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EVALUATION OF BIOACTIVE AND ANTI-NUTRITIONAL COMPOUNDS DURING SOYMILK FERMENTATION WITH *Lactobacillus plantarum* BAL-03-ITTG AND *Lactobacillus fermentum* BAL-21-ITTG

EVALUACIÓN DEL CONTENIDO DE COMPUESTOS BIOACTIVOS Y ANTINUTRICIONALES DURANTE LA FERMENTACIÓN DE LECHE DE SOYA CON *Lactobacillus plantarum* BAL-03 ITTG Y *Lactobacillus fermentum* BAL-21 ITTG

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Abstract

Soymilk is a food rich in proteins, phenolic compounds and lactose-free. However, due to organoleptic and anti-nutritional characteristics, soymilk is not very popular among the population. The objectives of this article are to evaluate the changes in the activity of α-galactosidase and β-glucosidase, as well as phytates, isoflavones, phenolic compounds contents and antioxidant activity of soymilk during the fermentation with *Lactobacillus plantarum* BAL-03-ITTG and *Lactobacillus fermentum* BAL-21-ITTG. Soymilk was fermented for 24 h with *L. plantarum* and *L. fermentum* separately, with an increase of 1.2 to 1.4 log CFU g^{-1} for both strains. The results showed that the strains are able to produce β-glucosidase, carrying out the conversion of β-glucosides in their corresponding aglycones, with an increase of 420 to 490%. In addition, the strains can decrease approximately 20% of the content of phytate in soymilk during fermentation. Therefore, both strains could be used in future works to obtain food from fermented soymilk.

Keywords: antioxidants, isoflavone, phytate.

Resumen

La leche de soya es un alimento rico en proteínas, compuestos fenólicos y libre de lactosa. Sin embargo, por sus características organolépticas y antinutricionales su consumo es poco popular entre la población. Los objetivos de este estudio fueron evaluar los cambios en la actividad de la α-galactosidasa y β-glucosidasa, así como del contenido de fitatos, isoflavonas, compuestos fenólicos y actividad antioxidante de la leche de soya durante la fermentación con *Lactobacillus plantarum* BAL-03-ITTG y *Lactobacillus fermentum* BAL-21-ITTG. La leche de soya fue fermentada durante 24 h con *L. plantarum* y *L. fermentum* por separado, con un incremento en el crecimiento de 1.2 a 1.4 log UFC g^{-1} para ambas cepas. Los resultados mostraron que ambas cepas son capaces de convertir los β-glucósidos en sus correspondientes agliconas, con un incremento del 420-490%. Además, pueden disminuir aproximadamente el 20% del contenido de fitatos durante la fermentación. Por lo anterior ambas cepas podrían ser utilizadas en futuros trabajos para la obtención de alimentos a partir de leche de soya fermentada.

Palabras clave: antioxidantes,isoflavonas, fitatos.

1 Introduction

Soybean (*Glycine max* L.) is a legume widely used for its edible bean. It is the most protein-rich plant when compared to any other cereal or legume (Grieshop *et al*., 2003). On average, dry soybean contains about 40 to 41% protein, 35% carbohydrates, 8 to 24% lipids, and 5% ash (Medic *et al*., 2014), and high levels of bioactive phenolic compounds have been quantified (Zhao & Shah, 2014). Isoflavones in their glucoside (genistin, daidzin and glycitin) or aglycone (genistein, daidzein and glycitein) forms, as well as benzoic, chlorogenic,

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gallic, cinnamic and ferulic acids, have also been reported as the main phenolic compounds in soybeans (Rodríguez-Roque *et al*., 2013; Zhao & Shah, 2014). Soybeans are also good sources of vitamins including B complex, C and E (Kim *et al*., 2006). These bioactive compounds confer antioxidant (Marazza *et al*., 2012), antihypertensive (Wang *et al*., 2015) and hypocholesterolemic (Belleville, 2002) properties. Moreover, soybean also has considerable amount of calcium (1.95 mg g^{-1}), iron (0.06 mg g^{-1}), magnesium (4.07 mg g^{-1}), phosphorus (4.69 mg g^{-1}) and potassium (23.87 mg g^{-1}), sodium (0.123 mg g^{-1}) and zinc (0.037 mg g^{-1}) (Hemmige-Natesh *et al*., 2017). For these reasons, soybean is considered an excellent alternative in developing functional foods (Granato *et al*., 2010; Day, 2013). Soybean products, however, can contain anti-nutritional factors, mainly phytates (García-Mantra *et al*., 2015), trypsin inhibitors (Gemede & Ratta, 2014) and oligosaccharides such as raffinose and stachyose (Hati *et al*., 2014). These oligosaccharides are generally associated with stomach aches, because they are not digested in the human intestinal tract due to the absence of α-galactosidase (Baú *et al*., 2015). Phytate may affect the bioavailability of minerals, solubility, functionality and digestibility of proteins and carbohydrates (García-Mantrana *et al*., 2015). Thus, new strategies to improve the attributes of these products must be developed.

