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Behavior study of *Bifidobacterium longum* using solid state fermentation from commercial soybean meal

Antonieta Rodríguez de Olmos [a], Oscar A. Garro [b], Marisa S. Garro [a,*]

[a] Centro de Referencia para Lactobacilos (CERELA-CONICET-CCT NOA Sur), Chacabuco 145, (T4000ILC), San Miguel de Tucumán, Tucumán, Argentina
[b] Universidad del Chaco Austral (UNCAUS)- INIPTA-CONICET, Cte. Fernandez 755, (3700)- Pcia, Roque Saenz Peña, Chaco, Argentina

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ABSTRACT

The development of alternatives to dairy products has gained great importance using plant-based foods. In this study, efforts have been made to develop vegetable alternatives from soybean paste without any supplement using single potential probiotic culture. Commercial soybean meal was inoculated with *Bifidobacterium* (*B.*) *longum* under previously fitted fermentation conditions by experimental design. Carbohydrates, organic acids, isoflavones, and amino acids profiles were studied in nine conditions of moisture and temperature, and also at several times in optimized conditions. Kinetics and technological parameters, effect of simulated gastric conditions on bacterium survival in optimized soybean paste, and their relationship with soybean matrix using scanning electron microscopy (SEM) were also evaluated. *B. longum* was able to use the main carbohydrates in soybean producing organic acids as was expected (acetic/lactic ~ 1.5) and to enhance bioactive isoflavones (maximum concentration: 32.6 mg/50 g of soybean paste). Furthermore, *B. longum* showed high tolerance at simulated saliva solution and gastric juice, but the pancreatic solution deeply affected its viability in MRSs and also in soybean paste. Soybean paste represents an excellent vegetable candidate to be used as potential probiotic carrier food for *B. longum* and an attractive alternative to develop new functional foods.

1. Introduction

Bifidobacteria are autochthonous members of the human intestinal microbiota. Although they represent only a minority component of the adult human intestinal microbial ecosystem (Arboleya, Watkins, Stanton, & Ross, 2016), their presence in the gastrointestinal tract (GT) has been associated with the concept of healthy microbiota (Arboleya et al., 2016; O'Callaghan & van Sinderen, 2016). Several health promoting effects have been directly related to the presence of bifidobacteria in the GT, such as maintenance of normal microbiota, protection of the host against pathogens by competitive exclusion, immunostimulation and immunomodulation, breakdown of non-digestible dietary carbohydrates, and reduction of serum cholesterol levels (Arboleya et al., 2016; O'Callaghan & van Sinderen, 2016). Due to these beneficial effects, some species of bifidobacteria have become common components in many dairy products and pharmaceuticals (Linares et al., 2017). However, studies using other substrates as alternatives to dairy products are limited.

Most of the studies about bifidobacteria and lactic acid bacteria (LAB) were done using submerged fermentation (Garro, Rivas, & Garro, 2021) and some studies have used alternative technologies such as solid state fermentation (SSF) (Bartkiene, Krungleviciute, Juodeikiene, Vidmantiene, & Maknickiene, 2015; Park, General, & Lee, 2012; Torino et al., 2013; Zhang et al., 2014). However, few of them have been based on optimization of kinetic and technological parameters of these cultures in SSF for the production of novel vegetarian foods.

Soybean meal is a by-product from dietary oil production with high quality of protein, and there is great availability in Argentina (FAO, 2017) representing an attractive alternative to obtain novel foods by SSF. Also, consumption of soybean has been linked to the prevention of several diseases and these health benefits are attributed to the presence of several bioactive compounds (Chen, Wang, Pan, Gao, & Chen, 2018; González-Montoya, Hernández-Ledesma, Silván, Mora-Escobedo, & Martínez-Villaluenga, 2018; Zaheer & Humayoun Akhtar, 2017). Soybean fermentation by probiotic microorganisms including *Lactobacillus* and *Bifidobacterium* may produce or increase some of these bioactive compounds, thus the functionalities of foods can be improved and also, offer a vegetarian alternative to dairy products as probiotic carrier food.

Rodríguez de Olmos, Bru, and Garro (2015) have preliminarily studied the effect of moisture and temperature on growth, proteolytic

* Corresponding author. CERELA-CONICET Chacabuco 145, (T4000ILC), San Miguel de Tucumán, Tucumán, Argentina.
E-mail address: mgarro@cerela.org.ar (M.S. Garro).

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activity, and β-glucosidase of *Bifidobacterium (B.) longum* CRL 849 by surface response methodology using commercial soybean meal. In this preliminary study, this strain showed good adaptation to solid soybean substrates, but was strongly affected by incubation temperature.

The aim of the current study was to analyze different compounds of interest from the fermentation of soybean paste by *B. longum* CRL 849 in several conditions of moisture and temperature fitted by experimental design, applying other methodologies. In addition, kinetic and technological parameters of the fermentation process were tested under optimal conditions. The effect of simulated gastric conditions on bacterium survival in optimized soybean paste and its relationship with soybean matrix was also evaluated using scanning electron microscopy.

2. Materials and method

2.1. Microorganisms and growth conditions

B. longum CRL 849 was obtained from the culture collection (CRL) of the Centro de Referencia para Lactobacilos (CERELA). This culture was selected by its ability to grow on soybean substrate using available nutrients and to produce β-glucosidase enzyme. Before experimental use, culture was propagated (2%, v/v) twice in MRS broth (Laboratorios Britania S.A., Argentina) supplemented with 1% sucrose, 0.00005% vitamin K and 0.0005% hemin, and incubated at 37 °C for 18 h in microaerophilic condition without agitation. All solutions were sterilized separately (0.22 μm filtration), and then added to the MRS broth. To obtain the inoculums for solid state fermentation, cells at the end of the exponential growth phase in supplemented MRS broth (MRSs) were collected by centrifugation, washed twice and suspended in sterile physiological solution (around to 10^9 CFU/mL).

