Impact of inulin and okara on *Lactobacillus acidophilus* La-5 and *Bifidobacterium animalis* Bb-12 viability in a fermented soy product and probiotic survival under *in vitro* simulated gastrointestinal conditions

Raquel Bedani<sup>a</sup>, Elizeu Antonio Rossi<sup>b</sup>, Susana Marta Isay Saad<sup>a,∗</sup>

<sup>a</sup> Departamento de Tecnologia Bioquímico-Farmacêutica, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, Av. Prof. Lineu Prestes, 580, 05508-000 São Paulo, SP, Brazil

<sup>b</sup> Departamento de Alimentos e Nutrição, Faculdade de Ciências Farmacêuticas, Universidade Estadual Paulista, Rod. Araraquara-Jaú, km 1, 14801-902 Araraquara, SP, Brazil

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A B S T R A C T

The effect of inulin and/or okara flour on *Lactobacillus acidophilus* La-5 and *Bifidobacterium animalis* Bb-12 viability in a fermented soy product (*FSP*) and on probiotic survival under *in vitro* simulated gastrointestinal conditions were investigated throughout 28 days of storage at 4 °C. Employing a 2<sup>4</sup> design, four *FSP* trials were produced from soymilk fermented with ABT-4 culture (La-5, Bb-12, and *Streptococcus thermophilus*); *FSP* (control); *FSP*-I (with inulin, 3 g/100 mL of soymilk); *FSP*-O (with okara, 5 g/100 mL); *FSP*-IO (with inulin + okara, ratio 3:5 g/100 mL). Probiotic viabilities ranged from 8 to 9 log cfu/g during the 28 days of storage, and inulin and/or okara flour did not affect the viability of La-5 and Bb-12. Bb-12 resistance to the artificial gastrointestinal juices was higher than for La-5, since the Bb-12 and La-5 populations decreased approximately 0.6 log cfu/g and 3.8 log cfu/g, respectively, throughout storage period. Even though the protective effect of inulin and/or okara flour on probiotic microorganisms was not significant, when compared to a fresh culture, the *FSP* matrix improved Bb-12 survival on day 1 of storage and may be considered a good vehicle for Bb-12 and could play an important role in probiotic protection against gastrointestinal juices.

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1. Introduction

The production of soy products has been emerging as an interesting alternative to dairy products and their incorporation into human diets is increasing due to their nutritional and functional properties (Xiao, 2008; Rivera-Espinoza and Gallardo-Navarro, 2010). Soy products, besides being good sources of protein, dietary fibre, oligosaccharides, trace mineral, and vitamins, may help to lower the incidence of cardiovascular diseases, type 2 diabetes, the risk of carcinogenesis, and lead to improved bone health and relief of menopausal symptoms (Bedani et al., 2006; Donkor et al., 2007a, b; Xiao, 2008; Bedani et al., 2010; Chen et al., 2010). Additionally, soy products contain isoflavones, phytoestrogens associated with various potential health benefits, such as anticarcinogenic activity and bone-sparing effects (Donkor et al., 2005).

The fermentation of soymilk for the production of a fermented soy product (*FSP*) may improve its flavour and texture, as well as enhance its beneficial health properties (Donkor et al., 2005). Several studies have shown that soy products, particularly soy yogurt, may be good vehicles for probiotic microorganisms (Donkor et al., 2007a, b; Farnworth et al., 2007; Champagne et al., 2009; Wang et al., 2009). Soymilk might be a good medium for *Bifidobacterium* spp. growth, due to the presence of raffinose and stachyose, which are fermented by most of the strains belonging to this genus. Strains of *Lactobacillus acidophilus* also were reported as presenting the ability to metabolize oligosaccharides during the fermentation of soymilk (Donkor et al., 2007a). Many probiotic strains possess α-galactosidase activity, which allows their growth in soymilk (Fung and Lione, 2010).

Among the most well-known probiotic microorganisms are strains belonging to the *Lactobacillus* and *Bifidobacterium* genera (Saxelin et al., 2005). *Bifidobacterium animalis* Bb-12 has been used in clinical trials either alone or together with other bacteria such as *L. acidophilus* La-5 or *Streptococcus thermophilus* (Larsen et al., 2006). Several beneficial effects have been attributed to *L. acidophilus* La-5 and *B. animalis* Bb-12, among them: prophylactic activity against infectious rotavirus diarrhoea in children (Saavedra et al., 1994; Weizman et al., 2005; Weichert et al., 2012); relief of clinical symptoms of atopic dermatitis in children (Isolauri et al.,...
intestinal microbiota modulation (Savard et al., 2011), and hipcholesterolemic effect (Abd El-Gawad et al., 2005). These strains are commercially available potential probiotic strains. In the development of probiotic foods, selecting suitable vehicles for the delivery of probiotics to ensure that they overcome the physical and chemical barriers found in the gastrointestinal tract (GIT) is extremely important (Wang et al., 2009; Martínez et al., 2011). Different strategies have been employed to obtain higher probiotic survival rates in the GIT, such as the co-administration with prebiotics, other probiotics and/or the use of a food matrix (Su et al., 2007; Martínez et al., 2011). Probiotic strains ought to endure consumption and transit through the GIT during the entire food storage period (Forssten et al., 2011). The resistance to simulated gastric and enteric juices is among the in vitro assays that are frequently suggested for the evaluation of the strain probiotic potential (Buriti et al., 2010; Ghassi et al., 2011). Checking the tolerance of probiotic microorganisms in the final product towards gastrointestinal conditions may help to select a suitable food matrix and contribute to probiotic survival and efficacy in the GIT (Schillinger et al., 2005; Buriti et al., 2010).