One of the foods commonly made from soybeans is soymilk: a liquid extract of soybeans produced by soaking, grinding and filtering (Day, 2013; Mishra & Mishra, 2013). The consumption of soymilk in Western countries, however, is limited because of the presence of undesirable "green", "beany" and "grassy" off-flavors (Kaneko *et al*., 2014). Soymilk has been reported to also contain anti-nutritional compounds as was previously mentioned (Abd EI-Gawad *et al*., 2015; García-Mantrana *et al*., 2015). However, soymilk has a significant concentration of isoflavones, which are phytoestrogen substances associated with the low risk of the so-called Western disease (Morales-de la Peña *et al*., 2018). To improve the flavor and digestibility of soymilk, the use of lactic acid bacteria (LAB) can be an alternative. The LAB are a heterogeneous group of bacteria that have been used for food preservation (Leroy & De Vuyst, 2004) and for improving the flavor, digestibility and acceptability of food (Granato *et al*., 2010, Mishra & Mishra, 2015; Riciputi *et al*., 2016). Lactobacillus has been used as a strategy to reduce the content of anti-nutritional factors (tannins, phytic acid and oligosaccharides) in cereals, because of the ability of these bacteria to produce enzymes like phytase, which catalyzes the sequential hydrolysis of phytate to phosphate (Sudarmadji & Markakis, 1977; Tang *et al*., 2010). In presence of the raffinose and stachyose, LAB are able to produce α-galactosidase and hydrolyze raffinose and stachyose (Hati *et al*., 2014; Baú *et al*., 2015). Furthermore, LAB play an important role in deglycosylation isoflavone conjugates during soy food fermentation, through the production of β-glucosidase, which hydrolyzes the β-glucosidic bonds of β-glucosides to release glucose and the corresponding aglycone (Zhao & Shah, 2014).

The fermentation of soymilk with LAB could be an alternative to increase the concentration of bioactive compounds and decrease the content of anti-nutritional compounds present in soymilk. Most experiments performed with the fermentation of soymilk with LAB were carried out using soy protein isolate whey protein and / or soymilk added with glucose (Hati *et al*., 2014; Zhao & Shah, 2014). Most of the earlier published works focused only on the growth of LAB and isoflavone content (Baú *et al*., 2015; Hati *et al*., 2015), but not on antioxidant capacity, phenolic content and the reduction of phytate content in fermented soymilk. Therefore, it is necessary to evaluate new strains for the production of fermented soy foods, which allow obtaining functional foods. In this way, in *in vitro* tests, *Lactobacillus plantarum* BAL-03-ITTG and *Lactobacillus fermentum* BAL-21-ITTG have been reported as potentially probiotic (González-Escobar, 2013). In *in vivo* test, *Lactobacillus plantarum* BAL-03-ITTG was able to reduce serum cholesterol and triglyceride levels by 33.9 and 15.88%, respectively (Ramírez-Torres, 2015). The potential of these microorganisms to develop fermented foods, however, has not been reported. Therefore, the objectives of this research were to evaluate the changes in α-galactosidase and β-glucosidase activity, as well as phytate, isoflavones, phenolic compounds contents and antioxidant activity in soymilk during fermentation with *Lactobacillus plantarum* BAL-03-ITTG and *Lactobacillus fermentum* BAL-21-ITTG.

2 Materials and methods

2.1 Soymilk preparation

Soymilk was prepared according to García-Mantrana *et al*. (2015) with some modifications. Washed soybeans were soaked in water in a 1:4 ratio (w:v) at 97±2 °C for 5 min, then drained and mixed with water in ratio of 1:7 (w:v) and liquefied 3 min with a blender. The slurry was filtered through two layers of muslin cloth, and approximately 480 mL of soymilk was obtained per 100 g of soybeans in wet weight. The soymilk was autoclaved at 112 °C for 12 min and stored at 4 °C until utilization.

2.2 Molecular identification of strains

The strains BAL-03-ITTG and BAL-21-ITTG were obtained from the research laboratory collection of cultures in the Tecnológico Nacional de México/Instituto Tecnológico de Tuxtla Gutiérrez, Chiapas, in Mexico.

Total genomic DNA was extracted using a Quick-DNA^{TM} Fungal/Bacterial Miniprep Kit (Zymo Research, Irvine, CA, USA), according to the manufacturer specifications. PCR was performed with bacterial universal 16S rRNA primers. The primers used were 27F (5'-AGA GTT TGA TCM TGG CTC AG-3 ') and 1492R (5'-TAC GGY TAC CTT GTT ACG ACT T-3'), which amplified products of approximately 1465 bases. The PCR products were purified using the PCR Product Purification System Kit from Roche® and sequenced in LANGEBIO, CINVESTAV, México. All sequences were compared with reference sequences obtained by a BLAST search (Altschul *et al*., 1990). The sequences were aligned using the CLUSTAL X (2.0) software with the default settings. The 16S rRNA gene sequence of strains BAL-03-ITTG and BAL-21-ITTG were deposited in the GenBank NCBI database under the accession numbers KY131967.1 and KY574532.1, respectively.