2.2. Solid state fermentation

SSF was performed with different moisture content (M: 50, 55, 65, 75, 80%) and incubated at several temperatures (T: 31, 33, 37, 41, 43 °C) that was proposed by an experimental design fitted by Rodríguez de Olmos et al. (2015). The levels of the independent variables were defined according to 2^2 full factorial Central Composite Design (CCD), comprising 11 experimental runs in 2 blocks: block 1 was conducted with 6 random assays (4 factorial points and 2 central points), and block 2 with 5 random assays (4 axial points and 1 central point). The resulted combinations of moisture (%) and temperature (°C) according to CCD were: 75%-33 °C; 75%-41 °C; 55%-33 °C; 55%-41 °C (factorial points), 80%-37 °C; 50%-37 °C; 65%-31 °C; 65%-43 °C (axial points) and 65%-37 °C (central point).

For each assay, SSF used 150 g of soybean paste (wet weight) that was prepared into 250 mL Erlenmeyer flask, from commercial soybean meal and distilled water to achieve the different moisture contents (M). After water was added, the pastes were homogenized and sterilized by autoclaving at 118 °C for 20 min. The soybean pastes were inoculated with 4% (*v/w*) of culture, corresponding c.a. 7.50×10^7 CFU/g of soybean paste. Then the pastes were homogenized and uniformly distributed into Petri dishes and incubated at the established temperatures (T) during 24 h. Uninoculated soybean paste treated in the same way was used as a control. Samples from each soybean paste at 0 and 24 h were taken.

2.3. Moisture and pH measures

A thermogravimetric infrared moisture analyzer with an automatic and programmable ceramic surface heater (SARTORIUS MA100C, Germany) was used to determine the moisture content of the soybean paste. Changes in pH were monitored during fermentation of soybean paste using a pH meter (SARTORIUS PT-10, Germany).

2.4. Microbial counts

Cell viability was determined by the plate dilution method using Reinforced Clostridium Medium agar (Biokar). Serial dilutions of each fermented soybean paste sample were plated in duplicate and the plates were incubated at 37 °C for 48–72 h. The results were expressed as colony forming units per gram (CFU/g).

2.5. Analysis of metabolites

2.5.1. Organic acids production and sugar consumption

For the evaluation of organic acids and residual sugars, proteins were removed from the samples (fermented and non-fermented pastes) and supernatants were stored at −20 °C until analysis (Ortiz, Fornaguera, Raya, & Mozzi, 2012). The production of lactic and acetic acid was determined by High Performance Liquid Chromatography (HPLC) according to Marazza, Nazareno, de Giori, & Garro, 2013. Stachyose, raffinose, sucrose, and their hydrolysis products (galactose, glucose, and fructose) were quantified by HPLC (Marazza, Garro, & de Giori, 2009). For the quantification of sugars and organic acids, calibration curves for each compound were performed using pure standards at different concentrations.

2.5.2. Free amino acid (FAA) determination

FAA content of non-fermented and fermented soybean paste was determined by RP-HPLC (Reversed-phase-HPLC). Samples were treated with 12.5% (*w/v*) TCA solution (sample: TCA, 1:3) to eliminate proteins and the amino acids were obtained from the supernatant. These supernatants were derivatized to *o*-phthaldialdehyde (OPA) derivatives, and the amino acids concentration was determined by RP-HPLC. The chromatographic separation was carried out using SHIMADZU Prominence with a Gemini C18 column (4.6 × 250 mm, particle size 5 μm) with an elution rate of 1 mL/min. Derivatized amino acids were determined with a fluorescence detector (excitation wavelength = 340 nm, emission wavelength = 450 nm). The binary solvent system consisted of solvent A: 40 mM sodium phosphate buffer (pH 6.4) and solvent B: acetonitrile, methanol, and water (45:45:10). Identification and quantification of amino acids were carried out by comparison with a standard mixture of amino acids (Sigma Aldrich). The LC solution 1.24 program was used for the analysis.

2.5.3. Isoflavones (IF) quantification

Soybean paste samples were stored at −20 °C until used for IF analysis. The extraction of IF from fermented and non-fermented soybean paste and their quantification by RP-HPLC were carried out according to Marazza et al. (2009). Standards of IF aglycones (Dai: daidzein and GEi: genistein) were obtained from Sigma Aldrich.

2.6. Characterization of optimized soybean paste fermented by B. longum CRL 849

2.6.1. Kinetics and technological parameters

Soybean substrate at 65% of moisture fermented by *B. longum* CRL 849 at 37 °C was selected to continue with kinetics and technological characterization of the fermentation process. The inoculum amount of bacterium was 2% (v/w) as it was previously optimized by Rodríguez de Olmos et al. (2015). Samples of fermented soybean paste were taken at several times after inoculation (0, 4, 8, 12, and 24 h) and the analysis of microbial count, pH measurement, carbohydrates, organic acids, β-glucosidase activity, FAA using OPA methodology and electrophoresis for protein analysis were performed. Unfermented samples were also taken as control of fermentation.

Microbial counts, pH measurement, carbohydrates consumption, and organic acids production were determined as previously was detailed.

β-glucosidase activity from *B. longum* CRL 849 and OPA methodology

in fermented soybean paste and control (uninoculated) were evaluated according to Rodríguez de Olmos et al. (2015).

Soybean protein extraction from control and fermented pastes was made according to the method of Gonzalez de Mejia, Vásconez, de Lumen, and Nelson (2004) with some modifications for solid samples. 0.5 g of soybean paste was taken and mixed with 3 mL of 0.05 M Tris/HCl buffer pH 8.2. The samples were then disintegrated using a sonicator (Digital Sonifier SLPt, Branson) for 10 min and centrifuged at 10000 *g* for 10 min. Supernatants were stored at −20 °C until used for protein quantification and electrophoresis determination.

The protein concentration in the different samples was determined according to Bradford (1976) using bovine serum albumin as standard.

Soybean protein profile was studied by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 4% stacking gel and 15% separating gel according to the methodology described by Shagger and von Jagow (1987). Aliquots of 20 μg of each protein sample were mixed with 5X Laemmli buffer (Laemmli, 1970) and loaded into the lanes. Molecular weight marker (MW) was loaded into the lane of SDS-PAGE. MW values of soybean protein fractions were estimated by using a Broad Range Protein Molecular Weight Marker (10–225 kDa) from Promega Corporation (Madison, WI. V8491). Electrophoresis was carried out in a vertical unit (BIORAD Mini PROTEAN 3 System, Hercules, USA), for 5 h to 30 mA. Coomassie Brilliant Blue R-250 was used to visualize the proteins bands.

