



## Food Microbiology

# Development and characterization of an innovative synbiotic fermented beverage based on vegetable soybean



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## ABSTRACT

Soymilk was produced from vegetable soybean and fermented by probiotics (*Lactobacillus acidophilus* La-5, *Bifidobacterium animalis* Bb-12) in co-culture with *Streptococcus thermophilus*. The composition of the fermented beverage and oligosaccharides content were determined. The effect of fructooligosaccharides and inulin on the fermentation time and viability of probiotic microorganisms throughout 28 days of storage at 5 °C were evaluated. The soymilk from vegetable soybeans was fermented in just 3.2 h, when pH reached 4.8. Fermentation reduced the contents of stachyose and raffinose in soymilk. Prebiotics had no effect on acidification rate and on viability of *B. animalis* and *S. thermophilus* in the fermented beverage. The viable counts of *B. animalis* Bb-12 remained above 10<sup>8</sup> CFU mL<sup>-1</sup> in the fermented soymilk during 28 days of storage at 5 °C while *L. acidophilus* La-5 was decreased by 1 log CFU mL<sup>-1</sup>. The fermented soymilk from vegetable soybeans showed to be a good food matrix to deliver probiotic bacteria, as well as a soy product with a lower content of non-digestible oligosaccharides.

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## Introduction

Soymilk has received increasing attention from consumers as an alternative to dairy products due to its protein quality, absence of cholesterol and lactose, and functional properties.<sup>1</sup>

However, the consumption of soymilk is limited due to the presence of non-digestible oligosaccharides, such as raffinose and stachyose, which are not hydrolyzed in the small intestine and may cause abdominal cramps, diarrhea and bloating.<sup>2,3</sup>

Lactic acid bacteria and probiotic microorganisms, for example, *Streptococcus thermophilus*, *Lactobacillus acidophilus*,

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and *Bifidobacterium*, can grow in soymilk and consume non-digestible oligosaccharides, decreasing or eliminating these anti-nutritional compounds, resulting in a healthier product for consumers.<sup>4–7</sup> A probiotic fermented soymilk combines the beneficial properties of soy with the health benefits of probiotic microorganisms. However, the acidification rate of pure probiotic cultures is usually low, and often off-flavors in the final product are produced. The solution to this problem is to mix the probiotic cultures with yogurt cultures to reduce the fermentation time and improve the sensory characteristics of the product.<sup>8,9</sup> Prebiotic ingredients, such as inulin and fructooligosaccharides (FOS), may also improve the activity and survival of probiotic bacteria in fermented soymilk.<sup>7,10,11</sup> Moreover, the combination of prebiotics and probiotics results in a synbiotic effect on gut microbiota.<sup>12</sup> Non-dairy synbiotic beverage<sup>13,14</sup> is also an alternative to consumer that are lactose intolerant or allergic to milk protein.

Vegetable soybean [*Glycine max* (L.) Merrill] is a popular food that is consumed in Asia, the United States and other countries, mainly as a snack, a vegetable for soups or stews, or in salads. It is a soybean that is harvested while the seeds are at approximately 80% of maturity, such that it has a green color and a soft texture.<sup>15,16</sup> These immature seeds have advantages over mature soybean, including improved sensory attributes and nutritional value, such as sweeter flavor and less contents of stachyose and raffinose, resulting in better digestibility.<sup>17,18</sup> Similar to mature soybean, vegetable soybean is rich in good quality protein, has a high mineral content and has the potential to prevent some diseases, including cancer, osteoporosis and menopausal symptoms due to its content in isoflavones.<sup>19</sup>

Numerous studies have been performed on the growth of probiotic cultures in soy beverage<sup>4,8,20</sup>; however, no information is available on the fermentation of soymilk from vegetable soybean. Thus, the aim of this study was to investigate the acidification rate of soymilk produced with vegetable soybeans supplemented with inulin and fructooligosaccharide (FOS), by a mixed culture of *L. acidophilus* La-5, *Bifidobacterium animalis* subsp. *lactis* Bb-12 and *S. thermophilus*. In addition, the survival of microorganisms during 28 days of storage at 5 °C and the contents of stachyose and raffinose in soymilk and fermented beverages were also evaluated.