2.3 Soymilk fermentation

Soymilk was fermented with BAL-21-ITTG and BAL-03-ITTG separately. Soymilk was inoculated with 1% of an inoculum containing between 7.2 and 7.4 log CFU g^{-1} of Lactobacillus cultures. The inoculated soymilk was incubated at 37 °C for 24 h. Samples were obtained at 0, 2, 4, 6, 8, 10, 12 and 24 h for the determination of viable cell count, pH, α-galactosidase and β-glucosidase activity, antioxidant activity, phytate and isoflavone contents. The samples for the determination of α-galactosidase and β-glucosidase activity, antioxidant activity, phytate and isoflavone contents determinations were previously lyophilized according to Enciso-Sáenz *et al*. (2018) by using a freeze dryer (Labconco FreeZone 2.5 L, Kansas City, MO, USA), at -50 °C for 24 h. Finally, the lyophilized samples were stored in glass amber bottles at -17 °C until use.

2.4 Determinations in fermented soymilk

2.4.1 Viable cell count and determination of pH

The viable cell count of LAB was determined in triplicate using the pour plate method in MRS (Peredo-Lovillo *et al*., 2019) agar incubated at 37 °C for 72 h. Results were expressed as log CFU g^{-1} of the fermented soymilk. The pH of the fermented milk was determined with a digital potentiometer (Oakton pH 1100 Series, VernonHills, IL, USA).

2.4.2 Determination of α-galactosidase and β-glucosidase activity

The enzyme extracts were obtained according to Baú *et al*. (2015). Briefly, 500 mg of lyophilized fermented soymilk were mixed with 5 mL of sodium acetate buffer (0.2 M, pH 4.8) and incubated for 1 h at 200 rpm at room temperature. The mix was centrifuged (3,500 rpm, 4 °C for 10 min) (Hermle Z 326K, Hermle Labortechnink, Wehingen, Germany), and the supernatant was filtered with a 0.45 μm Millipore filter (Millipore, Tullagreen, Ireland). The filtrate was used to determine α-galactosidase activity according to Scalabrini *et al*. (1998). The enzymatic activity assay was performed by the hydrolysis of *p*-nitrophenyl-α-D-galactopyranoside, determining the amount of *p*-nitrophenol released. One unit of enzyme activity (AU) was defined as the amount of enzyme necessary to release 1 μmol of *p*-nitrophenol per minute under the assay conditions. The results were expressed in AU per gram of dry sample (AU g^{-1}).

For β-glucosidase activity, 500 mg of lyophilized fermented soymilk was treated with 3 mL of citrate buffer (0.05 M, pH 4.5) containing NaCl (0.1 M) and incubated for 1 h at room temperature (Baú *et al*., 2015). Enzyme activity assay was performed according to Sanches de Lima and Ida (2014), determining the amount of *p*-nitrophenol released from *p*-nitrophenyl-β-D-glucopyranoside. One unit of

enzyme activity (AU) was defined as the amount of enzyme that releases 1 μmol of *p*-nitrophenol per minute under the assay conditions. The results were expressed in AU per gram of dry sample (AU g^{-1}).

2.4.3 *Isoflavones determination*

The extraction and HPLC quantification of daidzin, glycitin, genistin, daidzein, glycitein and genistein in fermented and unfermented soymilk were determined according to Zhao and Shah (2014) with some modifications. One gram of the lyophilized samples was mixed with 20 mL of 80% methanol and incubated in a water bath at 65 °C in darkness for 2 h. The samples were shaken every 10 min and cooled at room temperature. When the samples were cooled, 0.8 mL of NaOH (2 M) was added to the mixture and shaken (120 rpm) at room temperature for 10 min before 0.5 mL of glacial acetic acid was added. The mixture was filtered, and 8 mL of filtrate was added to 2 mL of 50% methanol. The solution was centrifuged at 4,500 rpm for 5 min and filtered (0.22 μM, Millipore) and analyzed by HPLC. For HPLC determinations, the isoflavones were separated using a Zorbax C18 column (250 mm x 4.6 mm x 5 μm; Agilent Technologies, Wilmington, DE, USA). A linear gradient was applied with a mixture of two solvents: (A) 88% water, 10% methanol, 2% acetic acid and (B) methanol at a flow of 0.8 mL min^{-1} using a High-Perfomance Liquid Chromatography (PerkinElmer, Norwalk, CT, USA). The gradient for each solvent started at 90:10 (A%: B%) and decreased linearly to 40:60 (A%: B%) for 25 min, then it was maintained for 8 min before returning to the initial conditions 90:10 (A%: B%). The detection wavelength was 260 nm. The injection volume was 20 μL. For calibration curves, daidzin, glycitin, genistin, daidzein, glycitein and genistein (Sigma-Aldrich Co., St. Louis, MO, USA) were used. The results were expressed in mg of isoflavones per g of sample on a dry basis (mg g^{-1}).