2.6.2. Sugar addition and prolongation of fermentation process

To evaluate whether the addition of an external carbon source or extension of fermentation time could enhance proteolytic or β-glucosidase activities of *B. longum* CRL 849, sucrose and glucose (2% *w/w*) were separately added into soybean substrate with 65% of moisture. The soybean paste was sterilized and inoculated with *B. longum* CRL 849 as it was previously described and incubated at 37 °C. All parameters of interest (pH, microbial count, organic acids, sugar consumption, β-glucosidase activity, and FAA) were also assayed at 24, 48, and 72 h. Unfermented soybean paste of each condition was taken as control of fermentation.

2.6.3. Tolerance to a simulated gastrointestinal digestion

To evaluate the possibility of using *B. longum* CRL 849 as a potential probiotic and the protection of the soybean matrix, the resistance to gastric and intestinal digestion was sequentially assayed with a protocol adapted from Zárate, Chaia, González, and Oliver (2000). Overnight cultures of *B. longum* from MRSs or fermented soybean paste were adjusted to 1×10^8 CFU/mL or 1×10^8 CFU/g with physiological solution (PS). A volume of 1.5 mL of cell suspension in PS was mixed with 1.5 mL artificial saliva solution (0.22 g/L $CaCl_2$, 6.2 g/L NaCl, 2.2 g/L KCl, 1.2 g/L $NaHCO_3$, 20 mg/L lysozyme, 2 mg/mL α-amylase, pH 6.5) and incubated 10 min at 37 °C with agitation. After that, 1.75 mL of a cell suspension of each sample was mixed with 2.25 mL of simulated gastric juice (125 mM NaCl, 7 mM KCl, 45 mM NaHCO3, 3 g/L pepsin, pH 2.0); the pH was adjusted to 2.0 with HCl and samples incubated at 37 °C with agitation for 90 min. Then, 3 mL of simulated intestinal juice (0.75% (*p/v*) bile salts, 2 mg/mL pancreatin, pH 8.0) were added and the mixtures were incubated at 37 °C during 90 min with agitation. Microbial count was done at initial and final of each treatment and the results were expressed as CFU/g.

2.6.4. Scanning electron microscopy (SEM)

To analyze the morphology of the bacterium used in this study and its arrangement on soybean matrix, SEM was used. For cultures from MRSs (8 h incubation at 37 °C), 1 mL aliquot was taken and centrifuged (8000 *g*, 10 min) to recover the cells and have a pellet of approximately 0.5 mm. For solid samples from fermented soybean paste (optimized conditions) and unfermented control, 0.2 g was taken. Subsequently, 1 mL of phosphate buffer (pH 7.4) containing 2.5% (*v/v*) of glutaraldehyde and 1% (*w/v*) of $CaCl_2$ was added to each sample, mixed by inversion and kept at 4 °C for 16 h. The samples were included in agar and fixed in phosphate buffer containing 1% (*w/v*) of osmium tetroxide at 20 °C for 2 h. Next, the samples were dehydrated using a series of solutions with increasing concentrations of ethanol (30, 50, 70, 85, 90, and 100%). The samples were placed on metal support and coated with a thin layer of gold using a cathodic ion spray (Jeol model jfc-1100). The microscopic observation was made with the Sweeping Microscope brand Zeiss model Supra 55vp, belonging to the CIME-CCT CONICET Tucumán-UNT (Integral Center of Electron Microscopy, dependent on CONICET and the National University of Tucumán).

2.7. Statistical analysis

Assays were carried out at least in duplicate, and results were expressed as mean values with standard deviations. Statistical analyses were performed using MINITAB 17 software (State College, PA, USA). Comparisons were accomplished by ANOVA general linear model followed by Tukey's and $p < 0.05$ was considered significant.

3. Results

3.1. Study of carbohydrates consumption, acid organic products, conversion of IF and FAA profile at different conditions of moisture and temperature (M-T)

To analyze in depth the SSF systems proposed by the experimental design of the previous study (Rodríguez de Olmos et al., 2015), carbohydrates consumption, the main fermentation products, and conversion of IF were determined, as well as the change in the amino acid content.

3.1.1. Sugar consumption and organic acid production by B. longum CRL 849 in SSF

Fig. 1 shows the fermentation products of *B. longum* CRL 849 at different conditions proposed by the experimental design at 0 h (control) and 24 h of fermentation. Fermentation products were acetic and lactic acid in a ratio close to the expected theoretical values (acetic/lactic ~ 1.5) in all conditions tested. However, the highest production of both acids was detected in soybean where the incubation temperature was 37 °C without significant differences between these samples ($p \geq 0.05$). For soybean paste with 50% of moisture, production of acetic acid was 116.4 ± 11.8 μmol/g and lactic acid 83.8 ± 7.6 μmol/g, whereas at 65% and 80% moisture, values were 93.1 ± 11.7 μmol/g and 68.9 ± 7.4, and 92.5 ± 10.9 μmol/g and 67.7 ± 6.9, respectively. In contrast, the concentration of organic acids was lower at higher temperature conditions where the bacteria showed loss of viability and less acidification (Rodríguez de Olmos et al., 2015), therefore temperature is an important factor for this type of bacterium in SSF.

Sucrose was present in commercial soybean meal with a percentage of 62.0 ± 1.4%, whereas other carbohydrates present were stachyose 24.0 ± 2.8%, raffinose 10.5 ± 0.7%, and glucose 4.5 ± 0.7%. The amount of each carbohydrate in the soybean pastes without inoculum (control) varied according to the substrate moisture (Fig. 1). For example, a scarce amount of glucose and galactose, and higher amounts of sucrose, raffinose, and stachyose can be seen in lower moisture conditions (where solids are more concentrated). *B. longum* CRL 849 used mainly sucrose as a carbon source in all tested conditions (Fig. 1), but this strain could not consume it completely at the end of the fermentation (24 h). The highest consumption (84.96%) was observed in soybean paste with 80% of moisture and fermented at 37 °C. Oligosaccharides (stachyose and raffinose) were also used by the strain in some conditions of fermentation. Products of the di, tri, and tetrasaccharides hydrolysis were detected in low quantity (galactose and glucose) or not detected (fructose), which suggests that they were consumed by the microorganism during growth.