## Materials and methods

### Production of soymilk

Vegetable soybeans, cultivar BRS-232, were supplied by Embrapa Soybean, Brazil. The plants were harvested mechanically and taken to the laboratory where the pods were removed and immediately bleached in boiling water for 3 min and cooled at 5 °C. Seeds were removed from the pods, packed in plastic bags, frozen at –18 °C and freeze-dried in a lab scale lyophilizer (Enterprise I; TERRONI, São Carlos, SP, Brazil).

Soymilk was produced by soaking fifty grams of freeze-dried vegetable soybeans in 455 g of water at room temperature for 10 min. The mixture was heated at 85 °C and blended for 3 min. The slurry was stirred at 85 °C for 5 min and filtered in a 0.5 mm conical sieve to obtain the soymilk.

### Fermentation of soymilk

Soymilk was supplemented with 40 g kg<sup>-1</sup> fructooligosaccharide, FOS (Orafti® P 95, Beneo Latinoamericana), 40 g kg<sup>-1</sup> inulin (Orafti® GR, Beneo Latinoamericana) or a mixture of 40 g kg<sup>-1</sup> FOS and 40 g kg<sup>-1</sup> inulin. Control fermented soymilk was prepared without the addition of the prebiotic ingredients. The four formulations were produced in triplicate. After the addition of the prebiotic ingredients, soymilks were pasteurized at 75 °C for 15 s, cooled at 37 °C, and inoculated with 0.02% of a freeze-dried ABT-4 culture (Christian Hansen, Denmark) containing *L. acidophilus* La-5, *B. animalis* subsp. *lactis* Bb-12 and *S. thermophilus*. Next, the soymilks were distributed in 50 mL sterile bottles and incubated at 37 °C until the pH reached 4.7–4.8. The fermented beverages were stored at 5 °C for 28 days.

### Chemical and physicochemical analyses

The chemical composition of lyophilized vegetable soybeans and soymilk without prebiotic ingredients was determined according to AOAC methods.<sup>21</sup> The moisture of the vegetable soybeans was determined by drying the sample in an oven at 105 °C until constant mass, based on AOAC method 925.09B, without vacuum utilization. The total solids content of the soymilks was determined according to AOAC method 990.20, and the moisture was calculated subtracting this value from 100. To determine the ash content, the sample was incinerated in a muffle at 550 °C (method 923.03 and method 945.46 for grains and soymilk, respectively, both from AOAC). The protein content was calculated by the measurement of total nitrogen using the micro Kjeldahl method, and the conversion factor applied was 6.25, based on AOAC method 979.09. The fat content was determined using the Soxhlet, based on AOAC method 920.39, using hexane as solvent (boiling point = 70 °C at 101,325 Pa). The total carbohydrate content was calculated by the difference.

The pH of the soymilk and fermented soymilk were measured using a pH meter (TEC-2; TECNAL, Piracicaba, SP, Brazil), according to AOAC method 981.12 (AOAC<sup>21</sup>).

Extraction of oligosaccharides (stachyose and raffinose) from vegetable soybeans was performed according to Oliveira et al.<sup>22</sup> with adaptations. The freeze-dried vegetable soybeans were ground to pass through a 0.5 mm sieve, and a sample of 2.50 g was mixed with 50 mL of an 80% ethanol solution and stirred for 2 min. The mixture was centrifuged at 5000 rpm for 10 min, and the content of the oligosaccharides was determined in the supernatant.

To analyze the oligosaccharides and organic acids contents in soymilk and fermented beverages, the samples were centrifuged at 5000 rpm for 5 min. The supernatant was centrifuged again under the same conditions, diluted in deionized water at a volumetric ratio of 50%, and neutralized with barium carbonate. Centrifugation was performed again at 5000 rpm for 5 min, and the oligosaccharides content in the supernatant was determined using HPLC.

The HPLC method described by Rivas et al.<sup>23</sup> was used to determine the contents of oligosaccharides. Samples of the liquors were filtered using 0.20 µm cellulose acetate membranes, neutralized with barium carbonate, and assayed by

HPLC for stachyose and raffinose using a 1100 series Hewlett-Packard chromatograph equipped with a refractive index detector operated at 50 °C and a 300 × 7.8 mm CARBOsep CHO 682 column (Transgenomic, Glasgow, UK) operating at 80 °C. Distilled water was used as the mobile phase (flow rate 0.4 mL min<sup>-1</sup>).

