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Influence of passion fruit by-product and fructooligosaccharides on the viability of *Streptococcus thermophilus* TH-4 and *Lactobacillus rhamnosus* LGG in folate bio-enriched fermented soy products and their effect on probiotic survival and folate bio-accessibility under *in vitro* simulated gastrointestinal conditions

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ABSTRACT

This study aimed to evaluate the influence of passion fruit by-product (PFBP) and fructooligosaccharides (FOS) on the viability of *Streptococcus thermophilus* TH-4 and *Lactobacillus rhamnosus* LGG in folate bio-enriched fermented soy products and their effect on probiotic survival and folate bio-accessibility under *in vitro* simulated gastrointestinal conditions during storage of the products at 4 °C for up to 28 days (at days 1, 14, and 28). Kinetic parameters and folate contents before and after fermentation were also evaluated. Four different bio-enriched soy products in which the two microorganisms were used in co-cultures were studied and PFBP and/or FOS were added at 1 g/100 g, except for the control product. No differences ($p < 0.05$) between the fermented soy products (FSP) were observed for the maximum acidification rate (V_{max}) and the time to reach the V_{max} (T_{max}) or pH 5.5 (T_f), indicating that the use of PFBP and/or FOS did not affect the fermentation kinetic parameters. Only *Lb. rhamnosus* LGG retained the desired viability (> 8 log CFU/mL) during storage, whereas *St. thermophilus* TH-4 populations decreased by day 14 reaching counts between 6.4 and 5.5 log CFU/mL by day 28. The folate content of all FSP increased after fermentation and the simultaneous presence of PFBP and FOS stimulated the co-culture to increase folate production. Folate content in all FSP decreased during storage. *Lb. rhamnosus* LGG was recovered at the end of the simulated digestion, but PFBP and/or FOS did not affect recovery. The folate content increased during the gastrointestinal assay for all FSP, especially for FSP without supplementation, suggesting an *in vitro* increase of folate bio-accessibility. Therefore, the bio-enriched probiotic FSP presented a great potential as an innovative functional food by delivering probiotic microorganisms and providing 14% of the recommended daily folate intake. The folate content of the FSP might be increased during gastrointestinal stress conditions, which could contribute to increase the folate bio-accessibility in the gut.

1. Introduction

The consumption of soy products has increased as an alternative to dairy products and also because these products are good sources of proteins, dietary fibres, vitamins, minerals and have been shown to possess functional properties (Bedani et al., 2013; Chen et al., 2010; Donkor et al., 2007). However, soy-based products are known to have unsavoury and anti-nutritional factors. In this sense, some researchers have suggested the use of lactic acid bacteria (LAB) to ferment soy products to improve their sensorial, nutritional, and health properties (Champagne et al., 2009; Farnworth et al., 2007; Marazza et al., 2013).

Soy-based matrix is a good substrate for the growth of LAB (Albuquerque et al., 2017; Battistini et al., 2017; Bedani et al., 2013). Some LAB strains, such as *Streptococcus thermophilus* and *Lactobacillus*

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spp., may produce α-galactosidase, which plays an important role on the metabolism of carbohydrates during fermentation of soy products (Albuquerque et al., 2017). This property contributes, for the improvement of soy unsavoury, the decrease in the anti-nutritional substrates present, and to the microbial production of several bioactive molecules, such as B-group vitamins (LeBlanc et al., 2017). Additionally, LAB are widely used by the food industry due to the technological importance and probiotic characteristics presented by some strains. Probiotics are "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014).

Streptococcus thermophilus is an important dairy starter culture also known for its ability to produce large amounts of folate during fermentation in a strain-dependent manner (Iyer et al., 2009; Laiño et al., 2012). The use of prebiotics, such as fructooligosaccharides (FOS) and other potential prebiotics like fruit by-products, as functional ingredient to stimulate folate production by microorganisms to bio-enrich foods has been investigated lately (Albuquerque et al., 2016; Albuquerque et al., 2017; Espírito-Santo et al., 2015; Vieira et al., 2017).