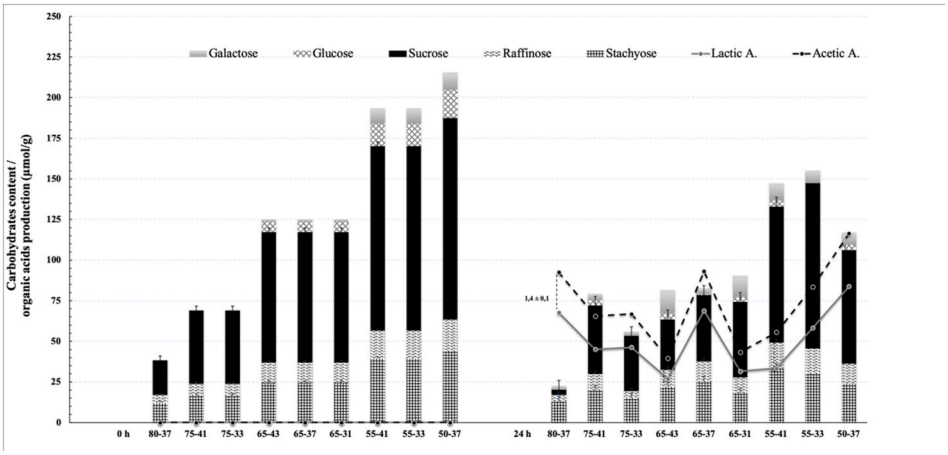

Fig. 1. Carbohydrates content and organic acids production at different conditions of moisture and temperature (M%-T °C) at 0 h in unfermented soybean paste (control) and 24 h soybean paste fermented by *Bifidobacterium longum* CRL 849.

3.1.2. FAA content in soybean paste fermented by B. longum CRL 849

FAA profile of fermented soybean paste at 50, 65, and 80% of moisture are shown in Fig. 2 and their respective moisture control (without inoculum). Other profiles (M-T: 75%-33 °C; 75%-41 °C; 55%-33 °C; 55%-41 °C; 65%-31 °C; 65%-43 °C) are summarized at supplementary data (Fig. S1).

The total concentration of FAA was lower than the initial one in most evaluated conditions, confirming the results previously obtained using OPA methodology (Rodríguez de Olmos et al., 2015).

Consumption of total amino acids was higher at fermentation conditions where temperature was optimum for *B. longum* (37 °C): 50-37; 65-37; 80-37 with a total decrease of 53.0; 33.2 and 52.7% respectively (Fig. 2). The greatest changes under these conditions were observed for Asp, Glu, Ala, Val, Ser, Phe, and Ile; decreasing more than 80.0%. In contrast, His increased its value with respect to the control after fermentation in all the analyzed conditions, suggesting that the synthesis is independent of the moisture and temperature used in the soy matrix (Fig. 2 and Fig. S1). Moreover, Lys showed little difference after the fermentation of the soybean pastes with *B. longum* at 37 °C (Fig. 2), however in other conditions, it was dependent on the temperature (lower temperatures favored its decrease) (Fig. S1).

3.1.3. IF aglycones in soybean paste

In the previous study, *B. longum* CRL849 showed β-glucosidase activity at all tested conditions of moisture and temperature (Rodríguez de Olmos et al., 2015). In this current study, it was demonstrated that this strain was able to increase the content of the IF aglycones with respect to each unfermented control. Higher concentrations found in fermented soybean paste were: 32.6 ± 6.5; 26.2 ± 9.8 and 25.5 ± 7.2 mg IF aglycones/50 g of soybean paste at 65%-43 °C; 65%-37 °C and 50%-37 °C, respectively, without significant differences between them ($p \geq 0.05$). Otherwise, the minimum values observed (13.8 ± 6.5, 13.2 ± 3.5, 10.8 ± 5.6 mg IF aglycones/50 g of soybean paste) were related to the high moisture content of soybean paste at 80 and 75%. Besides, fermented soybean paste showed a higher increment of GEi than DAi in most of the evaluated conditions. For high moisture contents (80 and 75%) the percentage of increase for DAi was between 37 and 39% and those of GEi between 63 and 61%. For lower moisture content (65, 55 and 50%) the percentages varied between 15 and 39% for DAi and 85-61% for GEi, with GEi/DAi ratios that varied between 6 and 1.5. Temperature had a slight influence on the GEi/DAi ratio.

3.2. Characterization of optimized solid state fermentation using B. longum CRL 849

According to Rodríguez de Olmos et al. (2015) and results found in this study, 65%-37 °C was selected to keep on with the following studies.

In this section kinetics and the main technological parameters of fermentation were analyzed. Besides, optimized soybean paste fermented by *B. longum* CRL 849 was characterized by SEM and its tolerance to gastrointestinal conditions was also evaluated.

3.2.1. Growth pattern

Fig. 3A shows *B. longum* CRL 849 growth, pH values, consumption of carbohydrates, and organic acid production at several times of fermentation. This bacterium reached 9.2 log CFU/g and a pH value of 4.8 ± 0.2 after 24 h of fermentation. The specific growth rate was 0.55 h^{-1}, calculated in the exponential phase of growth (between 4 and 8 h). At this same time, *B. longum* CRL 849 consumed 22 µmol/g of sucrose and produced 154 µmol of acetic acid and 97 µmol of lactic acid per gram of soybean paste. The ratio (acetic/lactic) obtained was 1.59 close to theoretical data expected considering the fermentation pathway of *Bifidobacterium*, as was previously observed. *B. longum* CRL 849 consumed only 28.4% of sucrose, glucose almost fully and fructose from sucrose was not observed at the end of fermentation process. However, oligosaccharides (stachyose and raffinose) consumption was scarcely evidenced. Regarding organic acids, the production of lactic acid began after 8 h of fermentation with a concomitant greater decrease in pH of the matrix.

