard deviation (SD). Multiple comparison procedure using least significant difference (LSD) was applied to determine that means are significantly different at $P < 0.05$ confidence level. The correlations were established using simple regression and analysis of variance models (ANOVA).

Results and discussion

β-glucosidase enzyme characterisation

The enzymatic activity of *B. longum* CRL849 was determined both in the culture supernatant and in CFE; the latter fraction being the only one displaying β-glucosidase activity. In order to study the enzyme localisation, CFE was ultra centrifuged and fractionated in membrane-wall fraction (FMW) and cytoplasmatic fraction (CP). Although the highest activity (154. 9 ± 0.6 UE mg^{-1} protein) was presented in CP, high levels of activity were detected in FMW (101.7 ± 0.5 UE mg^{-1} protein), which would indicate that the enzyme has a location both cytoplasmic and associated with membrane wall. In the following studies was used CFE. It was incubated in McIlvaine buffer at different pH values (3.0–7.6) to determine the effect of pH on the enzymatic activity. Fractions in a pH range between 5.2 and 6.4 (Fig. 1a) exhibited noticeable activity; the optimum pH value being 6.0 with a specific activity of 114.6 ± 7.8 UE mg^{-1} protein. These results are in agreement with those reported by Yang *et al*. (1996) who found the same value of optimal pH for β-glucosidase activity in *Bifidobacterium sp*. Previous studies (Garro *et al*., 2006) have shown that *B. longum* CRL849 (growing under pH-controlled conditions) reached the maximum specific activity both for α-galactosidase and β-glucosidase during the late exponential growth phase (10–12 h incubation), and optimum pH being 6.0. At pH values below 4.4 and above 7.0, β-glucosidase activity decreases about 42.7% and 52.6%, respectively, in comparison with the activity values determined at the optimal pH.

The effect of the temperature of incubation on the β-glucosidase activity of *B. longum* CRL849 is shown in Fig. 1b. CFE fractions showed high β-glucosidase activity at temperatures between 30 and 50 °C, the optimal temperature being 42 °C with a β-glucosidase activity value of 253.1 ± 11.1 UE mg^{-1} protein. Similarly, Nunoura *et al*. (1996) found that, the maximum β-glucosidase activity value was detected at 45 °C for *B. breve* 203. Differences in optimal temperature values for enzyme