Some countries like the USA, Canada, and Brazil have established mandatory programs to fortify foods of mass consumption with folic acid, which is the chemically synthesized form of folate. This vitamin is often not consumed in sufficient amounts and is related to essential metabolic processes of the human body such as replication, repair and methylation of DNA, and neural tube formation (Laiño et al., 2013). However, people with nutritionally balanced diets may consume potentially dangerous amounts of this vitamin due to this mandatory fortification of foods. This fact could cause some adverse effects including masking of the early haematological manifestations of vitamin B12 deficiency (Bailey and Ayling, 2009). The use of LAB to bio-enrich foods with natural vitamins is a cheap alternative to the use of synthetic vitamins to fortify foods. Besides, increasing vitamins by microbial fermentation may add value to the final product and improve the economy of food companies.

Studies suggest that fermented soy products (FSP) may contribute to human nutritional requirements and enhance human health (Bedani et al., 2014). According to Mo et al. (2013), during the processing of soybean, large amounts of nutrients are lost, including folates; however, this nutrient could be naturally increased by microbial fermentation. Previously, Albuquerque et al. (2017) bio-enriched soy milks supplemented with passion fruit by-product and/or FOS using different strains of *St. thermophilus* and *Lactobacillus* spp. In co-culture, *St. thermophilus* TH-4 and *Lactobacillus rhamnosus* LGG produced the highest amounts of folate, especially after fruit by-product and FOS supplementation.

Even though the scientific literature describes studies regarding FSP as vehicle of probiotics and prebiotics, concomitant information related to the use of FSP bio-enriched with natural folates to evaluate the bioaccessibility of the vitamin and the survival of the probiotic microorganisms during simulated gastrointestinal conditions is still lacking. Bio-accessibility is "the solubilized amount of a food compound or nutrient which becomes available for subsequent absorption in the gut after ingestion" (Guven et al., 2018). The use of *in vitro* simulated gastrointestinal models to determine folate bioaccessibility has been investigated (Mo et al., 2013; Ringling and Rychlik, 2017). Therefore, this study aimed to evaluate the influence of passion fruit by-product and/or FOS on the viability of *Streptococcus thermophilus* TH-4 and *Lactobacillus rhamnosus* LGG in folate bio-enriched fermented soy products and their effect on probiotic survival and folate bio-accessibility under *in vitro* simulated gastrointestinal conditions during storage of the products at 4 °C for up to 28 days (at days 1, 14, and 28). Kinetic parameters and folate contents before and after fermentation were also evaluated.

2. Material and methods

2.1. Microorganisms and growth conditions

Streptococcus (*St.*) *thermophilus* TH-4 and *Lactobacillus* (*Lb.*) *rhamnosus* LGG (Chr. Hansen, Hoersholm, Denmark) stored at −80 °C, respectively in Hogg-Jago (HJ) glucose broth (Blomqvist et al., 2006) and de Man, Rogosa, and Sharp (MRS) broth (Oxoid, Basingstoke, UK) supplemented with 20% (v/v) of glycerol, were grown in 12.5 mL of their respective broths at 37 °C for 24 h. Next, each microbial culture was mixed using a vortex, transferred to 250 mL of their respective fresh broths, and incubated at 37 °C for 24 h. Each final microbial culture (262.5 mL) was centrifuged (22,000*g* for 10 min) and washed using sterile saline solution (0.85% NaCl, w/v). This procedure was repeated twice to eliminate culture media residues and the *pellet* was inoculated into the different pasteurized soy mixtures (see Section 2.2), reaching a final inoculum of around 8 log CFU/mL.

2.2. Production of the fermented soy products

Three different batches of four formulations of fermented soy products (FSP) were prepared according to Bedani et al. (2014), with modifications. The production of FSP was performed in three steps: (1) mixture of ingredients and pasteurization of soy mixtures resulting in the pasteurized soy mixtures (PSM), (2) fermentation of PSM resulting in the fermented soy mixtures (FSM), and (3) addition of concentrated passion fruit juice to each fermented soy mixture and packaging of each FSP (FSP1, FSP2, FSP3, and FSP4). The ingredients and their respective quantities used to produce all FSP are presented in Table 1. The passion fruit (*Passiflora edulis* var. Flavicarpa) by-product was blanched, dried, processed to a fine powder (< 42 μm), and stored according to Albuquerque et al. (2016) until use. Fructooligosaccharides FOS P95® (Beneo, Orafti®, Oreye, Belgium) was used as prebiotic ingredient. Each soy mixture was prepared and pasteurized using the pilot-scale mixer Thermomix® (model TM31-127V, Vorwerk, Wollerau, Switzerland).