2.4.4 *Total phenolic content and hydrophilic and lipophilic antioxidant activity*

The total phenolic content was analyzed using the Folin-Ciocalteu method described by Morales-de la Peña *et al*. (2010). An aliquot of 0.5 mL of fermented soymilk was mixed with 0.5 mL of Folin-Ciocalteu reagent and 10 mL of Na_2CO_3 (20%). Samples were kept at room temperature for 1 h. Mixtures were then filtered (0.45 μm, Millipore), and the absorbance was measured at 725 nm with a UV-Vis spectrophotometer (Beckman Coulter Du® 73, Germany). Concentrations were determined by comparing the absorbance of the samples with a calibration curve constructed by using gallic acid (Sigma-Aldrich Co., St. Louis, MO, USA). Results were expressed as mg of gallic acid equivalents (GAE) per 100 g of beverage (mg GAE/100 g of fermented soymilk).

For hydrophilic and lipophilic fractions, the antioxidant activity of soymilk was determined by measuring the efficiency of inhibiting 1,1-diphenyl-2-picryl-hydrazyl (DPPH, Sigma®, Sigma-Aldrich Inc., St. Louis, MO, USA) radical, following the methodology described by Zhao and Shah (2014). Therefore, 2 g of fermented soymilk were mixed with 4 mL of 80% methanol and centrifuged at 4,500 rpm for 20 min at 4 °C. The supernatant was then considered as the hydrophilic fraction. On the other hand, to obtain lipophilic fraction, an aliquot of 2 g of fermented soymilk was mixed with 4 mL hexane and centrifuged at 4,500 rpm for 20 min at 4 °C. The supernatant was then considered as the hydrophilic fraction. Aliquots of 0.2 mL of hydrophilic or lipophilic extracts were mixed with 3.8 mL of methanolic solution of DPPH (0.025 g L^{-1}). The homogenate was shaken vigorously and kept in the dark for 30 min. Absorbance was measured at 517 nm against a methanol blank. Results were calculated and expressed as the percentage of inhibition of the DPPH radical (Eq. 1):

$$\text{DPPH inhibition (\%)} = \left(\frac{Ac - Asm}{Ac}\right) \times 100 \quad (1)$$

where *Ac* is the absorbance of the control (the methanolic solution of the DPPH radical without extract) and *Asm* is the absorbance of the sample.

2.4.5 *Phytate determination*

The extract of lyophilized soymilk was obtained using the methodology proposed by Frühbeck *et al*. (1995). Twenty mL of HCl (0.65 N) was added to the sample (0.5 g) and stirred for 2 h at room temperature. The mixture was centrifuged at 4,500 rpm for 30 min at 15 °C, and the supernatants were collected. For phytate determination, the pH of the extract was adjusted to 6.0 with NaOH (1 N), and then 10 mL were taken and transferred to the resin column (8 mm x 65 mm; Dowex® 1-X8 Anion Exchange Resin, 200-400 mesh, USA). The column was washed with 15 mL of NaCl (0.1 N). The phytate was eluted with 15 mL of NaCl

(0.7 N) and the purified extract was collected, 3 mL of deionized water were taken (used as blank), or 3 mL of purified extract to which the pH was previously adjusted to 3 and 1 mL of Wade reagent (0.03% $FeCl_3 \cdot 6H_2O$ plus 0.3% sulfosalicylic acid dissolved in deionized water) was added, and the absorbance was read at 500 nm. Concentrations were determined by comparing the absorbance of the samples with a calibration curve constructed by using sodium phytate (Sigma-Aldrich Co., St. Louis, MO, USA).

2.5 Statistical analysis

Treatments were conducted in triplicate. An analysis of variance (ANOVA) of the results was carried out in order to determine significant differences ($p \leq 0.05$) between treatments. A least significant difference (LSD) test was employed to determine differences between means. The data were analyzed using the Statgraphics Centurion XV software.

3 Results and discussion

3.1 Microorganism growth and pH changes during soymilk fermentation with LAB

Phylogenetic analysis based on the 16S rRNA gene, showed that the strain BAL-03-ITTG was closely related to *Lactobacillus plantarum* with a similarity of 99% and the BAL-21-ITTG to *Lactobacillus fermentum* with 99% similarity.