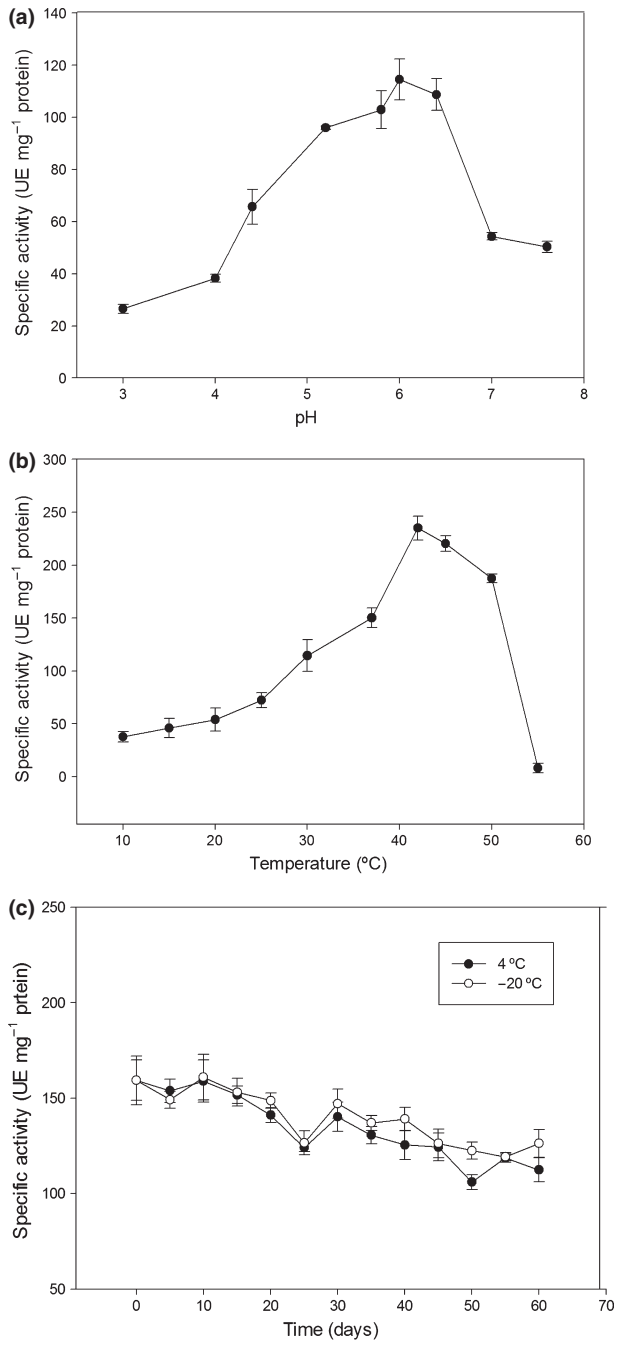

Figure 1 Enzymatic characterisation of β-glucosidase produced by *Bifidobacterium longum* CRL849. Effect of: (a) pH; (b) temperature incubation; (c) storage temperature.

activity may be assigned to the experimental conditions and strain characteristics.

Figure 1c shows β-glucosidase activity of CFE during 60 days storage at −20 and 4 °C for *B. longum* CRL849. In general, the β-glucosidase activity decreased similarly at both temperatures. After 30 days, the CFE lost between 8% and 12% of activity when it was stored at −20 and 4 °C, respectively. After 60 days of storage, the enzyme retained about 70% of residual activity when was stored at 4 °C, while the CFE fraction stored at −20 °C showed approximately 80% of residual activity in comparison with the initial value (159.3 ± 12.8 UE mg^{-1} protein). Otieno *et al*. (2005) reported that, in general, there was no significant difference ($P < 0.05$) in β-glucosidase activity and stability between storages at −80 °C and at 4 °C for *Bifidobacterium* and *Lactobacillus spp*. in fermented soymilk. These results suggest that low temperatures could be used to storage CFE and enzymatic activity can be preserved almost for 60 days. Kinetic studies were also carried out. The enzymatic activity was studied as a function of pNPGlu (0.35–5.30 mM), daidzin (DA) (0.05–0.48 mM) and genistin (GE) (0.05–0.48 mM) concentrations at 42 °C and pH 6.0. The yield of isoflavone glucosides hydrolysis was determined by HPLC. From the Lineweaver-Burk plot (l/velocity as a function of l/substrate concentration), the following values were calculated: *Km* = 0.7, 1.0 and 0.5 mM for pNPG, DA and GE, respectively. The *Vm* values were 5.1, 0.021 and 0.01 mmol per min mg^{-1} for pNPGlu, DA and GE, respectively (data not shown). The enzyme affinity was different among the substrates assayed, being higher for GE than for DA and pNPGlu. These values were in good agreement with those found in extracts from *Lentinula edodes* (Sun *et al*., 2010).

Soymilk fermentation

Growth pattern

The growth kinetics of *B. longum* CRL849 in soymilk at 37 °C is shown in Fig. 2. This microorganism grew well in this substrate. Moreover, their population showed an increase of 1.7 log CFU mL^{-1} up to 12 h of fermentation, reaching a value of 8.16 log CFU mL^{-1}. The highest specific growth rate ($\mu = 0.94$ h^{-1}) was attained during exponential growth phase (3–6 h). After 12 h, the strain showed a reduced growth rate; a maximum population of 8.26 log CFU mL^{-1} was reached at 24 h. Pyo *et al*. (2005) found an increase of 1.4 and 1.1 log CFU mL^{-1} of *B. breve* K-101 and *B. thermophilum* 00748, respectively, in soymilk after 48 h of fermentation. Pham & Shah (2008) studied the effect of lactulose supplementation on growth of *B. animalis* subsp. *lactis* bb12 and *B. longum* 20099 in soymilk during 24 h. This compound stimulated 2.4 log CFU mL^{-1} populations of both strains with values of 8.37 and 8.40 log CFU mL^{-1} for *B. animalis* subsp. *lactis* bb12 and *B. longum* 20099, respectively.