A volume of 1 L of an ultra-high temperature (UHT) treated commercial soy milk (Pura Soja, Mais Vita, Yoki®, Pouso Alegre, MG, Brasil)

Table 1
Ingredients used to produce the fermented soy products (FSP) with passion fruit flavour.

Ingredients (g/100 mL of commercial soy milk[a])	Soy mixture formulations[b]			
	SM1	SM2	SM3	SM4
Passion fruit by-product powder	–	1	–	0.5
FOS	–	–	1	0.5
Soy extract powder	2.5	2.5	2.5	2.5
Sugar	7.0	7.0	7.0	7.0
Dextrose monohydrate ST	1.0	1.0	1.0	1.0
Carrageenan gum	0.1	0.1	0.1	0.1

Ingredients (g/100 g of fermented soy mixture)	Fermented soy products (FSP)			
	FSP1	FSP2	FSP3	FSP4
Concentrated passion fruit juice	12.5	12.5	12.5	12.5

[a] Ultra-high temperature (UHT) treated commercial soy milk (Pura Soja, Mais Vita, Yoki®, Pouso Alegre, MG, Brasil); Water-soluble soy extract powder Mãe Terra (Mãe Terra®, São Paulo, Brazil); Sugar (Coopersucar-União, Limeira, SP, Brazil); dextrose monohydrate ST (Agargel Ind. e Com, Ltda, São Paulo, SP, Brazil); carrageenan gum (Agargel Ind. e Com. Ltda, São Paulo, SP, Brazil); concentrated passion fruit juice (Maguary, Araguari, MG, Brazil).

[b] Addition of the *pellets* containing viable cells of *St. thermophilus* TH-4 and *Lb. rhamnosus* LGG (Chr. Hansen, Hoersholm, Denmark).

was heated until 50 °C under agitation (500 rpm) when sugar (Coopersucar-União, Limeira, SP, Brazil) and dextrose monohydrate ST (Agargel Ind. E Com, Ltda., São Paulo, SP, Brazil) were added. After achieving 80 °C, soy extract powder (Mãe Terra®, São Paulo, Brazil) and carrageenan gum (Agargel Ind. e Com, Ltda) were added to the mixture. When the mixture achieved 90 °C, passion fruit by-product powder and/or FOS were added according to the soy mixture formulation (Table 1) and pasteurized for 5 min. The pasteurized soy mixture (PSM) was cooled at 37 °C using an ice bath. At this temperature, *St. thermophilus* TH-4 and *Lb. rhamnosus* LGG *pellets* (prepared using 262.5 mL of culture media with grown cultures according to Section 2.1) were added to each pasteurized soy mixture that achieved a final inoculum of around 8 log CFU/mL. Then, each PSM was transferred to two sterile flasks (500 mL) for fermentation at 37 °C using a water-bath equipment coupled to a CINAC system (*Cynetique d'acidification*, Ysebaert, Frépillon, France). The fermentation was performed until each PSM achieve pH 5.5 (T_f). Next, the fermented soy mixtures (FSM) were stored at 4 °C for 18 h approximately. Concentrated passion fruit juice (12.5% w/w) was added to the FSM and mixed using the pilot-scale mixer Thermomix® (model TM31-127V, Vorwerk). Aliquots of 35 g of each FSP were packaged in polypropylene plastic pots for food products (Tries Aditivos Plásticos, São Paulo, Brazil) and sealed with varnished metallic covers in a sealer (Delgo Nr. 1968, Delgo Metalúrgica, Cotia, Brazil). The pots containing the different FSP were stored at 4 °C for up to 28 days for analysis.

2.3. Kinetic parameters

Considering the fermentation of each FSP in the water-bath equipment coupled to a CINAC system (*Cynetique d'acidification*, Ysebaert), maximum acidification rate (V_{max}) was established as the time variation of pH (dpH/dT) and expressed as 10^{-3} pH units/min. Other kinetic parameters were also determined: time at which V_{max} was reached in each FSP (T_{max}) and time for each FSP to reach pH 5.5 (T_f). All kinetic parameters were calculated according to Oliveira et al. (2009).