3.2.2. FAA profile and β-glucosidase activity from B. longum CRL 849

Soybean proteins from fermented soybean paste by *B. longum* CRL 849 showed a decrease during 24 h. The initial total protein value was 9.2 ± 0.8 mg/mL (control: uninoculated soybean paste), after 24 h fermentation, it decreased reaching values of 5.0 ± 0.1 mg/mL. Despite this reduction of protein content, *B. longum* CRL 849 was not able to hydrolyze the soybean protein that was evidenced by SDS-PAGE (Fig. 3D), confirming the results previously obtained. No fraction of soybean protein was degraded by the strain during the fermentation process. However, this decrease could be related to the acidification of the soybean matrix by the action of organic acids present. Furthermore, consumption of FAA was gradually observed during fermentation progress, with lower values after 24 h of fermentation (Fig. 3B). These results are in concordance with those previously found in the previous study.

The production of β-glucosidase enzyme was higher after 8 h of fermentation with a maximum value close to 30 U/mL at 24 h (Fig. 3C).

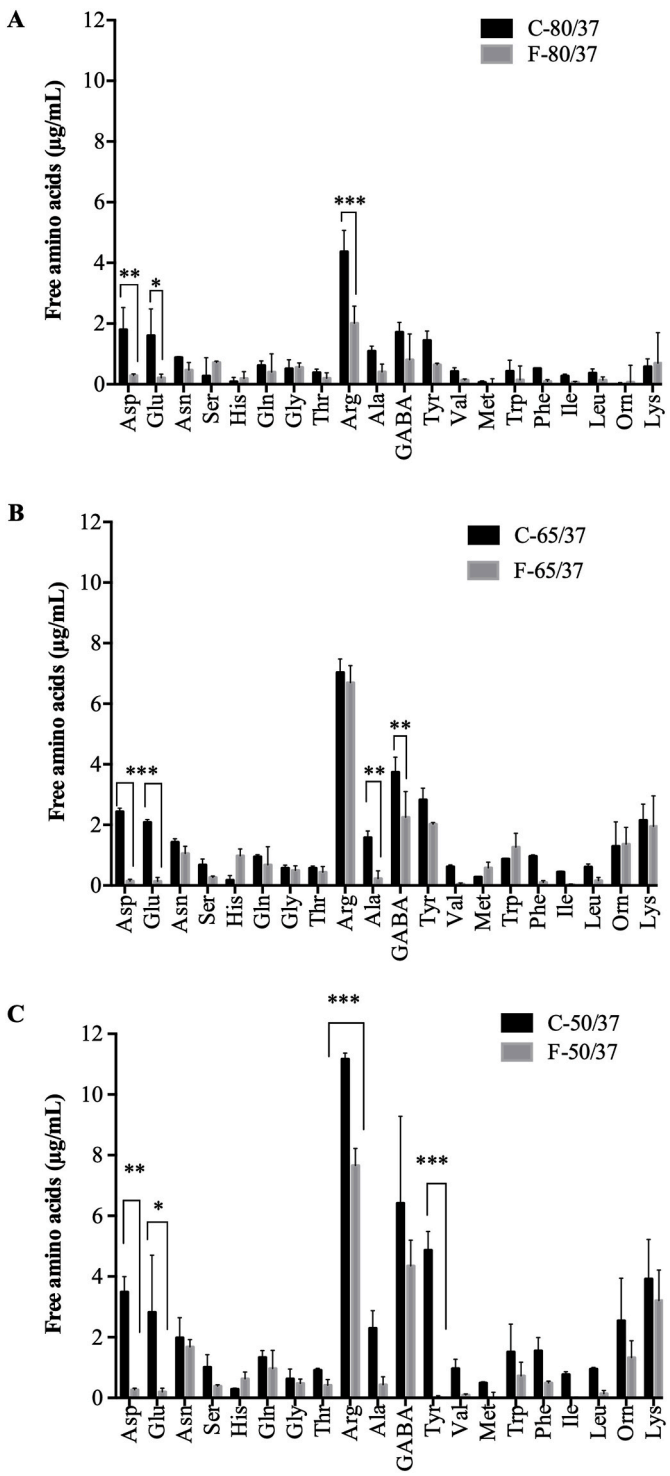

Fig. 2. Free amino acids profile in unfermented soybean paste (control: C black bars) and fermented soybean paste (F grey bars) by *Bifidobacterium longum* CRL 849 at 37 °C, for 24 h, in different moisture content (%). **A**: 80%; **B**: 65%; **C**: 50%. The amino acid content of fermented pastes that showed significant differences with respect to its unfermented control ($p < 0.05$) was marked with an asterisk (*).

At this time, the content of IF aglycones were also determined and their increase was at expense of decreasing their glycosylated precursors (Fig. S2). Control paste (unfermented) had a lower concentration of the IF aglycones: 5.6 ± 2.7 and 4.4 ± 0.7 mg/50 g of paste for DAi and GEi, respectively. In fermented soybean paste by *B. longum* CRL 849 at optimal conditions, the amounts of DAi and GEi were similar to those previously observed at 65% moisture and 37 °C.

3.2.3. Sugar addition and prolongation of fermentation process

Sugar addition (sucrose or glucose) or prolongation of fermentation time did not modify the parameters analyzed in this study (Fig. S3). In contradiction, the addition of 2% glucose showed a negative effect on most analyzed responses. In this condition bacterial growth was lower in contrast to the other two soybean pastes, showing 8.7 ± 0.2 log CFU/g. The pH value was 5.45 and, in correlation with these results, the acetic and lactic acids production was also decreased (data not shown).

FAA consumption trend was maintained under all conditions tested and at all fermentation times. Besides, an increase of β-glucosidase activity was observed with respect to the control for each soybean paste added with sucrose or glucose but not higher than basal soybean. This observed activity in each soybean paste was maintained over 72 h of fermentation (Fig. S3).

3.2.4. Tolerance to a simulated gastrointestinal digestion

Due to the characteristic GRAS (Generally Recognized as Safe) and potential probiotic effect of *B. longum* CRL 849, the tolerance to simulated gastrointestinal conditions was evaluated (Fig. 4).

The ability of the strain to resist these conditions was analyzed both in cultures from MRSs and from fermented soybean paste under previously optimized conditions. In addition, a refrigerated fermented soybean was included in the analysis.