Cell growth and pH profiles of *L. plantarum* BAL-03-ITTG and *L. fermentum* BAL-21-ITTG during soymilk fermentation can be observed in Figure 1. During fermentation, the cell population of *L. plantarum* increased from 7.2 to 8.6 log CFU g^{-1}, while *L. fermentum* counts increased from 7.4 to 8.6 log CFU g^{-1} after 24 h of fermentation. The growth kinetics of LAB showed that the adaptation phase was of 2 and 4 h for *L. fermentum* and *L. plantarum*, respectively. Between 2 to 8 h of fermentation, there was the logarithmic phase of growth. Similar results were reported by Zhao and Shah (2014) in the soymilk fermentation with *L. acidophilus*, *L. paracasei*, *L. zeae* and *L. rhamnosus*, in which the counts of LAB increased 1.5-2 log CFU mL^{-1} after 12 to 24 h of fermentation. The specific growth rate (μ_{max}) and doubling time for *L. plantarum* were 0.652±0.082 h^{-1} and 1.071±0.136 h, respectively. While the specific growth rate (μ_{max}) and doubling time were 0.394±0.020 h^{-1} and 1.763±0.093 h for *L. fermentum*. These values are lower than those reported by Yoon and Hwang (2008) in which the specific growth rate (μ_{max}) and doubling time were 1.35 h^{-1} and 0.513 h, respectively, during the soymilk fermentation with *L. curvatus*. During fermentation, the pH of soymilk dropped from 6.5 to 4.5 for both strains (Figure 1); this could be due to the production of lactic acid (Baú *et al*., 2015). A similar behavior in the decreased of pH was reported by Baú *et al*. (2015), reaching the lowest value (4.97) in the stationary phase of growth.

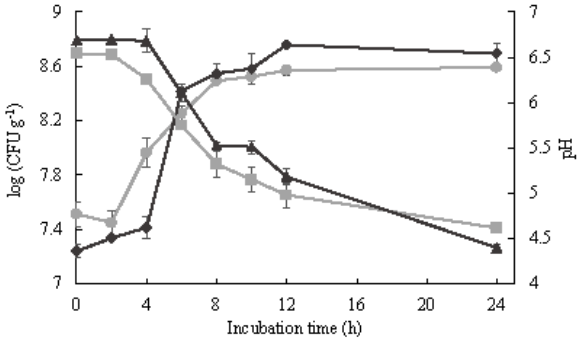

Fig. 1. Bacterial growth (♦) and pH (▲) change during soymilk fermentation with *L. plantarum*, bacterial growth (●) and pH (■) change during soymilk fermentation with *L. fermentum*.

3.2 α-Galactosidase activity in soymilk fermented with LAB

The α-galactosidase activity in the fermented soymilk varied from 0.0007 to 0.019 AU g^{-1} for *L. plantarum*, while in fermented soymilk with *L. fermentum*, the activity ranged from 0.001 to 0.020 AU g^{-1}. Furthermore, α-galactosidase activity showed similar profiles to cell growth, and the highest activity was obtained in the exponential growth phase (Figure 2A). These results indicated that microorganisms could hydrolyze the raffinose and stachyose in simple sugars that could be used for their growth. A maximum α-galactosidase activity was obtained after 8 h of fermentation, but this decreased slowly from 0.019 to 0.015 AU g^{-1} until the 24 h. Similar results were reported by Baú *et al*. (2015), who showed that α-galactosidase activity decreases after the exponential growth phase.

Table 1. Isoflavone content in soymilk fermented with *L. plantarum*

Incubation time (h)	β-Glucosides (mg g^{-1} d.b.)			Aglycones (mg g^{-1} d.b.)		
	Daidzin	Glycitin	Genistin	Daidzein	Glycitein	Genistein
0	1.650±0.14^{a}	0.223±0.01^{a}	1.230±0.09^{a}	0.085± 0.02^{d}	0.002±0.00^{c}	0.143±0.12^{e}
2	1.562±0.01^{ab}	0.259±0.03^{a}	1.245±0.14^{a}	0.095±0.02^{d}	0.002±0.00^{c}	0.126±0.01^{e}
4	1.500±0.06^{ab}	0.219±0.03^{a}	1.127±0.03^{a}	0.087±0.02^{d}	0.002±0.00^{c}	0.142±0.02^{e}
6	1.458±0.02^{b}	0.241±0.02^{a}	1.067±0.01^{ab}	0.205±0.04^{c}	0.003±0.00^{c}	0.261±0.04^{d}
8	1.105±0.09^{c}	0.275±0.09^{a}	0.922±0.02^{bc}	0.345±0.04^{b}	0.007±0.01^{bc}	0.318±0.01^{cd}
10	0.795±0.06^{d}	0.249±0.13^{a}	0.773±0.13^{c}	0.093±0.09^{b}	0.009±0.01^{bc}	0.388±0.05^{c}
12	0.668±0.02^{d}	0.280±0.12^{a}	0.731±0.09^{c}	0.592±0.03^{a}	0.015±0.00^{b}	0.493±0.01^{b}
24	0.388±0.07^{e}	0.260±0.15^{a}	0.410±0.08^{d}	0.673±0.08^{a}	0.037±0.01^{a}	0.661±0.05^{a}
LSD	0.163	0.204	0.2	0.115	0.01	0.071

Mean values with different letter in a column are significantly different ($p \leq 0.05$) according to LSD test.