Prebiotic ingredients like inulin may exert a protective effect, improving the survival and activity of probiotic bacteria during storage of probiotic food products, as well as during the passage through the GIT (Donkor et al., 2007c; Buriti et al., 2010; Hernández-Hernández et al., 2012). Other ingredients of the food matrix, such as whey proteins, resistant starch, β-glucan, stachyose, raffinose, and fructooligosaccharides may increase the resistance towards simulated gastrointestinal conditions (Perrin et al., 2000; Charalamopoulos et al., 2003; Martínez et al., 2011).

Okara is a byproduct of the soymilk industry. Raw okara is a white yellowish material consisting of the insoluble parts of soybean seeds, which remain in the filter sack when pure soymilk are filtered for the production of soymilk (Jiménez-Escrig et al., 2008). Although okara has a low market value, it contains protein, lipids, dietary fibre, and minerals, along with unspecified monosaccharides, and oligosaccharides (Jiménez-Escrig et al., 2008; Mateos-Aparicio et al., 2010). It also contains isoflavones and approximately 22% of these compounds are found in raw okara (Rossi et al., 2004; Jiménez-Escrig et al., 2008). Even though okara is frequently treated as an industrial waste, it might be a good source of nutrients for human consumption. Studies revealed that okara is a potential source of antioxidant components (Amin and Mukhrizah, 2006; Mateos-Aparicio et al., 2010). It might be useful as a weight-loss dietary supplement (Préstamo et al., 2007) and also protect the gut environment because of its antioxidant status and prebiotic effects (Jiménez-Escrig et al., 2008; Mateos-Aparicio et al., 2010). Studies have also suggested that okara consumption could lead to a beneficial effect on plasma lipid levels (Villanueva et al., 2011).

Therefore, the preparation of a FSP with probiotic cultures, inulin, and okara flour appears to be an interesting possibility for the development of a new probiotic product. Furthermore, no information is available on the effect of the supplementation of okara flour on the viability and resistance to simulated gastrointestinal conditions of L. acidophilus La-5 and B. animalis Bb-12 in FSP. The present study thus aimed to investigate the effect of inulin and/or okara flour on the viability and resistance to in vitro simulated gastrointestinal conditions of L. acidophilus La-5 and B. animalis Bb-12 incorporated into a FSP.

2. Materials and methods

2.1. Production of okara flour

Okara was supplied by the Development and Production Unit for Soybean Derivatives located at the Faculty of Pharmaceutical Sciences of the São Paulo State University. To obtain the flour, fresh okara was dried at 60 °C for 8 h in a forced air circulation oven (Quinis, Diadema, Brazil). Next, dried okara was minced in a ball mill for 12 h (Tecnal, Piracicaba, Brazil) and sieved through an industrial sieve (Produtest, Tecnal) for 10 min. A 10-mesh okara flour was obtained and employed in the production of the FSP.

2.2. Experimental design and fermented soy product manufacture

Employing a randomized 2-factorial design, four pilot-scale-making trials of FSP were produced, in triplicates (three different batches of the same trial, using new soymilk batch and new inoculum), from soymilk enriched with calcium (120 mg/100 mL of soymilk), according to Table 1. Soymilk enriched with calcium was used since soymilk may have low levels of this mineral compared to milk (Pathomrungsijoungul et al., 2010). The four types of products were fermented with an ABT-4 culture (Christian Hansen, Hørsholm, Denmark), containing the probiotic strains L. acidophilus La-5 and B. animalis subsp. lactis Bb-12, and the starter S. thermophilus: FSP (control), FSP-I (with inulin), FSP-O (with okara flour), and FSP-IO (with inulin and okara flour). The soy products were produced according to the procedure described by Rossi et al. (1999).