B. longum CRL 849 showed tolerance to simulated gastrointestinal conditions, but sensitivity to conditions that simulate pancreatic juice was observed. A decrease of 3 units log CFU/g was observed in samples from soybean paste without storage after pancreatic juice, similar results were observed in samples from MRSs. *B. longum* CRL 849 showed a lower tolerance in soybean paste after 7 days of storage at 4 °C in contrast with freshly fermented soybean paste, showing a decrease of 2 units log CFU/g after submit the samples to gastric solution and about 4 units log CFU/g after pancreatic conditions.

3.2.5. Scanning electron microscopy of fermented soybean paste

The optimized SSF system (65%-37 °C) and the microorganism under study were characterized using the scanning electron microscopy methodology. SEM was used for samples of fermented soybean paste and control (without inoculum) and also for samples of *B. longum* grown in MRSs. As shown in Fig. 5 soybean matrix (control) presented a rigid structure characteristic of vegetal substrates, showing "tubular and cylindrical forms" inside (Fig. 5A). After 16 h of fermentation, more fragmented structures were detected. The fermented soybean paste had the smallest cracked structures, some coagulated particles, and large holes. *B. longum* CRL 849 was visualized in a liquid medium (Fig. 5B) and also in soybean paste as bacilli with central or subterminal protuberance (Fig. 5C–D), a characteristic form of some bifidobacteria. The length of the bacilli in the liquid medium was found between 1.1 and 2.9 µm. In soybean paste, bifidobacteria were more difficult to visualize because few bacteria were found in the external face of matrix and others were inside the matrix. It seems some microorganisms did not adhere to the matrix but detached themselves from the soybean paste and clusters of bacteria were observed in some areas of the soybean paste, this effect could be due to the sample preparation methodology for SEM (agglomeration of microorganisms in some places after successive steps of dehydration and centrifugation of the sample) (Fig. 5C–D).

4. Discussion

Solid state fermentation was revalorized in recent years for its advantages over submerged fermentation and inexpensive substrates can be used to obtain value-added products or differentiated foods. In the present study, several compounds of interest obtained by SSF using

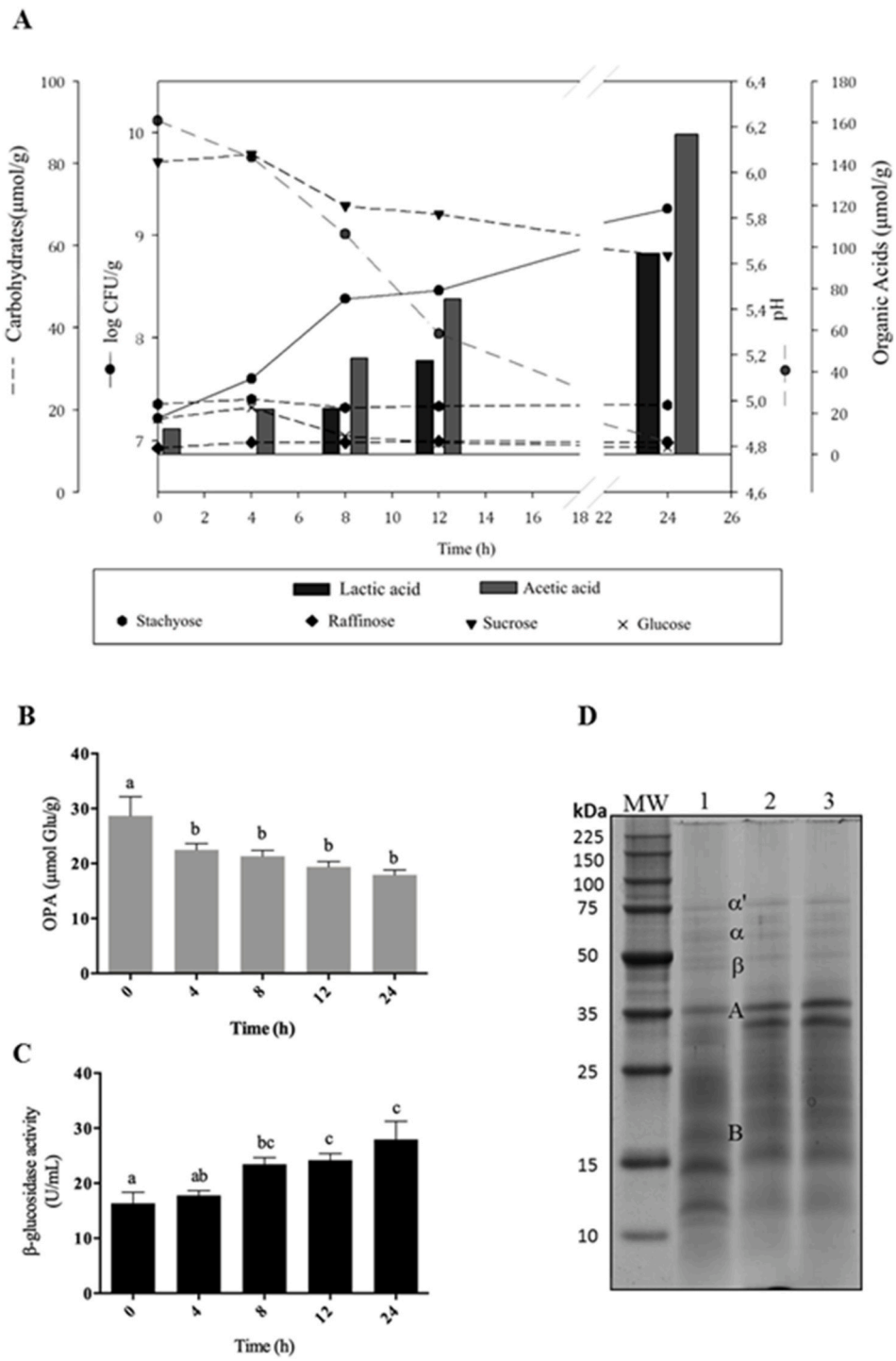

Fig. 3. Changes in cell viability of *Bifidobacterium longum* CRL 849, consumption of sugars, organic acids production and decrease in pH (**A**), determination of free amino acids (**B**), and β-glucosidase activity (**C**) at different times of fermentation in optimized conditions (65%-37 °C). SDS-PAGE of proteins extracted from control and fermented paste (**D**): (lane MW) Standard protein molecular weight marker; (lane 1) control without inoculation; (lane 2) fermented 4 h; (lane 3) fermented 24 h. Soybean protein subunits: β-conglycinin (α′,α, β), glycinin (A: acidic and B: basic). Bars with different letters are significantly different ($p < 0.05$).