2.4. Storage and sampling periods

The viability of *St. thermophilus* Th-4 and *Lb. rhamnosus* LGG and the pH values of each FSP were evaluated at days 1, 7, 14, 21, and 28 of storage at 4 °C. The folate concentration of each PSM (before fermentation) and FSM (after fermentation) was assessed. The folate content of each FSP was determined at days 1, 14, and 28 of storage (4 °C). The survival of *St. thermophilus* TH-4 and *Lb. rhamnosus* LGG and the bio-accessibility of folate during the *in vitro* simulated gastrointestinal conditions for each FSP were determined at days 1, 14, and 28 of storage (4 °C).

2.5. Viability of the microorganisms

In order to evaluate the viability of the microorganisms during the storage period of the fermented soy products and the survival of these microorganisms during simulated gastrointestinal conditions, *St. thermophilus* TH-4 and *Lb. rhamnosus* LGG were plate counted according to Albuquerque et al. (2017).

2.6. Determination of pH values and folate content

Triplicate FSP samples were used for the determination of pH (three different pots of the same batch, a total of 6 pots for each formulation), using a pH meter Orion (model Three Stars, Thermofisher Scientific, Waltham, MA, USA) equipped with a penetration electrode (model 2A04, Analyser, São Paulo, Brazil). Two batches were used for pH determination.

Folate content was determined using a microbiological assay with *Lb. casei* subsp. *rhamnosus* NCIMB 10463 as the indicator strain according to Albuquerque et al. (2017). Triplicates were used to determine the folate concentration of each sample. Values obtained from the standard curve were multiplied by the dilution factor to obtain the final folate concentrations expressed as ng/mL.

2.7. In vitro simulated gastrointestinal conditions

2.7.1. Survival of microorganisms and evaluation of folate bio-accessibility

The survival of *St. thermophilus* TH-4 and *Lb. rhamnosus* LGG during *in vitro* simulated gastrointestinal conditions were adapted from Liserre et al. (2007), according to procedures described by Buriti et al. (2010) and Bedani et al. (2013). Additionally, to evaluate the bio-accessibility of folate in the final product (0 h of assay) and from each gastrointestinal phase (gastric, enteric I, and enteric II, respectively, after 2, 4, and 6 h of assay), a sample (500 μL) was taken and processed according to Albuquerque et al. (2017), in order to determine the content of folate (according to Section 2.4) released from each FSP in each phase of the *in vitro* simulated gastrointestinal assay. The folate content of each enzymatic solution used during the simulated gastrointestinal assay was determined and subtracted from the final folate content obtained at each simulated gastrointestinal phase (data not shown).

Viable cell counts of *St. thermophilus* TH-4 and *Lb. rhamnosus* LGG were performed to determine the microorganisms' survival. Aliquots (1 mL) were collected from triplicate samples from each gastrointestinal phase, after 2 h, 4 h, and 6 h (for each time, three different flasks of each trial were used) and were pour-plated in specific culture media for each microorganism as described previously (Section 2.5). The adjustment of the dilutions was done properly and viable cell counts were expressed as log CFU/mL of FSP.

2.8. Statistical analyses

Three independent batches for each FSP were produced. The experiments were carried out in triplicates and results were presented as means ± standard deviations (SD). Minitab 17 Statistical Software® (MINITAB Inc., USA) using one-way ANOVA followed by a Tukey's post-hoc test was used for statistical analyses. Two different means were compared using the Student's *t*-test. Differences were considered as statistically significant for $p < 0.05$.

3. Results

3.1. Fermentation kinetics parameters

According to Table 2, the supplementation of soy mixtures with passion fruit by-product and/or FOS did not increase the maximum acidification rate (V_{max}); there was no significant difference between

Table 2
Maximum acidification rate (V_{max}), time at which V_{max} was reached (T_{max}), and time to reach pH 5.5 (T_f) during fermentation of different soy products supplemented with passion fruit by-products and/or fructooligosaccharides.