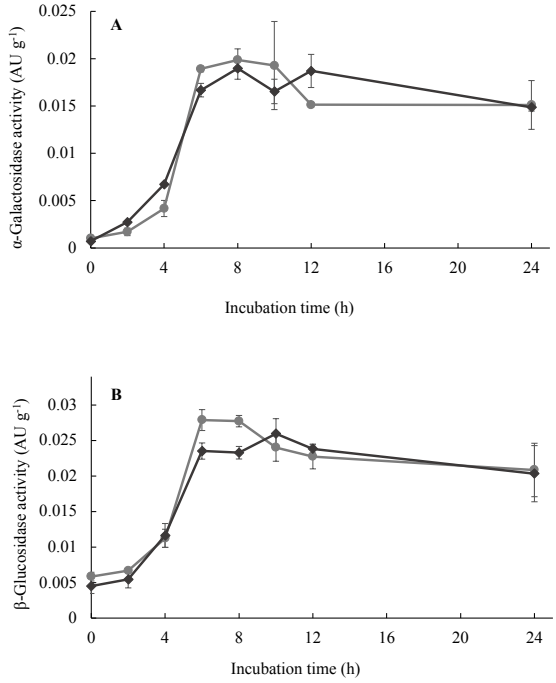

Fig. 2. α-Galactosidase (A) and β-glucosidase activity (B) in soymilk fermented with *L. plantarum* (♦) and *L. fermentum* (●).

3.3 β-Glucosidase activity and isoflavone content in soymilk fermented with LAB

The β-glucosidase activity in the fermented soymilk varied from 0.004 to 0.026 AU g^{-1} for *L. plantarum*, reaching the maximum activity at 10 h of fermentation, while in fermented soymilk with *L. fermentum*, it was between 0.006 and 0.027 AU g^{-1}. β-Glucosidase showed the highest activity after 8 h of fermentation, followed by a decrease after the exponential growth phase until the 24 h (Figure 2B). β-Glucosidase is responsible for catalyzing the hydrolysis of isoflavones β-glucosides to isoflavones aglycones (Baú *et al*., 2015). In soymilk fermented with *L. plantarum* and *L. fermentum*, the conversion of β-glucosides to aglycone was detected. Tables 1 and 2 show the content of isoflavones in fermented soymilk using *L. plantarum* and *L. fermentum*, respectively. Daidzin and genistin contents decrease significantly ($p \leq 0.05$) during fermentation, but the glycitin content was constant after 24 h. Daidzein and genistein, however, increased significantly after 24 h of fermentation. The isoflavones β-glucosides are converted into aglycones through the action of β-glycosidase, which catalyzes the hydrolysis the β-glycosidic bonds to release more bioactive aglycone counterparts (Rodríguez-Roque *et al*., 2013; Baú & Ida, 2015).

The aglycones isoflavones are absorbed in the human small intestine more easily than glycosylated conjugates because their molecular weight improves diffusion and absorption (Rekha & Vijayalakshmi, 2011). In soymilk fermented after 24 h with *L. plantarum*, the content of isoflavones aglycones increased 490%, while in soymilk fermented with *L. fermentum* an increase of 420% was observed. Our results were higher than those of Zhao and Shah (2014), who observed that in soymilk containing glucose (1%, w/v) after 12 to 24 h of fermentation with *L. acidophilus*, *L. paracasei*, *L. zeae* and *L. rhamnosus* the content of isoflavones increases 309 to 337%. In the soymilk fermented with *L. plantarum* and *L. fermentum*, the total content of aglycone isoflavone was 1.371 mg g^{-1} and 1.27 mg g^{-1} respectively, while

Table 2. Isoflavone content in soymilk fermented with *L. fermentum*.