soybean meal and a *Bifidobacterium* strain were analyzed. *B. longum* CRL 849 belongs to a group of bacteria that are characterized by their nutritionally demanding and sensitivity to oxygen (El Enshasy et al., 2016; Mozzetti et al., 2010), which often makes it difficult to use in SSF. In this study, the ability to grow well of *B. longum* CRL 849 in SSF using only carbon and nitrogen sources present in soybean meal was demonstrated. Pre-treatments of the substrate with enzymes or fermentation to enhance subsequent growth of bifidobacteria or in co-culture with another microorganism were not necessary as it has been done in other studies (Zhang et al., 2014; Zhang, Li, Zhang, Shi, & Wang, 2015). However, the temperature was the main factor with the most influence in the development of microorganisms and products formation.

B. longum CRL 849 has mainly used sucrose from the substrate, with acetic and lactic acid production. The molar ratio (acetic/lactic) was close to 1.5 in all conditions tested coinciding with the expected theoretical data considering the unique fermentation pathway of bifidobacteria via the fructose-6-phosphate route (Scardovi & Trovatelli, 1965), by which 3 mol of acetic and 2 mol of lactic acid are produced per 2 mol of glucose used. Contrarily, the acetic/lactic molar ratio was variable according to fermentation time when this strain was used in submerged fermentation using soymilk (Marazza, Nazareno, et al., 2013). The results obtained in this study are consistent with Hou, Yu, and Chou (2000) who observed similar values in soymilk fermented by *B. infantis* CCRC 14633 and *B. longum* B6.

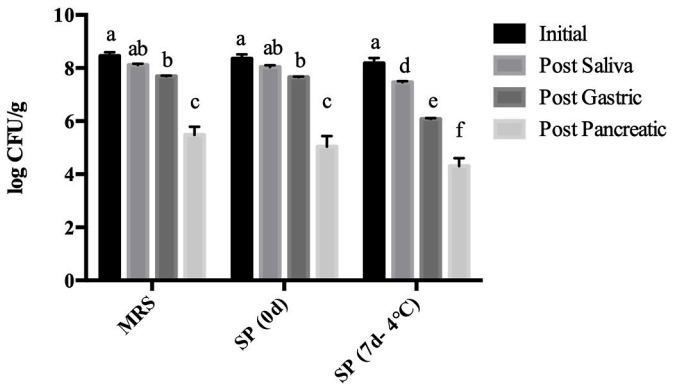

Fig. 4. Tolerance to simulated gastrointestinal conditions of *Bifidobacterium longum* CRL 849 grown in liquid medium, and fermented soybean paste without storage (0d) and stored 7 days at 4 °C (7d). Bars with different letters are significantly different ($p < 0.05$).

The microorganism showed a preference for sucrose instead of stachyose and raffinose present in soybean substrate. Similar results were found by Garro, de Valdez, and de Giori (2004) using the same strain in soymilk under different temperature conditions, despite having alpha-galactosidase activity. Also, Chen, Wei, & Chi. (2011) have demonstrated a higher intake of sucrose than oligosaccharides by *B. longum* strain BCRC 14661 using soybean meal.

Furthermore, *B. longum* CRL 849 did not show proteolytic activity, but amino acids intake was evidenced in all conditions of moisture and temperature tested. However, Lys amount remained unaltered after fermentation at 37 °C. This is an interesting result because the fermentation product could be used to supplement cereals where Lys is a limiting essential amino acid (EAA) (Yang et al., 2017). As an EAA, Lys cannot be synthesized by humans or farm animals and represents an indicator of other dietary EAAs (Cline, Tsai, Stelzleni, Dove, & Azain, 2016). For optimized soybean paste fermented by *B. longum* CRL 849, a net consumption of amino acids was also found at several times of fermentation. These results are in concordance with Zhang et al. (2014) who have studied the proteolytic activity of *Bifidobacterium (B.) animalis* 937 in combination with *Bacillus subtilis* natto in SSF using soybean as a matrix. However, *B. animalis* 937 used the peptides released by the partial hydrolysis of the soybean protein by *Bacillus subtilis* natto. In contrast, Zhang et al. (2015) observed that *B. animalis* subsp. *lactis* V9 used in SSF with 65% moisture was able to hydrolyze the protein of oats used as a substrate, releasing peptides. Regarding IF, β-glucosidase enzyme plays a key role in the hydrolysis of the β-glucoside bond in the glycosylated IF to release the IF aglycones that have been related to the biological properties (Hati, Vij, Singh, & Mandal, 2015; Marazza, Nazareno, et al., 2013; Marazza, LeBlanc, de Giori, & Garro, 2013). Several studies have demonstrated this property in lactic cultures using mainly submerged fermentation (Baú, Garcia, & Ida, 2015; Hati et al., 2015; Marazza et al., 2009, 2013a). In this study, β-glucosidase enzyme was detected in all conditions of moisture and temperature fitted by experimental design. Also, this enzyme was detected at several times of fermentation in optimized conditions with the highest values after 8 h of fermentation. Similar results were found by Marazza, Nazareno, et al. (2013) who used the same strain in soymilk; the total IF aglycones concentration was 129.3 mg/100 g of dried soymilk at 24 h after the freeze-drying process. In this study, the drying process was not necessary; 52.4 mg/100g was the amount of total IF aglycones obtained with wet soybean paste. Optimized soybean paste had 65% of moisture, so the total IF aglycones amount per 100 g of dried paste was higher than that obtained by soymilk fermentation (149.7 mg/100g). Taking into account that to ensure beneficial effects of IF a daily intake of 50 mg is necessary (Cassidy, Binham, & Setchell, 1994), the consumer should thus incorporate at least 1 L of fermented milk per day. in their diets. However, solid products ensure the same daily amount of bioactive IF with a lower product intake which clearly shows the advantage of SSF. Other authors also found an improvement of soybean IF aglycones in SSF by lactic cultures (Chen, Wei, & Chi, 2011; Correa Deza, Rodríguez de Olmos, & Garro, 2019).