Formulation	V_{max} (10^{-3} pH units/min)	T_{max}	T_f (min)
FSP1	14.5 ± 0.2^{A}	57 ± 11^{A}	127 ± 11^{A}
FSP2	13.3 ± 0.1^{A}	73 ± 8^{A}	143 ± 20^{A}
FSP3	14.9 ± 0.2^{A}	63 ± 23^{A}	151 ± 19^{A}
FSP4	13.8 ± 0.1^{A}	75 ± 7^{A}	141 ± 18^{A}

FSP1: fermented soy product. FSP2: fermented soy product supplemented with 1% (w/v) of passion fruit by-product powder. FSP3: fermented soy product supplemented with 1% (w/v) of fructooligosaccharides. FSP4: fermented soy product supplemented with 0.5% (w/v) of passion fruit by-product powder and 0.5% (w/v) of fructooligosaccharides. Values are expressed as mean ± standard deviation. ^{A,B}Different superscript capital letters in the same column denote significant differences ($p < 0.05$).

Table 3
Viable cell counts of *St. thermophilus* TH-4 and *Lb. rhamnosus* LGG in different fermented soy products during storage at 4 °C for up to 28 days.

Microorganisms	Time (days)	Populations of microorganisms (log CFU/mL)			
		FSP1	FSP2	FSP3	FSP4
Streptococcus thermophilus TH-4	1	8.2 ± 0.3^{Aa}	8.7 ± 0.1^{Aa}	8.5 ± 0.4^{Aa}	8.5 ± 0.1^{Aa}
	7	8.2 ± 0.1^{Aa}	8.4 ± 0.5^{Aba}	8.2 ± 0.1^{Aba}	8.2 ± 0.2^{Aa}
	14	7.9 ± 0.2^{Aab}	8.0 ± 0.2^{Bab}	7.8 ± 0.0^{Bb}	8.5 ± 0.5^{Aa}
	21	6.6 ± 0.3^{Bb}	6.7 ± 0.1^{Cb}	6.7 ± 0.1^{Cb}	7.4 ± 0.3^{Ba}
	28	6.4 ± 0.3^{Ba}	5.5 ± 0.2^{Db}	5.9 ± 0.0^{Db}	6.7 ± 0.3^{Ca}
Lactobacillus rhamnosus LGG	1	8.6 ± 0.2^{Aa}	8.4 ± 0.3^{Aa}	8.4 ± 0.2^{Aa}	8.6 ± 0.4^{Aa}
	7	8.5 ± 0.3^{Aa}	8.6 ± 0.2^{Aa}	8.4 ± 0.2^{Aa}	8.6 ± 0.1^{Aa}
	14	8.7 ± 0.1^{Aa}	8.6 ± 0.2^{Aa}	8.2 ± 0.1^{ABb}	8.3 ± 0.2^{Ab}
	21	8.7 ± 0.1^{Aa}	8.7 ± 0.2^{Aa}	7.7 ± 0.2^{Bc}	8.3 ± 0.2^{Ab}
	28	8.6 ± 0.1^{Aa}	8.4 ± 0.3^{Aa}	8.3 ± 0.3^{Aa}	8.2 ± 0.3^{Aa}

FSP1: fermented soy product. FSP2: fermented soy product supplemented with 1% (w/v) of passion fruit by-product powder. FSP3: fermented soy product supplemented with 1% (w/v) of fructooligosaccharides. FSP4: fermented soy product supplemented with 0.5% (w/v) of passion fruit by-product powder and 0.5% (w/v) of fructooligosaccharides. Values are expressed as mean ± standard deviation. ^{A,B}Different superscript capital letters in a column denote significant differences for each microorganism during different storage periods ($p < 0.05$). ^{a,b}Different superscript lowercase letters in a row denote significant differences between formulations ($p < 0.05$).

the four FSM ($p < 0.05$) at the end of the fermentation period. No significant difference ($p < 0.05$) was observed between the time that each PSM achieved V_{max} (T_{max}) or the time that each PSM reached pH 5.5 (T_f) when the fermentation was stopped. According to Brazilian microbiological parameters for food (ANVISA, 2001), all FSP presented satisfactory microbiological conditions indicating that the production of the fermented soy products was carried out under satisfactory hygiene conditions (data not shown).