Each formulation of FSP was manufactured in batches of 4 L. Lactose (1.0 g/100 mL of soymilk) (Alibra®, Alibra Ingredientes, Campinas, Brazil) and soybean oil (0.8 g/100 mL of soymilk) (Liza, Cargill Agrícola, Uberlândia, Brazil) were added to calcium-enriched soymilk (Mais Vita, Yoki Alimentos, São Bernardo do Campo, Brazil) and mixed for 5 min using a blender (Magiclean, Arno, São Paulo, Brazil). The mixture was then heated to 50 °C for the addition of sugar (8.0 g/100 mL of soymilk) (da Barra®), Cosan, Barra Bonita, Brazil) and up to 80 °C for the incorporation of skimmed milk powder (2.5 g/100 mL of soymilk) (Molico, Nestlé, Barra Bonita, Brazil). For the preparation of the product, FSP-O and FSP-IO formulations. The mixture was again put into a blender for 5 min and heated to 95 °C for the addition of okara flour (5.0 g/100 mL of soymilk) in the case of FSP-O and FSP-IO formulations. Afterwards, the mixture was cooled to 37 °C for the addition of the ABT culture (Christian Hansen) at 0.5 g/100 mL of soymilk in order to achieve an inoculation level of 8 log cfu/g of each probiotic strain used (L. acidophilus La-5 and B. animalis Bb-12). Fermentation took place at 37 °C until reaching pH 5.0. Next, the product was cooled in an ice-bath and refrigerated (4 ± 1 °C) for 24 h. Portions of 50 g of FSP were packaged in appropriate polypropylene plastic pots for food products (68 mm diameter, 32 mm high, 55 mL total

<table>
<thead>
<tr>
<th>Ingredients (g/100 mL of soymilk)</th>
<th>FSP</th>
<th>FSP-I</th>
<th>FSP-O</th>
<th>FSP-IO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inulin (I)</td>
<td>0.00</td>
<td>3.00</td>
<td>3.00</td>
<td>3.00</td>
</tr>
<tr>
<td>Okara flour (O)</td>
<td>0.00</td>
<td>0.00</td>
<td>5.00</td>
<td>5.00</td>
</tr>
<tr>
<td>Lactose</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Skimmed milk powder</td>
<td>2.50</td>
<td>2.50</td>
<td>2.50</td>
<td>2.50</td>
</tr>
<tr>
<td>Sugar</td>
<td>8.00</td>
<td>8.00</td>
<td>8.00</td>
<td>8.00</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>0.80</td>
<td>0.80</td>
<td>0.80</td>
<td>0.80</td>
</tr>
<tr>
<td>Gelatin</td>
<td>0.30</td>
<td>0.30</td>
<td>0.30</td>
<td>0.30</td>
</tr>
<tr>
<td>Probiotic culture (×10^7 cfu/g)</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
</tr>
</tbody>
</table>

4 ABT-4 culture (containing the probiotic strains Lactobacillus acidophilus La-5 and Bifidobacterium animalis subsp. lactis Bb-12, and the starter Streptococcus thermophilus Christian Hansen, Hørsholm, Denmark) with around 10^7 cfu/g of L. acidophilus La-5 and 10^11 cfu/g of B. animalis Bb-12.
volume, Três Aditivos Plásticos, São Paulo, Brazil) and sealed with metallic covers with varnish in a sealer (Delgo Nr. 1968, Delgo Metalúrgica, Cotia, Brazil). The batches were stored under refrigeration (4 ± 1 °C) for 28 days, according to the shelf-life already reported by other authors for soy-based products (Donkor et al., 2005, 2007b). Products from each trial were used to determine the viability of the probiotic and starter cultures, pH, and the probiotic resistance to simulated gastrointestinal conditions after 1, 7, 14, 21, and 28 days of refrigerated storage.

2.3. Determination of pH and probiotic viability during storage

The pH values of the FSP were determined for quintuplicate samples (five different pots of the same batch, totalling 15 pots for each trial) with a pH meter Orion, Three Stars model (Thermofisher Scientific, Waltham, MA, USA), equipped with a penetration electrode model 2A04 (Analyser, São Paulo, Brazil).

In order to determine the probiotic viability in the products during storage, 25 g portions of FSP duplicate samples (two different pots of the same batch, totalling 6 pots for each trial at each storage point) were collected aseptically, blended with 225 mL of 0.1% peptone water using a Bag Mixer 400 (Interscience, St. Nom, France) and submitted to serial dilutions. Lactic acid bacteria (LAB) populations were determined by pour-plating 1 mL of each dilution in formulated DeMan-Rogosa-Sharp (MRS) agar, modified through the substitution of glucose for maltose (Difco, Le Pont de Claux, France), according to the International Dairy Federation (IDF, 1995), after 48 h of aerobic incubation at 37 °C.

Bifidobacterium animalis Bb-12 populations were counted by pour-plating 1 mL of each dilution in MRS agar (Oxoid, Basingstoke, UK) containing sodium propionate (3 g/L) (Sigma–Aldrich, St. Louis, MO, USA) and lithium chloride (2 g/L) (Merck, Darmstadt, Germany) (LP-MRS agar), after 72 h of anaerobic incubation (Anaerobic System Anaerogen, Oxoid) at 37 °C (Lapiere et al., 1992; Vinderola and Reinheimer, 1999).