Lactic and acetic acids were determined according to Rivas et al.<sup>23</sup> Samples of liquors were filtered using 0.20 µm membranes and used for direct HPLC determination of lactic and acetic acid using an Agilent 1200 series HPLC instrument with a refractive index (RI) detector (Agilent, Waldbronn, Germany) operated at 50 °C. The other analysis conditions were as follows: Aminex HPX-87H column (BioRad, Hercules, CA, USA); mobile phase, 0.003 M H<sub>2</sub>SO<sub>4</sub>; flow, 0.6 mL min<sup>-1</sup>.

### Microbiological analysis

To determine the survival of microorganisms in fermented vegetable soymilk during the storage period (1, 4, 7, 14, 21 and 28 days), 10 g of each sample were blended with 90 g of a 8.5 g L<sup>-1</sup> sterile saline solution for 1 min at 260 rpm in a Stomacher (Seward, Worthing, UK) and subjected to serial decimal dilutions with the same diluent. On each sampling day, a new bottle containing fermented soymilk from the same batch was used for the analysis.

Viable cells numbers of *S. thermophilus* was determined by surface-plating 20 µL of each dilution in M17 agar supplemented with 5 g L<sup>-1</sup> lactose. Plates were incubated at 37 °C for 48 h under aerobic conditions.<sup>24</sup>

*B. animalis* Bb-12 viability was monitored by surface-plating 20 µL of each dilution in De Man Rogosa Sharpe (MRS) agar containing 0.2 g L<sup>-1</sup> of bile salts, 0.3 g L<sup>-1</sup> of sodium propionate, 0.5 g L<sup>-1</sup> of cysteine-HCl and 0.2 g L<sup>-1</sup> of lithium chloride. Plates were incubated at 37 °C for 48 h under anaerobic conditions (GasPak™, BD BBL™, EUA).<sup>25</sup>

*L. acidophilus* La-5 population was determined by surface-plating 20 µL of each dilution in M-MRS agar (formulated MRS agar containing 20 g L<sup>-1</sup> of maltose instead of glucose). Plates were incubated at 37 °C for 48 h under aerobic conditions.<sup>25</sup>

### Experimental design and statistical analysis

The effects of two independent variables, namely FOS and inulin contents, on vegetable soymilk physical and chemical characteristics were studied using a 2<sup>2</sup> factorial design replicated in three randomized blocks, which totaled four treatments and twelve trials. These results were expressed as the mean ± standard deviation (SD). Differences between means during the experimental period were analyzed using ANOVA followed by Tukey test upon verification of normality and homogeneity (*p* < 0.05). Minitab software, version 16, was utilized for the statistical analysis.

## Results

### Chemical composition of vegetable soybeans and soymilk

The composition of vegetable soybean cultivar BRS 232 used in this study and the soymilk produced with the same cultivar is

presented in Table 1. The contents of raffinose and stachyose in dry seeds of vegetable soybean BRS 232 were 2.8 ± 0.5 mg g<sup>-1</sup> and 4.8 ± 0.1 mg g<sup>-1</sup>, respectively.

The yield of the soymilk extraction process was 6.35 ± 0.09 kg of soymilk per kg of freeze-dried vegetable soybeans. The protein content in soymilk was 23 ± 2 mg g<sup>-1</sup>, and the protein recovery was 37.8 ± 0.5%.

### Changes in pH, organic acids and oligosaccharides during fermentation and refrigerated storage of soymilk

The addition of prebiotics, i.e., FOS, inulin or their combination to soymilk did not affect the fermentation time (*p* > 0.05). The time required to reach a pH of 4.8 ranged from 3.13 to 3.33 h, and the pH decreased at a mean rate of 0.39 ± 0.01 units (U) per hour (Table 2).

The pH, lactic and acetic acid values of fermented soymilks during refrigerated storage are shown in Table 2. All of the beverages presented a small but significant (*p* < 0.05) reduction in pH and an increase in lactic acid content during 28 days of storage. However, the greatest reduction in pH (0.14 U) and the highest content of lactic acid (2.59 g L<sup>-1</sup>) were observed in beverages with inulin supplementation. The acetic acid content in all of the fermented soymilk had no significant (*p* > 0.05) increase during storage.