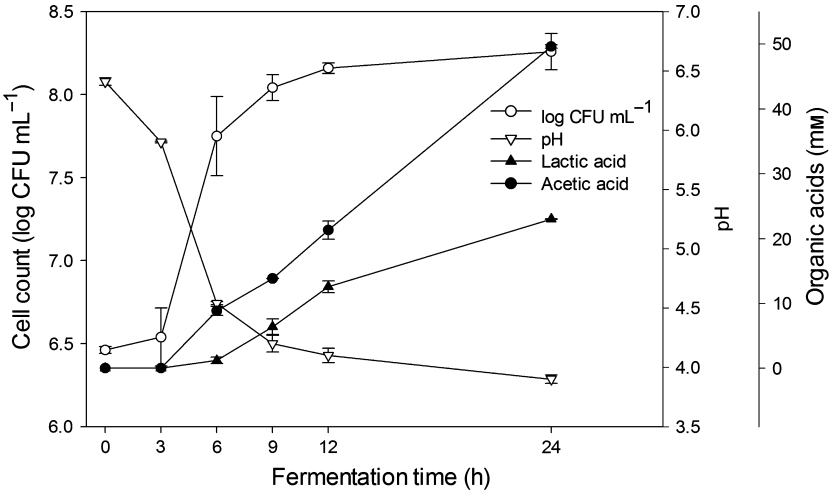

Figure 2 Changes in cell viability, pH and organic acids during soymilk fermentation by *Bifidobacterium longum* CRL849 at 37 °C for 24 h.

In our studies, *B. longum* CRL849 exhibited higher cell population (8.26 log CFU mL^{-1}) than that found by Pyo *et al.* (2005), but similar to those reported by Pham & Shah (2008). These results could be due to the presence of sucrose and α-galactosyl oligosaccharides (raffinose and stachyose) in soymilk that are rapidly metabolised by invertase and α-galactosidase enzymes enhancing *B. longum* CRL849 growth (Garro *et al.*, 1998, 1999, 2004; LeBlanc *et al.*, 2004a,b,c).

The drop in pH was more pronounced between 3 and 6 h of fermentation (from 5.9 to 4.5) while %TA increased during fermentation reaching a value of 0.84% at 24 h (data not shown). These results were higher than those reported by Pyo *et al.* (2005) for *B. breve* K101 and *B. thermophilum* 00748. Moreover, *B. longum* CRL849 showed 2.3 and 1.2-fold higher % TA than for *B. longum* B6 and *B. infantis* CCRC 14633 used by Hou *et al.* (2000). A low pH is a desirable condition in fermented products because organic acids contribute to the flavour development and shelf-life of the final products.

It is well known that certain lactic acid bacteria and bifidobacteria are able to produce high amounts of lactic and acetic acids during fermentation of soymilk (Hou *et al.*, 2000; Wang *et al.*, 2003; Garro *et al.*, 2004). In this study, *B. longum* CRL849 produced 49.63 ± 0.25 mM of acetic acid and 23.02 ± 0.05 mM of lactic acid at the end of fermentation (24 h) (Fig. 2). The acetate/lactate molar ratio changed throughout the fermentation period, between 7.3 to 2.2 at 6 and 24 h, respectively. These results are 1.5-fold higher than for the strains *B. infantis* CCRC 14633 and *B. longum* B6 studied by Hou *et al.* (2000) in soymilk after 48 h. The organic acid formation in fermented soy milk was due to the ability of *B. logum* strain to express high α-galactosidase activity to degrade efficiently the soymilk α-galactosyl oligosaccharides (Garro *et al.*, 1998, 1999, 2004; LeBlanc *et al.*, 2004a,b,c).