S. thermophilus viability was monitored by pour-plating 1 mL of each dilution in M17 agar with sterile lactose solution lactose (10% w/v) (Oxoid), after 48 h of incubation at 37 °C (Richter and Vedamuthu, 2001).

2.4. Survival of L. acidophilus and B. animalis under simulated gastrointestinal conditions

2.4.1. Preparation of fresh probiotic culture to be used as a control

An ABT-4 freeze-dried culture (Christian Hansen) was grown in MRS broth (Oxoid) at 37 °C for 18 h. Cells were harvested by centrifugation (Sorvall Instruments model RC 5C, Wilmington, USA) at 10,000 rpm, at 4 °C, for 10 min and resuspended in sterile 0.5% (w/v) NaCl solution. This suspension was employed as a control, not incorporated into the FSP, to determine L. acidophilus La-5 and B. animalis Bb-12 survival as a fresh culture towards simulated gastrointestinal conditions as described in item 2.4.2. The preparation of fresh culture was performed in triplicates.

2.4.2. Determination of simulated transit tolerance

The evaluation of probiotic survival in fresh culture and in refrigerated FSP submitted to simulated gastric and enteric conditions was carried out according to Liserre et al. (2007), with the modifications suggested by Buriti et al. (2010). Other adjustments were necessary and proceeded as follows.

Ten millilitres from each triplicate dilution of FSP in 0.5% (w/v) NaCl solution was transferred to 3 sterile flasks, with a total of 9 flasks containing the samples (3 dilutions carried out with 3 different samples of the same batch in the same period of storage), and pH was adjusted to 2.3–2.6 with 1N HCl (Merck). The same procedure was followed for the fresh culture suspension. Pepsin (from porcine stomach mucosa, Sigma–Aldrich) and lipase (Amano lipase C, from Penicillium camemberti, Sigma–Aldrich) solutions were added to samples to reach a concentration of 3 g/L and 0.9 mg/L, respectively. Flasks were incubated at 37 °C, with agitation of 150 rpm (Metabolic Water Bath Dubnoff MA-095, Marconi, Piracicaba, Brazil) during 2 h, leading to the simulated gastric phase. In the next step, the pH of samples was increased to 5.4–5.7 using an alkaline solution [150 mL of 1 N NaOH (Synth, Diadema, Brazil) and 14 g of PO4H2Na2H2O (Synth)] and distilled water up to 1 L. Bile (bovine bile, Sigma–Aldrich) and pancreatin (pancreatin from porcine pancreas, Sigma–Aldrich) were added to reach a concentration of 10 g/L and of 1 g/L, respectively. Samples were incubated again at 37 °C for 2 h under agitation, leading to simulated enteric phase 1. In the last step, the pH was increased to 6.8–7.2 using the same alkaline solution, bile and pancreatin were adjusted to maintain the concentration of 10 g/L and 1 g/L, respectively, and the samples were incubated again at 37 °C for 2 h under agitation, leading to simulated enteric phase 2 and reaching 6 h of assay. Enumeration of L. acidophilus and B. animalis was carried out in aliquots collected from triplicate samples after 2 h, 4 h, and 6 h (three different flasks of the same trial for each time). Aliquots of 1 mL were pour-plated in MRS agar modified through substitution of glucose for maltose (IDF, 1995) and LP-MRS agar (Lapiere et al., 1992; Vinderola and Reinheimer, 1999) for enumeration of L. acidophilus and B. animalis, respectively, as described in Section 2.3. The results were presented as log cfu/g of fresh probiotic culture or FSP.

2.5. Statistical analysis

The results were expressed as mean ± SD. Initially, data were checked regarding homogeneity of variances using the Levene test. Differences between trials during the experimental period were statistically analysed using repeated measures ANOVA, followed by Tukey test (P < 0.05). The nonparametric Kruskall–Wallis test, followed by Dunn’s multiple comparison procedure (P < 0.05), were employed to compare the trials and Friedman test with Bonferroni correction for comparison between days, for data showing non-homogenous variance. Statistical analysis was performed using XLSTAT 2011 (Addinsoft, USA) and MINITAB 14 (MINITAB Inc., USA).