The estimation of main effect, that was obtained by the difference in process performance caused by a change from the low to the high level of the corresponding factor, showed that FOS addition decreased the lactic acid content by 0.1 g L<sup>-1</sup> (*p* < 0.05), while inulin addition increased the content of lactic acid by 0.25 g L<sup>-1</sup> in fermented soymilk. No significant (*p* > 0.05) interaction between factors was observed.

Acetic acid content in fermented soymilk supplemented with prebiotics was lower (*p* < 0.05) than in control soymilk however, the differences were in general less than 0.1 g L<sup>-1</sup> (Table 2).

The concentration of oligosaccharides in soymilk without prebiotic before fermentation was 0.63 g L<sup>-1</sup> and 0.38 g L<sup>-1</sup> for stachyose and raffinose, respectively (Fig. 1). Due to the reduced content of oligosaccharides in vegetable soybean, the soymilk obtained in our study also presented a lower content of stachyose and raffinose compared with soymilk from mature soybean.

After fermentation of soymilk, the content of stachyose and raffinose significantly decreased (*p* < 0.05) to 0.45 g L<sup>-1</sup> and 0.23 g L<sup>-1</sup>, respectively.

**Table 1 – Chemical composition of the vegetable soybeans from cultivar BRS 232 and of soymilk.**

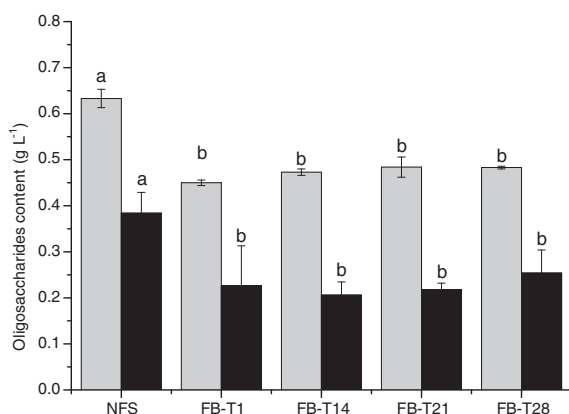
Parameter	Soybean (mg g <sup>-1</sup> )	Soymilk (mg g <sup>-1</sup> )
Moisture	696 ± 3	927 ± 1
Protein	127 ± 3	23 ± 2
Fat	65 ± 2	13.0 ± 0.6
Ash	16.0 ± 0.4	5.3 ± 0.1
Carbohydrates <sup>a</sup>	96 ± 4	32 ± 5

<sup>a</sup> Carbohydrate content determined by difference.

**Table 2 – Fermentation time, acidification rate, pH, lactic and acetic acid content of fermented soymilk after 1 and 28 days of storage at 5 °C.**

Parameter	Soymilk formulation <sup>a</sup>			
	C	F	I	FI
Time to reach pH 4.8 (h)	3.33 <sup>a</sup> ± 0.22	3.22 <sup>a</sup> ± 0.13	3.19 <sup>a</sup> ± 0.10	3.13 <sup>a</sup> ± 0.05
Acidification rate (pH U/h)	0.41 <sup>a</sup> ± 0.03	0.38 <sup>a</sup> ± 0.05	0.38 <sup>a</sup> ± 0.08	0.39 <sup>a</sup> ± 0.01
pH (day 1)	4.56 <sup>a</sup> ± 0.12	4.56 <sup>a</sup> ± 0.11	4.54 <sup>a</sup> ± 0.11	4.53 <sup>a</sup> ± 0.10
pH (day 28)	4.48 <sup>a</sup> ± 0.05	4.46 <sup>a</sup> ± 0.09	4.40 <sup>a</sup> ± 0.06	4.42 <sup>a</sup> ± 0.04
Lactic acid, gL <sup>-1</sup> (day 1)	2.09 <sup>a</sup> ± 0.13	1.79 <sup>b</sup> ± 0.04	2.11 <sup>a</sup> ± 0.14	2.13 <sup>a</sup> ± 0.06
Lactic acid, gL <sup>-1</sup> (day 28)	2.24 <sup>b</sup> ± 0.03	2.28 <sup>b</sup> ± 0.16	2.59 <sup>a</sup> ± 0.12	2.42 <sup>a</sup> ± 0.07
Acetic acid, gL <sup>-1</sup> (day 1)	0.54 <sup>a</sup> ± 0.03	0.46 <sup>ab</sup> ± 0.05	0.43 <sup>b</sup> ± 0.01	0.47 <sup>ab</sup> ± 0.03
Acetic acid, gL <sup>-1</sup> (day 28)	0.58 <sup>a</sup> ± 0.05	0.46 <sup>a</sup> ± 0.07	0.47 <sup>a</sup> ± 0.04	0.41 <sup>a</sup> ± 0.07