3.2. pH values and viability of microorganisms in fermented soy products during storage

Before fermentation, the pH of each pasteurized soy mixture was determined (PSM1, 6.95 ± 0.13; PSM2, 6.95 ± 0.19; PSM3, 7.25 ± 0.03; PSM4, 7.02 ± 0.07). At the end of storage (day 28), all FSP presented a slight pH decrease that was statistically different from the initial pH at day 1 ($p < 0.05$) (data not shown). The final pH values ranged from 4.6 to 4.4 except for FSP4 (from 4.6 to 4.5), which was slightly higher and significant different ($p < 0.05$) from the other FSP samples at day 28. A slight post-acidification during storage was observed.

The viability of *St. thermophilus* TH-4 and *Lb. rhamnosus* LGG, present in each FSP, during the storage period evaluated is presented in Table 3. At day 1, both microorganisms presented counts above 8 log CFU/mL, very similar to the initial inoculum used for each PSM before fermentation. Along the storage period evaluated, a decrease in the *St. thermophilus* populations was observed for all FSP, particularly after 21 days of storage. FSP4 presented the highest *St. thermophilus* populations during storage ($p < 0.05$). *Lb. rhamnosus* LGG was viable during the entire period with counts always above 8 log CFU/mL for all FSP.

3.3. Folate content and its stability in fermented soy products during storage

Before fermentation, the pasteurized soy mixture with FOS (PSM3) presented the highest folate content (227 ± 0 ng/mL), followed by the pasteurized soy mixture with passion fruit by-product (PSM2, 180 ± 8 ng/mL), pasteurized soy mixture (PSM1, 169 ± 4 ng/mL), and pasteurized soy mixture with passion fruit by-product and FOS (PSM4, 160 ± 7 ng/mL) (Fig. 1). At the end of the fermentation of the four pasteurized soy mixtures (at pH 5.5), the fermented soy mixture with both passion fruit by-product and FOS (FSM4) presented the highest folate content (617 ± 58 ng/mL) and was significant different ($p < 0.05$) from FSM3 (376 ± 10 ng/mL), FSM1 (333 ± 23 ng/mL), and FSM2 (317 ± 30 ng/mL). The folate content of the last three fermented soy mixtures was not statistically different ($p > 0.05$).

In general, during the storage period (days 1, 14, and 28), the folate content of all FSP decreased (Fig. 1). After mixing the fermented soy mixtures with concentrated passion fruit juice, the folate content of all FSP was determined in the following day (day 1) and FSP4 presented

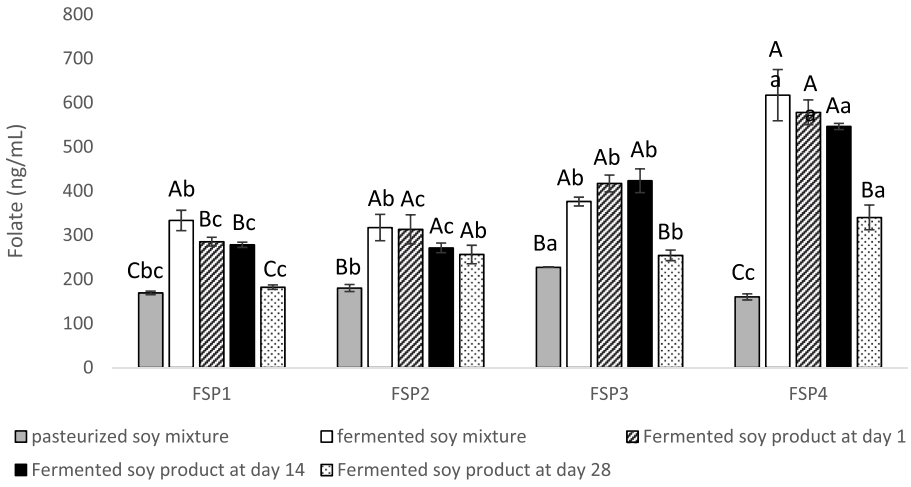

Fig. 1. Folate concentrations in fermented soy products during production and shelf-life at 4 °C. FSP1: fermented soy product. FSP2: fermented soy product supplemented with 1% (w/v) of passion fruit by-product powder. FSP3: fermented soy product supplemented with 1% (w/v) of fructooligosaccharides. FSP4: fermented soy product supplemented with 0.5% (w/v) of passion fruit by-product powder and 0.5% (w/v) of fructooligosaccharides. ^{A,B}Different capital letters denote significant differences between the folate content among the different times by each fermented soy product ($p < 0.05$). ^{a,b}Different small letters denote significant differences between all fermented soy products considering each time of analysis ($p < 0.05$).