3. Results and discussion

3.1. pH values and probiotic viability

The pH values of FSP during refrigerated storage are shown in Table 2. Throughout the whole period studied, all products presented a small but significant pH reduction (P < 0.05). This decrease

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Products</th>
<th>FSP</th>
<th>FSP-I</th>
<th>FSP-O</th>
<th>FSP-IO</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.56 ± 0.05a&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>4.70 ± 0.06&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>4.61 ± 0.03&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>4.73 ± 0.04&lt;sup&gt;AB&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>4.50 ± 0.03b&lt;sup&gt;A&lt;/sup&gt;</td>
<td>4.55 ± 0.08b&lt;sup&gt;A&lt;/sup&gt;</td>
<td>4.43 ± 0.12b&lt;sup&gt;A&lt;/sup&gt;</td>
<td>4.59 ± 0.04b&lt;sup&gt;A&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>4.33 ± 0.12b&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>4.57 ± 0.01b&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>4.38 ± 0.12b&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>4.55 ± 0.04b&lt;sup&gt;AB&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>4.28 ± 0.08b&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>4.43 ± 0.06b&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>4.37 ± 0.02b&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>4.52 ± 0.06b&lt;sup&gt;AB&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>4.31 ± 0.06b&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>4.40 ± 0.05b&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>4.32 ± 0.03b&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>4.46 ± 0.06b&lt;sup&gt;AB&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD. A,B Different superscript capital letters in a row denote significant differences between trials (P < 0.05); a,b,c Different lowercase superscript letters in a column denote significant differences during storage period (P < 0.05). FSP: fermented soy product (control); FSP-I: FSP with inulin; FSP-O: FSP with okara flour; FSP-IO: FSP with inulin and okara flour.
is probably related to the product post-acidification, due to the continuous production of organic acids resulting from fermentation of carbohydrates by probiotic and starter cultures (Farnworth et al., 2007; Wang et al., 2009). According to Wang et al. (2009), soymilk has a low buffering ability, due to composition and physicochemical properties of soy proteins. This fact probably contributed for the pH reduction in the present study.

Currently, there is a tendency to use starter cultures without Lactobacillus delbrueckii subsp. bulgaricus associated with probiotic cultures, for example ABT (L. acidophilus, Bifidobacterium, and S. thermophilus), in order to inhibit the post-acidification of products. In the ABT cultures, S. thermophilus is the main microorganism responsible for fermentation, and the three bacteria present show a lower proteolytic activity than L. delbrueckii subsp. bulgaricus (Shihata and Shah, 2000).

The viability of L. acidophilus, B. animalis, and S. thermophilus obtained from all trials of FSP is shown in Table 3. Populations of L. acidophilus and B. animalis remained above 8 log cfu/g between the first and 28th day of storage in different soy products. In general, the addition of inulin and okara flour in FSP did not influence the probiotic viability during the storage period. Even though certain variations in the La-5 populations were observed, these changes were of little microbiological significance, since they were always below 0.5 log cfu/g.

Many factors may affect the Lactobacillus spp. and Bifidobacterium spp. viability in food, including the probiotic strains used, the pH, the presence of hydrogen peroxide and dissolved oxygen, the concentration of metabolites such as lactic and acetic acids, the medium buffering capacity, as well as the storage temperature (Donkor et al., 2006). However, the main factors for the loss of probiotic viability are the decrease in the medium pH and the accumulation of organic acids resulting from growth and fermentation (Shah, 2000). Apparently, the pH obtained in the present study did not affect the viability of L. acidophilus La-5 and B. animalis Bb-12, since the pH reduction was similar in all trials.

A number of studies have shown that the use of probiotics in the production of soy-based products is very promising in terms of the maintenance of suitable probiotic viability. Similarly to what was observed for the FSP here described, Chang et al. (2010) reported that the viabilities of Bifidobacterium breve K-110, S. thermophilus 3781, and L. acidophilus Q509011 in a soy yogurt on day 0 were, respectively, 8.75, 8.43, and 8.96 log cfu/g, and these populations did not change during 15 days of storage at 4 °C. Additionally, Donkor et al. (2007b) verified that the viability of L. acidophilus L10, B. animalis subsp. lactis B94, and Lactobacillus casei L26 was maintained above 8 log cfu/g of soymilk. Moreover, supplementation with 2% inulin and 1% raffinose + 1% glucose led to an improved viability during fermentation and storage, possibly due to α-galactosidase activity in the presence of oligosaccharides (Donkor et al., 2007a). On the other hand, in a study with refrigerated or frozen milk-based guava mousse with La-5, the addition of inulin did not improve viability of this microorganism during shelf life (Buriti et al., 2010). The differences found in those studies and in the present work might be attributed to probiotic strain-specific response (Paseephol and Sherkat, 2009).

Thus, the probiotic populations here recorded are in agreement with the Brazilian legislation, which recommends a minimum population ranging from 10^9 up to 10^10 cfu per daily serving portion of product for beneficial health effects in the gut (ANVISA, 2008). This would mean 10^9 up to 10^10 cfu/g for a daily serving portion of 100 g of FSP.

S. thermophilus population remained stable (approximately 9 log cfu/g) during refrigerated storage in the different trials of FSP. Farnworth et al. (2007) verified that S. thermophilus growth during a 6 h incubation period was higher in a soy yogurt formulation than in milk. S. thermophilus can grow in soy-based products due to its ability to metabolize sucrose (Chumchuere and Robinson, 1999) and soy oligosaccharides (Donkor et al., 2007a).