<sup>a</sup> C (without prebiotic), F (with 40 g kg<sup>-1</sup> of fructooligosaccharide, FOS), I (with 40 g kg<sup>-1</sup> of inulin), FI (with 40 g kg<sup>-1</sup> of FOS and 40 g kg<sup>-1</sup> of inulin). Mean ± standard deviation of three runs. Means in the same row with different letters are significantly different ( $p < 0.05$ ).



**Fig. 1 – Oligosaccharides content, stachyose (grey bars) and raffinose (black bars), in the non-fermented soymilk (NFS) and in the soymilk fermented beverages (FB) during the storage period (T1, T14, T21 and T28). For each oligosaccharide, means with different letters are significantly different ( $p < 0.05$ ).**

#### Changes in microbiological counts during the storage of fermented soymilk

The population of *S. thermophilus*, *L. acidophilus* La-5 and *B. animalis* Bb-12 in fermented soymilks throughout storage is shown in Table 3. The addition of FOS and/or inulin in soymilk had no significant ( $p > 0.05$ ) effect on *S. thermophilus* and *B. animalis* viability in fermented beverages. Although inulin had increased lactic acid content during the storage of fermented soymilk, no significant enhancement in *S. thermophilus* population was observed. A no significant ( $p > 0.05$ ) decrease, below 0.3 log CFU mL<sup>-1</sup>, in *S. thermophilus* and *B. animalis* populations was observed in almost all the fermented beverages during 28 days of storage. The *B. animalis* Bb-12 viability in fermented vegetable soymilk was greater than *L. acidophilus* La-5. At the end of 28 days of storage, the viable cell number of *B. animalis* was higher than 8 log CFU mL<sup>-1</sup> in all of the fermented beverages, regardless of the presence or absence of prebiotic, while the viable cell number of *L. acidophilus* was reported between 5.6 and 6.4 log CFU mL<sup>-1</sup>.

The decrease in *L. acidophilus* population in fermented soymilk during storage was significant ( $p < 0.05$ ) and higher than 1 log CFU mL<sup>-1</sup> for all treatments. In general, beverages with inulin and/or FOS presented significantly ( $p < 0.05$ ) lower counts compared to beverages without prebiotics.

#### Discussion

The results obtained for the composition of vegetable soybean BRS 232 (Table 1) were consistent with previous reports for other cultivars. Song et al.<sup>18</sup> and the USDA<sup>26</sup> reported 655 mg g<sup>-1</sup> and 675 mg g<sup>-1</sup> of moisture, 153 mg g<sup>-1</sup> and 130 mg g<sup>-1</sup> of protein, 44.9 mg g<sup>-1</sup> and 68 mg g<sup>-1</sup> of fat, 20 mg g<sup>-1</sup> and 17 mg g<sup>-1</sup> of ash, and 127 mg g<sup>-1</sup> and 110 mg g<sup>-1</sup> of carbohydrates in immature soybeans. The differences observed between those studies and the results reported in this study may be related to different vegetable soybean genotypes.

For mature seeds from the same cultivar, Oliveira et al.<sup>22</sup> reported three-fold and eight-fold higher concentrations, namely, 8.5 mg g<sup>-1</sup> and 37.0 mg g<sup>-1</sup> in raffinose and stachyose, respectively. This difference in sugar content between the different maturation stages of soybeans has been previously reported by other studies.<sup>16,27</sup> Within one week, the contents of stachyose and raffinose might duplicate or triplicate depending on the cultivar.<sup>15</sup>

The protein recovery (37.8%) in vegetable soymilk was low compared with 67.4% to 78.8% reported by Vishwanathan et al.<sup>28</sup> for soymilk extracted from mature soybeans. These authors employed hot extraction, with the same temperature used in the present study (between 80 and 95 °C), and thus, the discrepancy between our studies may be related to the soybean particle size,<sup>29</sup> the genotype<sup>30</sup> and with the maturation stage of the seeds.