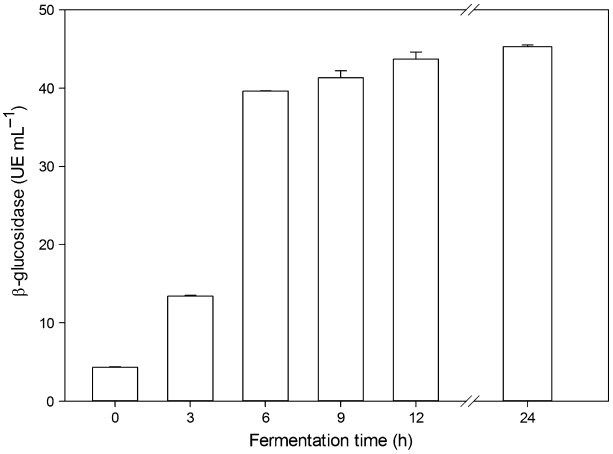

Figure 3 Production of β-glucosidase enzyme during soymilk fermentation by *Bifidobacterium longum* CRL849 at 37 °C for 24 h.

β-glucosidase activity and isoflavone bioconversion

β-Glucosidase activity during soymilk fermentation with *B. longum* CRL849 was studied at 37 °C for 24 h (Fig. 3). The strain, which produced high levels of β-glucosidase activity during fermentation, displayed a marked β-glucosidase increase between 3 and 6 h (from ca. 13.4 to 39.6 UE mL^{-1}) coincident to the maximum growth rate, while at 24 h, a slight activity increase was observed, being 1.1-fold higher than that found at 6 h. A similar behaviour was reported by Pyo *et al.* (2005) and Chien *et al.* (2006).

β-Glucosidase plays a key role in breaking down the β-glucosidic bond in isoflavone glucosides to release the biologically active aglycones (Esaki *et al.*, 2004).

Table 1 Changes in soymilk isoflavone contents during fermentation with *Bifidobacterium longum* CRL849 at 37 °C

		Isoflavone (mg per 100 g^{-1} soymilk)			
		Glucosides		Aglycones	
Soymilk/Strain	Fermentation Time (h)	Daidzin	Genistin	Daidzein	Genistein
Control*	0–24	38.21 ± 0.80[a]	91.64 ± 0.85[a]	11.31 ± 0.26[d]	11.97 ± 0.16[e]
B. longum CRL849	0	38.90 ± 2.30[a]	91.51 ± 1.50[a]	12.22 ± 0.75[d]	12.21 ± 0.21[e]
	3	35.62 ± 0.20[a]	87.09 ± 0.60[a,b]	14.28 ± 0.90[d]	16.83 ± 1.50[d]
	6	30.38 ± 1.40[b]	68.05 ± 0.65[b]	16.65 ± 0.17[c]	25.28 ± 1.40[c]
	9	25.80 ± 0.50[c]	49.01 ± 0.32[c]	23.40 ± 0.20[b]	33.50 ± 1.30[c]
	12	22.82 ± 1.03[c]	31.10 ± 0.06[c]	32.88 ± 0.93[a]	69.44 ± 1.20[b]
	24	9.94 ± 0.05[d]	14.57 ± 1.03[d]	36.03 ± 0.17[a]	83.29 ± 1.15[a]

*noninoculated soymilk.

Values in each column having the same letter are not significantly different ($P > 0.05$).

The high β-glucosidase activity produced by *B. longum* CRL849 throughout the fermentation seemed to be biomass associated if the increase on β-glucosidase activity with the increment of population is considered.