### 3.2. Survival of L. acidophilus and B. animalis under simulated gastrointestinal conditions

The L. acidophilus and B. animalis survival in the ABT-4 fresh culture exposed to in vitro simulated gastrointestinal conditions is shown in Table 4. Comparing the survival of L. acidophilus La-5 and B. animalis Bb-12 in freshly prepared probiotic culture and in soy-based products on day 1 (Figs. 1 and 2), the presence of FSP matrix improved the survival for both strains, particularly for B. animalis Bb-12. This is in line with observations made by Wang et al. (2009), who reported that the survival rate of L. casei Zhang for gastric juices with pH 2 and pH 2.5 was higher when this microorganism was present in fermented soymilk than as a pure culture.

Madureira et al. (2011) tested three probiotic strains (L. casei LAFTI L26, L. acidophilus LAFTI L10, and B. animalis B80) against

### Table 3

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Time (days)</th>
<th>Products</th>
<th>FSP</th>
<th>FSP-I</th>
<th>FSP-O</th>
<th>FSP-IO</th>
</tr>
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<tbody>
<tr>
<td>L. acidophilus</td>
<td>1</td>
<td></td>
<td>8.47 ± 0.12&lt;sup&gt;A&lt;/sup&gt;</td>
<td>8.32 ± 0.26&lt;sup&gt;B&lt;/sup&gt;</td>
<td>8.56 ± 0.19&lt;sup&gt;Ab&lt;/sup&gt;</td>
<td>8.49 ± 0.14&lt;sup&gt;ab&lt;/sup&gt;</td>
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<td>8.56 ± 0.20&lt;sup&gt;A&lt;/sup&gt;</td>
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<td>8.43 ± 0.07&lt;sup&gt;B&lt;/sup&gt;</td>
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<td>21</td>
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<td>8.20 ± 0.09&lt;sup&gt;B&lt;/sup&gt;</td>
<td>8.27 ± 0.13&lt;sup&gt;Ab&lt;/sup&gt;</td>
<td>8.48 ± 0.11&lt;sup&gt;Ab&lt;/sup&gt;</td>
<td>8.47 ± 0.14&lt;sup&gt;ab&lt;/sup&gt;</td>
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<td>8.00 ± 0.15&lt;sup&gt;B&lt;/sup&gt;</td>
<td>8.15 ± 0.07&lt;sup&gt;Ab&lt;/sup&gt;</td>
<td>8.45 ± 0.08&lt;sup&gt;Ab&lt;/sup&gt;</td>
<td>8.37 ± 0.14&lt;sup&gt;ab&lt;/sup&gt;</td>
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<td>8.86 ± 0.11&lt;sup&gt;Ab&lt;/sup&gt;</td>
<td>8.97 ± 0.10&lt;sup&gt;Ab&lt;/sup&gt;</td>
<td>8.95 ± 0.23&lt;sup&gt;ab&lt;/sup&gt;</td>
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<td>8.89 ± 0.11&lt;sup&gt;Ab&lt;/sup&gt;</td>
<td>8.93 ± 0.16&lt;sup&gt;Ab&lt;/sup&gt;</td>
<td>8.94 ± 0.07&lt;sup&gt;ab&lt;/sup&gt;</td>
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<td>8.90 ± 0.12&lt;sup&gt;ab&lt;/sup&gt;</td>
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<td>8.86 ± 0.15&lt;sup&gt;Ab&lt;/sup&gt;</td>
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<td>8.84 ± 0.10&lt;sup&gt;Ab&lt;/sup&gt;</td>
<td>8.81 ± 0.19&lt;sup&gt;Ab&lt;/sup&gt;</td>
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<td>9.31 ± 0.08&lt;sup&gt;Ab&lt;/sup&gt;</td>
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<td>9.31 ± 0.04&lt;sup&gt;ab&lt;/sup&gt;</td>
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<td>9.25 ± 0.08&lt;sup&gt;Ab&lt;/sup&gt;</td>
<td>9.26 ± 0.04&lt;sup&gt;ab&lt;/sup&gt;</td>
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</table>

Values are expressed as mean ± SD. <sup>A,B,C</sup> Different superscript capital letters in a row denote significant differences between trials (P < 0.05); <sup>a,b</sup> Different lowercase superscript letters in a column for each microorganism denote significant differences during storage period (P < 0.05). FSP: fermented soy product (control); FSP-I: FSP with inulin; FSP-O: FSP with okara flour; FSP-IO: FSP with inulin and okara flour.
simulated gastrointestinal conditions in MRS medium and when incorporated into cheese. The authors concluded that *B. animalis* Bo was more resistant to the gastrointestinal conditions assayed than the other strains studied. Furthermore, a protective effect conferred by the cheese as a food matrix was verified, which was more evident for the lactobacilli strains, contrary to that observed in the present study. In a previous study, Madureira et al. (2005) had already reported that *B. animalis* Bb-12 and Bo and *Lactobacillus brevis* LMG 6906 presented greater survival in simulated gastric and enteric conditions when incorporated into cheese than *L. acidophilus* LAC-1 and Ki, *Lactobacillus paracasei* LCS-1 and *B. animalis* BLC-1, suggesting that this characteristic is strain-dependent.