Fermentation of soymilk from vegetable soybean was very fast, in 3.2 h pH reached 4.8 and no significant difference ( $p > 0.05$ ) was observed with the addition of inulin or FOS. Our results differed from those of Rinaldoni et al.,<sup>11</sup> who reported that ultrafiltered soymilk concentrate fermented by *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus* without inulin did not reach a pH lower than 4.9, and soy yogurt was not obtained. These authors also observed that independent of the concentration of inulin

**Table 3 – Viable counts of *S. thermophilus*, *L. acidophilus* La-5 and *B. animalis* Bb-12 in fermented soymilk during storage period for 28 days at 5 °C.**

Microorganism	Time (days)	LogCFU mL <sup>-1a</sup>			
		C	F	I	FI
<i>S. thermophilus</i>	1	8.22 ± 0.13 <sup>Aa</sup>	8.16 ± 0.06 <sup>Aa</sup>	8.13 ± 0.05 <sup>Aa</sup>	8.18 ± 0.03 <sup>Aa</sup>
	4	8.23 ± 0.04 <sup>Aa</sup>	8.21 ± 0.06 <sup>Aa</sup>	8.26 ± 0.14 <sup>Aa</sup>	8.18 ± 0.14 <sup>Aa</sup>
	7	8.16 ± 0.04 <sup>Aa</sup>	8.11 ± 0.10 <sup>Aa</sup>	8.15 ± 0.08 <sup>Aa</sup>	8.22 ± 0.10 <sup>Aa</sup>
	14	8.16 ± 0.04 <sup>Aa</sup>	8.26 ± 0.11 <sup>Aa</sup>	8.16 ± 0.20 <sup>Aa</sup>	8.20 ± 0.19 <sup>Aa</sup>
	21	8.13 ± 0.07 <sup>Aa</sup>	8.12 ± 0.10 <sup>Aa</sup>	8.07 ± 0.19 <sup>Aa</sup>	8.06 ± 0.04 <sup>Aa</sup>
	28	7.90 ± 0.10 <sup>Aa</sup>	7.90 ± 0.13 <sup>Aa</sup>	8.04 ± 0.13 <sup>Aa</sup>	8.03 ± 0.18 <sup>Aa</sup>
<i>L. acidophilus</i>	1	7.56 ± 0.26 <sup>Aa</sup>	7.38 ± 0.19 <sup>Aa</sup>	7.35 ± 0.19 <sup>Aa</sup>	7.30 ± 0.16 <sup>Aa</sup>
	4	7.55 ± 0.24 <sup>Aa</sup>	7.39 ± 0.13 <sup>Aa</sup>	7.38 ± 0.09 <sup>Aa</sup>	7.30 ± 0.16 <sup>Aa</sup>
	7	7.37 ± 0.09 <sup>Aa</sup>	7.29 ± 0.18 <sup>Aa</sup>	7.32 ± 0.15 <sup>Aa</sup>	7.28 ± 0.07 <sup>Aa</sup>
	14	7.17 ± 0.14 <sup>Aa</sup>	6.98 ± 0.27 <sup>Aa</sup>	6.94 ± 0.16 <sup>Aa</sup>	6.85 ± 0.18 <sup>ABa</sup>
	21	7.02 ± 0.18 <sup>Aa</sup>	6.24 ± 0.07 <sup>Bb</sup>	6.20 ± 0.02 <sup>Bb</sup>	6.20 ± 0.47 <sup>BCb</sup>
	28	6.18 ± 0.28 <sup>Bab</sup>	6.05 ± 0.35 <sup>Bab</sup>	5.63 ± 0.29 <sup>Cb</sup>	6.42 ± 0.08 <sup>BCa</sup>
<i>B. animalis</i>	1	8.58 ± 0.17 <sup>Aa</sup>	8.50 ± 0.09 <sup>Aa</sup>	8.50 ± 0.08 <sup>Aa</sup>	8.49 ± 0.08 <sup>Aa</sup>
	4	8.48 ± 0.01 <sup>ABa</sup>	8.49 ± 0.07 <sup>Aa</sup>	8.31 ± 0.27 <sup>Aa</sup>	8.39 ± 0.03 <sup>ABa</sup>
	7	8.34 ± 0.13 <sup>ABa</sup>	8.39 ± 0.07 <sup>Aa</sup>	8.44 ± 0.01 <sup>Aa</sup>	8.51 ± 0.07 <sup>Aa</sup>
	14	8.13 ± 0.06 <sup>Ba</sup>	8.28 ± 0.12 <sup>Aa</sup>	8.47 ± 0.05 <sup>Aa</sup>	8.47 ± 0.09 <sup>Aa</sup>
	21	8.34 ± 0.03 <sup>ABa</sup>	8.25 ± 0.19 <sup>Aa</sup>	8.25 ± 0.23 <sup>Aa</sup>	8.21 ± 0.24 <sup>ABa</sup>
	28	8.30 ± 0.05 <sup>ABa</sup>	8.33 ± 0.14 <sup>Aa</sup>	8.24 ± 0.35 <sup>Aa</sup>	8.00 ± 0.15 <sup>Ba</sup>