In general, the bioconversion of isoflavone glucosides (daidzin and genistin) into the bioactive aglycone forms (daidzein and genistein) (Table 1) followed the same pattern of β-glucosidase activity (Fig. 3) during the *B. longum* strain incubation. A total isoflavone content of 153.13 ± 1.45 mg per 100 g^{-1} dry sample was found in the unfermented soymilk (control). The contents of isoflavone glucosides, daidzin and genistin, biologically inactive forms, were higher (84.8%) than their respective aglycone isoflavones (15.2%). During soymilk fermentation with *B. longum* CRL849, a concomitant enzymatic hydrolysis of isoflavone glucosides occurred leading to changes in the concentration of all the isoflavone forms (Table 1). At 24 h, 81.2% of the isoflavone glucosides were hydrolysed into the aglycone form, the genistein concentration being 2.3-fold higher than that of daidzein (83.29 ± 1.15 and 36.03 ± 0.17 mg per 100 g^{-1}, respectively). Similar increasing trend in aglycone concentration during incubation and the concomitant reduction in β-glucoside concentration for other probiotic strains were also reported (Esaki *et al*., 2004; Chien *et al*., 2006). Lactulose is considered a bifidogenic factor able to enhance the β-glucosidase activity of *Bifidobacterium* (Gonzales *et al*., 2003). Lactulose supplementation on the biotransformation of isoflavone glucosides in soymilk fermented with *B. animalis subsp. lactis* bb12 and *B. longum* 20099 has been reported to increase 1.2-fold isoflavone hydrolysis in respect to the unsupplemented soymilk (Pham & Shah, 2008). Similarly, in our study, sucrose supported the growth and β- glucosidase production of *B. longum* CRL849.

Total isoflavone content, including glucoside and aglycone, in soymilk prepared from soybean, was slightly higher (1.6-fold) than that observed by Pham & Shah (2008) for soymilk prepared from soy protein isolate (SPI). In respect to the biotransformation of isoflavones in soymilk by lactic acid bacteria and bifidobacteria, Tsangalis *et al*. (2002) reported that 57.75% of isoflavone glucosides of soymilk were hydrolysed by *B. pseudolongum*-a while 9.79% by *B. pseudolongum*-b after 24 h fermentation. Chien *et al*. (2006) also studied this process and reported that only 6.42% glucosides were hydrolysed by *B. longum* after 32 h of incubation. In our study, *B. longum* CRL849 was more efficient (81.2 % hydrolysis) showing approximately 24% and 75% higher hydrolysis extents than those reported by Tsangalis *et al*. (2002) and Chien *et al*. (2006), respectively. Also, fermentation time required to isoflavone glucosides bioconversion was shorter (24 h) than that determined by Chien *et al*. (2006) (32 h).

Determination of antioxidant activity

The antioxidative potential of isoflavone extracts from fermented soymilk samples was determined by three different methods, the results are shown in Table 2.

Antiradical activity (ARA) was determined by the DPPH radical-scavenging method in the isoflavone extracts from soymilk fermented with *B. longum* 849. This activity (%ARA) slowly increased during fermentation process, reaching values about 3.2 times higher than the control (isoflavone extracts from uninoculated soymilk incubated under the same experimental conditions), resulting in an activity of 30.2% ± 4.0% at 24 h of fermentation. The ability to scavenge a free radical is directly related to the number and location of aromatic hydroxyl groups that are able to donate a hydrogen atom (Rice-Evans *et al*., 1997), so that the levels and type of aglycones present in the fermented soymilk play an important role. In this sense, genistein presents three hydroxyl groups located in positions 5,

Table 2 Antiradical, antioxidant and DNA protective activities of isoflavone extracts from soymilk fermented with *Bifidobacterium longum* CRL849 for 24 h at 37 °C

Fermentation time	% ARA	% AOA	% I-pDNA-O
Control	8.1 ± 1.7[a]	18.6 ± 2.7[ab]	9.0 ± 0.8[a]
0 h	9.5 ± 2.4[ab]	17.3 ± 3.7[a]	9.5 ± 2.7[a]
3 h	18.5 ± 4.5[b]	17.7 ± 1.0[a]	12.0 ± 3.9[ab]
6 h	18.0 ± 2.1[b]	21.4 ± 0.8[bc]	13.0 ± 2.5[ab]
9 h	18.6 ± 1.4[b]	23.0 ± 1.8[c]	20.0 ± 3.6[bc]
12 h	27.9 ± 3.8[c]	31.8 ± 0.9[d]	24.0 ± 1.9[c]
24 h	30.2 ± 4.0[c]	55.6 ± 0.9[e]	27.3 ± 4.1[c]

Values in each column having the same letter are not significantly different ($P > 0.05$).
Control: soymilk without inoculation.
% ARA: percentage antiradical activity, determined by the DPPH radical method.
% AOA: percentage antioxidant activity by β-carotene bleaching assay.
% I-pDNA-O: percentage inhibition the plasmid DNA oxidation induced by Fenton's reagent.