The survival of *L. acidophilus* and *B. animalis* in the different trials of FSP submitted to *in vitro* simulated gastrointestinal conditions is presented in Figs. 1 and 2, respectively. In general, *L. acidophilus* La-5 population showed a considerable reduction from 0 to 6 h of assay (average reduction between 3.20 and 4.71 log cfu/g) for all the 5 storage periods evaluated. The highest decrease in this strain

<table>
<thead>
<tr>
<th>Exposure time (h)</th>
<th>Microorganisms (log cfu/g)</th>
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<tr>
<td></td>
<td><em>L. acidophilus</em> La-5</td>
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<tr>
<td>0</td>
<td>8.70 ± 0.12</td>
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<tr>
<td>2</td>
<td>5.86 ± 0.13</td>
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<td>6</td>
<td>3.68 ± 0.29</td>
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<td></td>
<td><em>B. animalis</em> Bb-12</td>
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<tr>
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<td>8.85 ± 0.18</td>
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<td>4</td>
<td>3.90 ± 0.14</td>
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</tr>
<tr>
<td>6</td>
<td>4.62 ± 0.17</td>
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</table>

Values are expressed as mean ± SD.

The survival of *L. acidophilus* La-5 and *B. animalis* Bb-12 in the ABT-4 fresh culture in MRS broth at 37 °C for 18 h before (0 h) and during exposure to *in vitro* simulated gastric (2 h) and enteric (4 h and 6 h) conditions.

Table 4

![Fig. 1. Survival of *L. acidophilus* La-5 (log cfu/g) in fermented soy products during storage for 1, 7, 14, 21, and 28 days (i, ii, iii, iv, and v, respectively) before (0 h) and during exposure to *in vitro* simulated gastric (2 h) and enteric (4 h and 6 h) conditions. For the same storage period, A, B, C Different superscript capital letters denote significant differences between trials for the same sampling period of the *in vitro* assay (*P* < 0.05); a, b, c, d Different superscript lowercase letters denote significant differences between different sampling periods of the *in vitro* assay for the same trial (*P* < 0.05). FSP: fermented soy product (control); FSP-I: FSP with inulin; FSP-O: FSP with okara flour; FSP-IO: FSP with inulin and okara flour.](image-url)
viability was observed in the gastric phase, suggesting its high sensibility towards simulated gastric juice containing HCl and pepsin. On the other hand, it seems that the refrigerated storage period (28 days) was not a determining factor for *L. acidophilus* *La-5* survival, since the reduction of *L. acidophilus* *La-5* population from 0 to 6 h of assay was similar for all soy products in the storage periods evaluated.

The okara flour improved the *L. acidophilus* *La-5* survival in the gastric phase (2 h), particularly on days 7, 14, and 21 of storage. Nevertheless, no significant differences were observed between FSP and FSP-I after 2 h of assay for all storage periods evaluated (P > 0.05), suggesting that the presence of inulin in the FSP did not protect the strain against the simulated gastric juice adverse effect. Even though the presence of okara flour in FSP-O and FSP-IO has been shown to confer a protective effect on *L. acidophilus* *La-5* during the gastric phase of the assay (2 h), it did not prevent the decreased *L. acidophilus* *La-5* survival after the enteric phase (6 h). In fact, final *L. acidophilus* *La-5* viability was very similar to the control (FSP) on days 7, 14, and 21 of storage. A tendency towards protection conferred by okara was verified after 6 h of assay on days 1 and 28 (Fig. 1).

*L. acidophilus* was also susceptible to the assay phases containing bile, since a significant reduction (P < 0.05) in this probiotic population was observed for most samples between the gastric and enteric phases. Similar results were observed by Buriti et al. (2010), using the same strain (*L. acidophilus* *La-5*) incorporated into guava mousses. Bile affects the phospholipids and proteins of bacterial cell membranes, disrupting cellular homeostasis and Gram-positive bacteria seem to be more susceptible to the deleterious effects of bile than Gram-negative do. However, the tolerance to the

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**Fig. 2.** Survival of *B. animalis* Bb-12 (log cfu/g) in fermented soy products during storage for 1, 7, 14, 21, and 28 days (i, ii, iii, iv, and v, respectively) before (0 h) and during exposure to in vitro simulated gastric (2 h) and enteric (4 h and 6 h) conditions. For the same storage period, A,B,C Different superscript capital letters denote significant differences between trials for the same sampling period of the in vitro assay (P < 0.05); a,b,c,d Different superscript lowercase letters denote significant differences between different sampling periods of the in vitro assay for the same trial (P < 0.05). FSP: fermented soy product (control); FSP-I: FSP with inulin; FSP-O: FSP with okara flour; FSP-IO: FSP with inulin and okara flour.
bile is a strain-dependent characteristic that should not be general-ized in terms of species (Begley et al., 2005).