<sup>a</sup> C (without prebiotic), F (with 40 g kg<sup>-1</sup> of fructooligosaccharide, FOS), I (with 40 g kg<sup>-1</sup> of inulin), FI (with 40 g kg<sup>-1</sup> of FOS and 40 g kg<sup>-1</sup> of inulin). Mean ± standard deviation of three runs. Means in the same row with different lowercase letters are significantly different ( $p < 0.05$ ). Means in the same column, for the same microorganisms, with different uppercase letters are significantly different ( $p < 0.05$ ).

added to the soymilk (20–70 g L<sup>-1</sup>), the pH reached 4.65 within 4 h of fermentation. Since they used as food matrix a concentrated soymilk, with protein content between 40 and 58 g L<sup>-1</sup>, almost twice compared with the soymilk from vegetable soymilk, the higher protein content increased the buffering capacity, and thus, reduced the fermentation rate. On the other hand, Pandey and Mishra<sup>31</sup> observed that higher FOS concentrations (20–100 g L<sup>-1</sup>) in soymilk resulted in a shorter fermentation process. The effect of inulin on the reduction in fermentation time in milk has also been reported by previous studies.<sup>12,32,33</sup>

The acidification rate of soymilk from vegetable soybean by probiotics strains in co-culture with *S. thermophilus* (Table 2) was faster than in milk when compared with results reported by Casarotti et al.<sup>34</sup> In milk fermented by the combination of *S. thermophilus* and *L. acidophilus* La-5, acidification rate was 0.34 pH U/h, whereas in the present study, values ranged from 0.38 to 0.41 pH U/h. This difference in acidification rate may be explained due to the lower buffering capacity of soymilk as reported by Champagne et al.<sup>8</sup> who also observed that acidification of soy beverage by *S. thermophilus* alone or in combination with *Lactobacillus helveticus* or *Bifidobacterium longum* was faster than in milk.

Data in the literature shows that *S. thermophilus* can grow well in soy beverages; however, the acidification rate is a function of the selected strain and the presence of other cultures, such as probiotics.<sup>6,20</sup> Our results demonstrated that *S. thermophilus* in the presence of *L. acidophilus* La-5 and *B. animalis* Bb-12 was able to ferment soymilk from vegetable soybean in a short time period.

The production of lactic acid during fermentation of soymilk can be attributed mainly to *S. thermophilus*, which is a homofermentative species that produces lactic acid to a higher extent than probiotic bacteria in soymilk.<sup>6</sup> As beverages with inulin presented a higher content of lactic acid, the presence of this ingredient may have enhanced *S. thermophilus* metabolism.

Acetic acid is produced mainly by bifidobacteria in which carbohydrate metabolism results in the production of acetic and lactic acids in a molar ratio 3–2. Thus, our results showed that the addition of prebiotics to soymilk did not enhance bifidobacteria metabolism in vegetable soymilk beverage.