Table 4
Comparison of changes (from pasteurized soy mixture to fermented soy mixture and from pasteurized soy mixture to days 14 and 28) in the folate content produced by the co-culture (*St. thermophilus* Th-4 and *Lb. rhamnosus* LGG) during preparation and storage of different fermented soy products.

Soy mixture (PSM or FSM) or fermented soy product (FSP)	ΔFolate (ng/mL)* $Folate_{FSM} - Folate_{PSM}$	ΔFolate (ng/mL)** $Folate_{FSP\ D14} - Folate_{PSM}$	ΔFolate (ng/mL)*** $Folate_{FSP\ D28} - Folate_{PSM}$
F1	152 ± 4^{Ba}	110 ± 4^{Cb}	13 ± 4^{Cc}
F2	141 ± 8^{Ba}	92 ± 8^{Cb}	74 ± 8^{Bb}
F3	149 ± 0^{Bb}	196 ± 0^{Ba}	27 ± 0^{Cc}
F4	458 ± 7^{Aa}	386 ± 7^{Ab}	180 ± 7^{Ac}

FSM: fermented soy mixture; PSM: pasteurized soy mixture; FSP D14: fermented soy product at day 14 of storage; FSP D28: fermented soy product at day 28 of storage. F1: soy mixture or fermented soy product (control); F2: soy mixture or fermented soy product supplemented with 1% (w/v) of passion fruit by-product; F3: soy mixture or fermented soy product supplemented with 1% (w/v) of fructooligosaccharides; F4: soy mixture or fermented soy product supplemented with 0.5% (w/v) of passion fruit by-product and 0.5% (w/v) of fructooligosaccharides. Values are expressed as mean ± standard deviation. ^{A,B}Different superscript capital letters in the same column denote significant differences ($p < 0.05$). ^{a,b}Different superscript lowercase letters in the same line denote significant differences ($p < 0.05$).

* ΔFolate = $Folate_{FSM}$ (ng/mL) − $Folate_{PSM}$ (ng/mL), the net amount of folate produced during fermentation.

** ΔFolate = $Folate_{FSP\ D14}$ (ng/mL) − $Folate_{PSM}$ (ng/mL), the net amount of folate until day 14 of FSP storage.

*** ΔFolate = $Folate_{FSP\ D28}$ (ng/mL) − $Folate_{PSM}$ (ng/mL), the net amount of folate until day 28 of FSP storage.

the highest folate content (578 ± 28 ng/mL), followed by FSP3 (417 ± 19 ng/mL), FSP2 (313 ± 33 ng/mL) and FSP1 (285 ± 10 ng/mL). At day 14, although a significant decrease in the folate content of FSP4 was observed when compared to day 1 ($p < 0.05$), this FSP still presented the highest level of the vitamin (547 ± 7 ng/mL). For this period, the lowest folate content was observed for FSP1 (278 ± 6 ng/mL) and FSP2 (271 ± 11 ng/mL). FSP2 showed no significant ($p < 0.05$) difference on its folate content from day 1 to day 28. At the end of storage (day 28), the folate content obtained for FSP4 (340 ± 28 ng/mL) and FSP2 (256 ± 21 ng/mL) was significantly higher ($p < 0.05$) than that presented by their respective unfermented pasteurized soy mixtures. In contrast, at day 28, FSP3 (254 ± 12 ng/mL) and FSP1 (182 ± 5 ng/mL) were not statistically different when their folate concentrations were compared to their respective pasteurized soy mixtures, before fermentation. All FSP presented higher folate content at days 1 and 14 of storage when compared to their respective pasteurized soy mixtures ($p < 0.05$).