	β-Glucosides (mg g^{-1} d.b.)			Aglycones (mg g^{-1} d.b.)		
Incubation time (h)	Daidzin	Glycitin	Genistin	Daidzein	Glycitein	Genistein
0	1.749±0.03^{a}	0.255±0.00^{a}	1.310±0.00^{a}	0.092± 0.0^{d}	0.002±0.00^{c}	0.137±0.00^{c}
2	1.588±0.02^{a}	0.220±0.00^{b}	1.164±0.00^{a}	0.090±0.00^{d}	0.003±0.00^{c}	0.137±0.02^{c}
4	1.315±0.03^{b}	0.183±0.01^{cd}	0.918±0.00^{b}	0.151±0.02^{d}	0.002±0.00^{c}	0.224±0.04^{c}
6	1.178±0.15^{b}	0.191±0.02^{bc}	0.820±0.11^{b}	0.317±0.02^{c}	0.014±0.00^{b}	0.371±0.01^{b}
8	0.750±0.01^{cd}	0.152±0.001^{e}	0.547±0.01^{c}	0.538±0.00^{b}	0.029±0.00^{a}	0.544±0.00^{a}
10	0.816±0.16^{c}	0.169±0.02^{cde}	0.633±0.12^{c}	0.588±0.07^{ab}	0.032±0.00^{a}	0.561±0.07^{a}
12	0.700±0.14^{cd}	0.157±0.02^{de}	0.574±0.11^{c}	0.586±0.07^{ab}	0.032±0.01^{a}	0.545±0.08^{a}
24	0.576±0.08^{d}	0.164±0.00^{e}	0.559±0.05^{c}	0.646±0.06^{a}	0.033±0.00^{a}	0.591±0.06^{a}
LSD	0.228	0.031	0.165	0.099	0.008	0.104

Mean values with different letter in a column are significantly different ($p \leq 0.05$) according to LSD test.

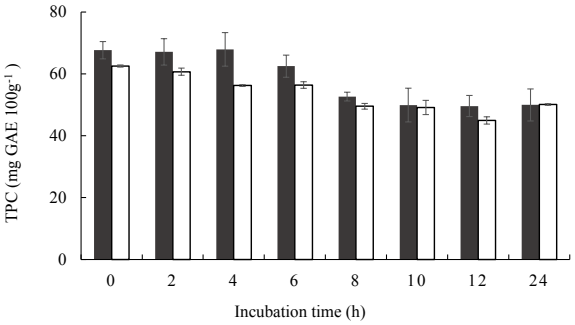

Fig. 3. Total phenolic content (TPC) of soymilk fermented with *L. plantarum* (■) and *L. fermentum* (□).

that of the unfermented soymilk was 0.23 mg g^{-1}. This result showed that this lactic acid bacteria during soymilk fermentation produces the β-glucosidase and has the ability to produce soymilk with more aglycone content, which has greater beneficial effects than other types of isoflavones (Messina & Messina, 2000).

3.4 Total phenolic content and antioxidant capacity of fermented soymilk

The total phenolic content in soymilk fermented with *L. plantarum* and *L. fermentum* is shown in Figure 3. Unfermented soymilk contains 62 to 67 mg GAE per 100 g of sample. At the end of the fermentation, the samples showed a significant decrease ($p<0.05$) in the content of total phenols. The phenol content decreases mainly during the exponential growth phase (the first 8 h), which suggests that the microorganisms could be consuming some of these phenolic compounds or could be used to avoid the oxidation of soymilk compounds. Similar results were reported by Zhao and Shah (2014), who reported in soymilk fermented with *L. zeae* that the total phenol content decreased. The principal phenolic acids reported in soymilk are gallic acid, 4-hydroxybenzoic acid, *p*-coumaric acid, ferulic acid, cinnamic acid and chlorogenic acid and catechin (Rodríguez-Roque *et al*., 2013; Zhao & Shah, 2014). *L. plantarum* possesses phenolic acid decarboxylases (PAD) inducible in the presence of *p*-coumaric acid and ferulic acid. PAD decarboxylates ρ-coumaric acid and ferulic acid to corresponding vinyl derivatives (4-vinyl phenol and 4-vinyl guaiacol) (Rodríguez *et al*., 2009). Through the action of the PAD and the reductase enzymes, *L. plantarum* could metabolize caffeic acid to vinyl catechol and ethyl catechol (Cavin *et al*., 1997). In addition, phenolic compounds could be delaying or inhibiting the oxidation processes, avoiding the formation of volatile decomposition products (aldehydes and ketones) and causing the decrease in the concentration of these phenolic compounds (Alamed *et al*., 2009). Virtanen *et al*. (2006) reported that during soymilk fermentation with LAB when the proteolysis is observed, antioxidant peptides and antioxidant amino acids could be obtained. This could explain that in fermented soymilk with *L. fermentum* after 24 h a slight increase in the content of total phenols was observed.

Antioxidant activity of hydrophilic and lipophilic extract during the fermentation of soymilk was determined (Figure 4). During the fermentation of soymilk, antioxidant activity of the hydrophilic extract (Figure 4A) was in the range of 16 to 24% (expressed as a percentage of the inhibition of the DPPH radical). For the hydrophobic extract, however, the percentage of inhibition varied between 0.2 and 1.7% (Figure 4B).

Table 3. Accumulate phytate reduction during soymilk fermentation with *L. plantarum* and *L. fermentum*.