Due to the reduced content of oligosaccharides in vegetable soybean, the soymilk obtained in our study also presented a lower content of stachyose and raffinose compared with soymilk from mature soybean (Fig. 1). The literature data show that in soymilk extracted from mature soybean, the stachyose content can vary from 2.1 to 8.8 g L<sup>-1</sup> and that raffinose varied from 1.44 to 2.2 g L<sup>-1</sup>.<sup>35,36</sup>

The hydrolysis of these  $\alpha$ -galacto-oligosaccharides requires the enzyme  $\alpha$ -galactosidase, which hydrolyses  $\alpha$ -galactoside bonds.<sup>37</sup> *Bifidobacterium* spp., *L. acidophilus* and *S. thermophilus* have  $\alpha$ -galactosidase activity; however, the utilization of these sugars varies with the culture species and strains employed.<sup>6,20</sup> Donkor et al.<sup>4</sup> reported that *S. thermophilus* reduced raffinose in soymilk by 64.5%, whereas stachyose was metabolized by over 40% for most of the strains evaluated. Hou et al.<sup>5</sup> observed that *Bifidobacterium* reduced raffinose by 39% and stachyose by 65% after 48 h of

fermentation of soymilk. In the present study, the reduction of raffinose and stachyose was 39.5% and 28.5%, respectively (Fig. 1). The short fermentation time in our study may have resulted in a lower reduction in oligosaccharides content.

Many studies have shown that soymilk is a good vehicle for probiotic bacteria<sup>1,4,6,10,33</sup>; however, no information is available for soymilk from vegetable soybean. Our results showed that viability of *B. animalis* Bb-12 remained high, above  $10^8$  CFU mL<sup>-1</sup> during 28 days of storage of fermented vegetable soymilk. However, at the same time, cell counts of *L. acidophilus* La-5 reduced more than 1 log. Apparently, the low pH and the accumulation of organic acids was not responsible for the loss of probiotic viability, since Bedani et al.<sup>10</sup> verified that cell number of *L. acidophilus* La-5 remained above  $10^8$  CFU mL<sup>-1</sup> in a fermented soy product, with a similar pH reported herein. Nevertheless, in their study, lactose, sucrose and skimmed milk powder was added to the soymilk before fermentation. Thus, the increase in sugar that is easily fermented and in amino acids content may have contributed to a higher count of *L. acidophilus* during the storage when compared to the soymilk from vegetable soybeans.<sup>10</sup>

There have been some contradictory reports regarding the use of FOS and inulin to stimulate the growth and survival of probiotic strains. Yeo and Liong<sup>7</sup> verified that soymilk supplemented with inulin or FOS did not affect the population of *L. acidophilus* ATCC 4356, *B. longum* FTDC 8643 and *Bifidobacterium* FTDC 8943, but it did enhance the growth of *L. acidophilus* FTDC 8033, suggesting that the effect was strain-dependent. Thus, the fact that the addition of FOS and inulin to vegetable soymilk did not enhanced the survival of *L. acidophilus* during refrigerated storage might be attributed to probiotic strain response.

The present study demonstrated that a serving portion of 100 mL of fermented soymilk from vegetable soybean has more than  $10^{10}$  CFU of probiotics. Considering that a probiotic dose of  $10^8$  CFU per day is needed for beneficial effects in the gut,<sup>38</sup> the fermented soymilk from vegetable soybean is a good potential vector, during a shelf-life of 28 days, for viable probiotic microorganisms to promote health benefits to the host coupled with better nutritional quality.

## Conclusions

The soymilk from vegetable soybeans (cultivar BRS 232) was fermented in a short time, 3.2 h, by a mixed culture containing *L. acidophilus* La 5, *B. animalis* Bb 12 and *S. thermophilus*, demonstrating an important technological property for industrial purpose. Fermentation decreased the content of stachyose and raffinose. The viability of *B. animalis* in fermented soymilk remained stable over 28 days of storage at above  $10^8$  CFU mL<sup>-1</sup>, while *L. acidophilus* decreased by 1 log in the same period to values close to  $10^6$  CFU mL<sup>-1</sup>. The addition of FOS or inulin had no effect on acidification rate and also on the viability of *B. animalis* and *S. thermophilus*. Taken together, these results demonstrated that fermented soymilk from vegetable soybean is a very promising product as a vehicle for probiotic bacteria, as well as soy product with a lower content of nondigestible oligosaccharides.

## Conflicts of interest

The authors declare no conflicts of interest.

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