On the other hand, Marazza, Nazareno, et al. (2013) have found that IF aglycones have a higher antioxidant activity than IF glucosides obtained by fermentation of soymilk using *B. longum* CRL 849. Therefore,

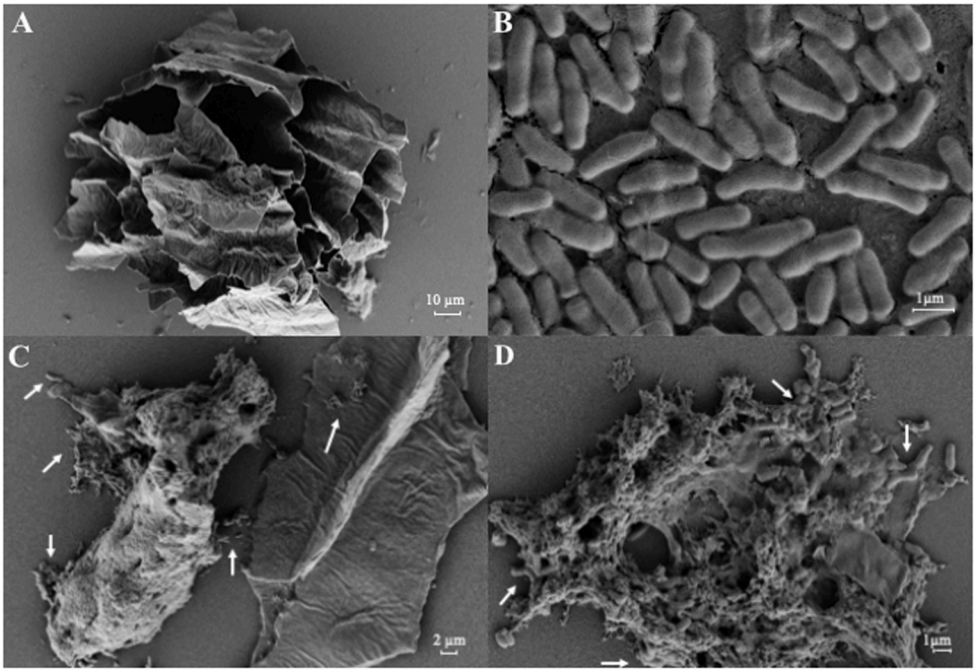

Fig. 5. Characterization of optimized solid state fermentation by scanning electron microscopy: structure of the soybean matrix (control without inoculum) (**A**); *Bifidobacterium longum* CRL 849 in liquid medium (MRSs, 8 h at 37 °C) (**B**), and in soybean paste in optimized conditions (65%-37 °C, 16 h) (**C-D**). Representative photographs were obtained at different magnifications.

the high IF aglycone levels found in the soybean paste fermented by the same strain could have the same activity and may be beneficial to design new solid functional foods. More studies are needed to confirm the functional activity of this fermented soybean paste.

In order to increase β-glucosidase and evaluate if sugar addition or prolongation time were able to stimulate proteolytic activity, sucrose and glucose were added to soybean paste and time prolongation was also assayed. However, *B. longum* CRL 849 did not exhibit proteolytic activity, the β-glucosidase activity was maintained in all tested conditions of sugar addition and these results were maintained up to 72 h. Thus, it would not make sense to add external sugar or extend the fermentation time; rather 16 h could be an adequate time to obtain the desired results with this bacterium.

There are numerous studies in which the tolerance to in vitro gastrointestinal tract of LAB and bifidobacteria was evaluated due to its potential as a probiotic, mainly in milk-derived foods. Dairy products, mainly yoghurts, are ideal as a matrix for the use of probiotics, due to their high acceptance by consumers and the excellent viability of most of these microorganisms. However, certain compounds such as cholesterol content, milk protein allergy, and lactose intolerance have led to the exploration of other types of non-dairy matrices such as cereals, oilseeds, fruits, and vegetables (Bansal, Mangal, Sharma, Yadav, & Gupta, 2016; Chen, Lu, Yu, Chen, & Tian, 2019; Vera-Pingitore et al., 2016). In the present study, it was demonstrated that soybean paste can be used as a carrier substrate for potentially probiotic dairy cultures as *B. longum* CRL 849. This strain showed good tolerance to some of simulated gastrointestinal solutions with a negative effect on its viability after challenge with pancreatic juice. This tolerance could be increased using additional protective agents. Also, optimized SSF was characterized by SEM showing the structure of soybean paste and characteristic forms of this bacterium. Other authors have also used SEM to characterize the system using soybean and bifidobacteria strains (Mao, Pan, Hou, Yuan, & Gao, 2018; Matias, Padilha, Bedani, & Saad, 2016).

5. Conclusions

Several systems of SSF using *B. longum* CRL 849 were characterized and optimal fermentation conditions were: 65% of moisture, 37 °C, without addition of external sugar and 16 h of fermentation to obtain the desired soybean product. In these conditions, *B. longum* CRL 849 grew well, consumed sucrose, and produced organic acids. Lys amounts were maintained and bioactive isoflavones were increased after fermentation. The nutritional value of the obtained soybean paste, the high IF aglycone levels found in fermented soybean paste could have the same antioxidant activity previously observed in soymilk.

Sustainable technologies such as SSF could be used to design new solid functional soybean-based foods with attractive nutritional and functional properties. Also, soybean paste represents an excellent vegetable candidate to be used as potential probiotic carrier food, offering new products to the vegan market which is constantly growing.

CRediT authorship contribution statement

Antonieta Rodríguez de Olmos: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization, Writing – review & editing. **Oscar A. Garro:** Conceptualization, Visualization, Writing – review & editing. **Marisa S. Garro:** Conceptualization, Methodology, Software, Data curation, Writing – original draft, Visualization, Investigation, Writing – review & editing, Resources, Supervision, Project administration, Funding acquisition, All authors have read the manuscript and approved it.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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