Buriti et al. (2010) also reported that inulin improved L. acidophilus La-5 tolerance to gastrointestinal conditions during the first week of synbiotic guava mousse refrigerated storage. In the present study, the protective effect of inulin was not observed in different storage periods evaluated. In the food matrix, inulin binds to available water, producing a gel made up of a tridimensional network of microcrystals that interact, forming small aggregates that occlude a great amount of water. This protection is probably associated with the resistance of inulin to hydrolysis by the GIT enzymes and with its degree of polymerization (DP) around 25 for long-chain inulin (Franch, 2008; Buriti et al., 2010) like the Beneo® HP-Gel employed in the production of mousse by Buriti et al. (2010). This structure might have involved the bacterial cells inside the food matrix, contributing to physical protection. However, the food matrix tested and the proportion and type of inulin (Beneo® GR, DP around 10) employed were different in the present study.

Comparing the different test-trials (FSP-I, FSP-O, and FSP-IO) with the control (FSP) after 6 h of the in vitro assay, the presence of inulin and okara flour in the FSP increased B. animalis Bb-12 survival under simulated gastrointestinal conditions on day 1 of storage (P < 0.05). On days 7 and 21, the okara showed a tendency towards B. animalis Bb-12 protection.

In the present study, both the B. animalis and the L. acidophilus strains survived very well when incorporated into the FSP tested, maintaining mean populations always above 8 log cfu/g during 28 days of storage. Nevertheless, the L. acidophilus La-5 strain was very sensitive to the harsh test conditions during the in vitro tests, since its viability usually fell to below 5 log cfu/g after the various stages of the assay. On the other hand, B. animalis Bb-12 was very robust in the harsh test conditions, maintaining mean populations of above 7 log cfu/g up to the end of the assays, for all the products after all the storage test periods. In fact, studies have shown that strains of B. animalis are the most resistant to gastric juice (Mainville et al., 2005; Matto et al., 2006; Madureira et al., 2011). This could explain the higher tolerance of B. animalis Bb-12 to simulated gastrointestinal conditions in the present study, when compared to L. acidophilus La-5. Indeed, some strains of bifidobacteria may have acid stress adaptation strategies (Collado and Sanz, 2007). Thus, the intrinsic characteristics of the strains could be decisive for this tolerance.

The components of a diet or the food ingredients may influence probiotic survival during the passage through the GIT (Sanders and Marco, 2010). Thus, the microorganism might not be greatly exposed to bile in certain microenvironments produced in the presence of a food matrix or ingredient, since these components of a diet could bind to bile acids, preventing them from exerting their toxicity on the probiotics present (Begley et al., 2005). The okara flour employed in some of the tested FSP is rich in proteins, lipids, and fibres (Jiménez-Esrig et al., 2008). It is possible that these components could have improved the probiotic tolerance to gastric and enteric juice action. Nevertheless, this effect was only observed in L. acidophilus La-5 strain against the gastric juice action and in B. animalis Bb-12 on day 1 of storage.

Studies to verify the influence of different food matrices on probiotic resistance to gastrointestinal conditions are important to select suitable vehicles for the delivery of these microorganisms, since the food format has the potential to affect their survival, physiology, and the potentially efficacy of probiotic strains (Sanders and Marco, 2010). Additionally, the present study represents an effort to enhance the use of okara, a byproduct of soymilk industry, thereby helping to reduce the accumulation of agricultural waste.

4. Conclusions

The present study showed that the viability of L. acidophilus La-5 and B. animalis Bb-12 in the four formulations of FSP tested was satisfactory until the 28th day of refrigerated storage, ranging from 8 to 9 log cfu/g for both microorganisms, and was not affected by the presence of inulin and/or okara flour and by the storage period. The resistance to artificial gastrointestinal juices, whilst high for B. animalis Bb-12, maintaining mean populations of above 7 or 8 log cfu/g, was lower for L. acidophilus La-5 (below 5 log cfu/g). Even though the protective effect of inulin and/or okara flour on probiotic microorganisms was not significant, the FSP matrix improved survival for both strains, particularly B. animalis Bb-12, when compared to a freshly prepared culture submitted to the same conditions. Therefore, the FSP matrix may be considered good vehicle for the probiotics tested and could play an important role in their protection against gastrointestinal juices. Further studies, involving the complete nutritional characterization and the sensory features comparison of the fermented soy products are required.

Acknowledgements

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References