	Accumulate phytate reduction (%)	
Incubation time (h)	*L. plantarum*	*L. fermentum*
0	0 ± 0.0^{d}	0 ± 0.0^{d}
2	0.11 ± 0.15^{d}	0.86 ± 0.46^{d}
4	0.96 ± 0.33^{d}	4.57 ± 1.18^{c}
6	10.00 ± 1.25^{c}	11.87 ± 0.13^{b}
8	16.00 ± 0.59^{b}	20.26 ± 0.35^{a}
10	19.82 ± 1.55^{a}	20.59 ± 0.05^{a}
12	20.69 ± 0.07^{a}	20.52 ± 0.27^{a}
24	20.73 ± 0.19^{a}	20.91 ± 0.49^{a}
LSD	1.73	1.15

Mean values with same letter in a column are not significantly different ($p > 0.05$) according to LSD test.

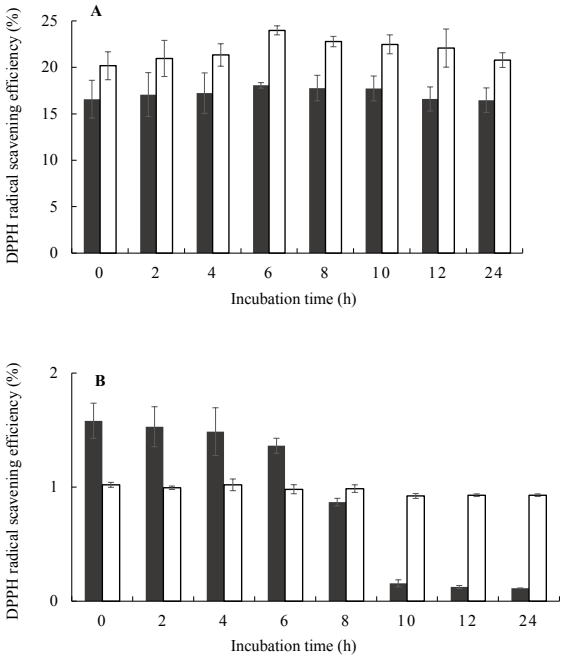

Fig. 4. Changes in hydrophilic (A) and lipophilic (B) antioxidant activity in soymilk during fermentation with *L. plantarum* (■) and *L. fermentum* (□).

These values were similar to those reported by Zhao and Shah (2014), who showed that during the fermentation of soybean milk with *L. acidophilus*, *L. paracasei*, *L. zeae* or *L. rhamnosus*, the DPPH radical inhibition percentage oscillated in a range of 14 to 19.6%. Although the phenols content decreased during fermentation, the antioxidant activity remained constant during fermentation. This could be due to the increase of aglycones during fermentation, which could have an antioxidant effect.

3.5 Phytate content during fermentation of soymilk

The phytic acid molecule has six radicals (it is a molecule with multiple negative charges) that show a strong capacity to chelate several cations (positive charges), and therefore, the molecule has a certain anti-nutritive activity. For that, residual phytate content in the foods is very important. The initial content of phytate was 2.58-2.60 mg g^{-1} of soymilk, but this decreased significantly in the first 6 and 4 h of fermentation with *L. plantarum* and *L. fermentum*, respectively (Table 3). After 24 h of fermentation, the phytate reduction was 20%. Several authors have reported that different strains of lactic acid bacteria have the ability to reduce the levels of phytates in food (Raghavendra & Halami, 2009; Fischer *et al*., 2014). Saraniya and Jeevaratnam (2014) reported that *L. plantarum* and *L. pentosus* decrease 47 and 66% the phytate content in soymilk fermented after 18 h compared to the unfermented soymilk. García-Mantrana *et al*. (2015) reported that in soymilk fermented with *L. casei* the phytate content remained constant throughout fermentation. In soymilk supplemented with phytate fermented with different strains of LAB, the initial phytate content did not decrease significantly during fermentation and storage (Tang *et al*., 2010). Therefore, the ability to reduce the phytate concentration could depend on the origin of the strain or culture conditions used.

Conclusions

The results of this investigation demonstrate that both LAB produced α-galactosidase during soymilk fermentation at 37 °C for 24 h, which suggests that these strains could hydrolyze raffinose and stachyose, undesirable sugars in soymilk. *L. plantarum* BAL-03-ITTG and *L. fermentum* BAL-21-ITTG can potentially reduce the phytate content (anti-nutritional compounds) and increase the formation of aglycones in fermented soymilk by action of β-glucosidase. The phenols total content decrease little during fermentation of soymilk using *L. plantarum* and *L. fermentum* but antioxidant activity remained constant. This study provides an evaluation of the soymilk fermentation with *L. plantarum* BAL-03-ITTG and *L. fermentum* BAL-21-ITTG, two autochthons lactic acid bacteria, for their possible application to the development of soy-based foods.

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