7 and 4' while that daidzein only two, in positions 7 and 4'. Due to this structural difference, genistein have greater ability to scavenge the DPPH radical than daidzein. On the other hand, the hydrolysis reaction leaves the hydroxyl group free from its glycosidic bond and therefore increases the antiradical capacity. This would explain the high ARA activity of the isoflavone extract sample with 24 h of fermentation with respect to the isoflavone extract of control, which besides containing high content of total aglycones, the concentration of genistein was greater than daidzein (Table 1).

β-Carotene bleaching assay

The antioxidant activity of isoflavone extracts of fermented soymilk samples remained constant for the first 3 h, and after 9 h, it increased 1.3 times with respect to the control, reaching a value of 23% approximately. The major differences were observed between 12 and 24 h, the maximum value of AOA (55.6% ± 0.9%) being reached after 24 h of fermentation. This increase was ascribed to the aglycones released from their glucosides by the β-glucosidase enzyme action of the strain used in the soymilk fermentation (*B. longum* CRL 849). These results are consistent to those reported by Rao & Muralikrishna (2002), indicating that the aglycones have higher activity than their corresponding glucosides. In general, the glucose bound to the aglycones reduced 50 to 100 times the AOA (Naim *et al.*, 1976). Besides, the main antioxidant studies of isoflavones are reported using the pure forms of these compounds instead extracts obtained from different matrix. Burda & Oleszek (2001) evaluated the antioxidant activity of several pure natural compounds including daidzein and genistein (isoflavone aglycones) and reported activities of 32.9% and 24.6%, respectively. In the present research, samples of soymilk with 12 h of fermentation showed similar results. However, when the fermentation time reached 24 h, the AOA was enhanced 1.7 times in respect to the AOA reported by Burda & Oleszek (2001). The results obtained show that it is possible to improve the antioxidant capacity of soymilk using a probiotic strain (*B. longum*) able to convert isoflavones during fermentation process.

Protection towards Oxidative DNA strand breakdown

The ability to inhibit the oxidation of DNA induced by Fenton's reagent was evaluated in the isoflavone extracts of soymilk samples (Table 2). During fermentation time, a slight increase in this ability was observed. The maximum value of 27.3% ± 4.1% was found at 24 h.

The results presented in this research demonstrate that isoflavone extracts of soymilk fermented with *B. longum* are capable of scavenge free radicals and inhibit lipid oxidation induced by a pro-oxidant enzyme. These properties are very important because, in many serious diseases (Alzheimer's disease, diabetes, arthritis, cancer), which involve free radicals and lipid oxidation (Beckman & Ames, 1998; Shahidi, 2004). However, many other illnesses arise as a result of the DNA damage caused by an oxidative state. The protection of the DNA against oxidative stress will help to prevent or lessen the chances of developing many genetic disorders. On the basis of this background, it was strongly demonstrated that soymilk enriched by fermentation in isoflavone aglycones had, also, the ability to protect DNA against the exposure of an oxidising agent.

Conclusions

In the present study, the β-glucosidase system of *B. longum* CRL849 was preliminarily characterised. This microorganism was able to increase more than 80% of bioactive isoflavones aglycones during soymilk fermentation in shorter times compared other studies previously reported. In this way, it was demonstrated that this enzyme plays a key role in the bioavailability of isoflavones, as it is responsible for releasing the bioactive aglycone forms. The antiradical and antioxidant capacities as well as the DNA protective ability of the isoflavone aglycone extracts increased during the soymilk fermentation. The probiotic *Bifidobacterium* strain used in this study shows great potential to produce fermented soymilks enriched in bioactive isoflavones with demonstrated antioxidant capacity, and thus, improving the functional health benefits of this food product. In addition, further detailed studies for the development and design of functional beverage of

soybean with a differential impact in the host, are necessary.

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