Aiming to evaluate which fermented soy product presented the highest amounts of folate after fermentation by the co-culture TH-4 + LGG, we presented the net amounts of folate (comparison of changes in the folate values) in Table 4 where the amount of folate produced during the fermentation process of the pasteurized soy mixtures is given and shows vitamin production by the microorganisms in co-culture. In addition, the comparison of changes in the folate content of the fermented soy products at day 1 and their respective unfermented pasteurized soy mixtures showed no significant difference from the net folate values presented by the comparison of changes of the soy mixtures after fermentation (FSM) and their respective pasteurized soy mixtures [*ΔFolate = $Folate_{FSM}$ (ng/mL) − $Folate_{PSM}$ (ng/mL)] ($p > 0.05$). According to Table 4, the net folate content after fermentation of PSM4 and during its entire storage period was statistically higher than for the other FSPs for the same periods of analysis ($p < 0.05$). Depending on the FSP, the folate content may increase approximately 3.5-fold when compared to the unfermented pasteurized soy mixtures and FSP4 presented the highest bio-enrichment with the highest increase in the folate content ($p < 0.05$).

3.4. Survival of microorganisms during in vitro simulated gastrointestinal conditions

The survival of *Lb. rhamnosus* LGG during *in vitro* simulated gastrointestinal conditions was evaluated at days 1, 14, and 28 of storage (4 °C) and the results are presented in Fig. 2. In general, the highest reductions for all FSP were observed during the gastric and the enteric I phase, followed by an increase in the recovery of *Lb. rhamnosus* LGG in the end of the simulated digestion (after the enteric II phase). At days 1, 14, and 28, the highest recovery of *Lb. rhamnosus* LGG was observed for FSP3, FSP1, and FSP2, respectively ($p < 0.05$).

It was not possible to detect any viable cells of *St. thermophilus* TH-4 during the *in vitro* gastrointestinal assay. The same result was observed when a fermented milk supplemented with barley and containing *St. thermophilus* TH-4 was evaluated for the same gastrointestinal conditions (data not shown). Considering the entire period of analysis, different populations of *Lb. rhamnosus* LGG were recovered in the end of the *in vitro* simulated gastrointestinal digestion depending on the FSP formulation (Table 5). At day 1, a slight decrease in the lactobacilli population from FSP3 (a reduction of 0.7 ± 0.4 log CFU/mL) was observed from the time 0 h to 6 h of digestion, whereas for FSP4, the reduction in the lactobacilli population (4.6 ± 0.3 log CFU/mL) was the highest one. There was no significant difference regarding the reduction of *Lb. rhamnosus* LGG at the end of the *in vitro* gastrointestinal assay between FSP1 (2.3 ± 0.5 log CFU/mL) and FSP2 (1.7 ± 0.6 log CFU/mL) for this period (day 1). At day 14, FSP1 presented the lowest reduction in the lactobacilli population (1.2 ± 0.8 log CFU/mL) when compared to the other FSP. There was no significant difference among the reductions of *Lb. rhamnosus* LGG at the end of the *in vitro* gastrointestinal assay for the other FSP (FSP2, FSP3, and FSP4) on day 14. Regarding day 28, FSP2 presented the lowest reduction in the lactobacilli population (0.5 ± 0.6 log CFU/mL) in the end of the simulated gastrointestinal conditions. For this period, the highest lactobacilli population reduction was observed for FSP4.

3.5. Determination of folate bio-accessibility during simulated gastrointestinal conditions

The results regarding the amount of folate released from each FSP in the products before treatment and in each gastrointestinal phase (gastric, enteric I, and enteric II) of the *in vitro* gastrointestinal assay are presented in Fig. 3. In general, there was an increase in the folate content during the simulated gastrointestinal phases considering all FSP formulations during the entire storage period. The only exception was FSP1 at day 1, which did not show any significant difference for the folate content at the end of the *in vitro* assay. At day 1, the release of folate was higher for FSP3 in all *in vitro* gastrointestinal phases, while at day 14, FSP1 presented the highest contents of released folates in all simulated gastrointestinal phases when compared to the other FSP. At day 28, both FSP1 and FSP3 presented a similar concentration of folate at each gastrointestinal phase.

Table 6 presents the net values of folate regarding the enteric I and enteric II phases of the *in vitro* gastrointestinal assay for all FSP formulations during the entire period of analysis. All FSP presented a high folate release at the enteric I and enteric II phases during storage, except for FSP1 during the enteric I phase at day 1 and FSP1 and FSP4 at the enteric II phase at day 1. The highest amounts of folate were released by FSP1 at day 14, followed by the same FSP1 and FSP3 at day 28 for each enteric I and enteric II phases. Depending on the formulation of FSP, the folate released during the *in vitro* simulated

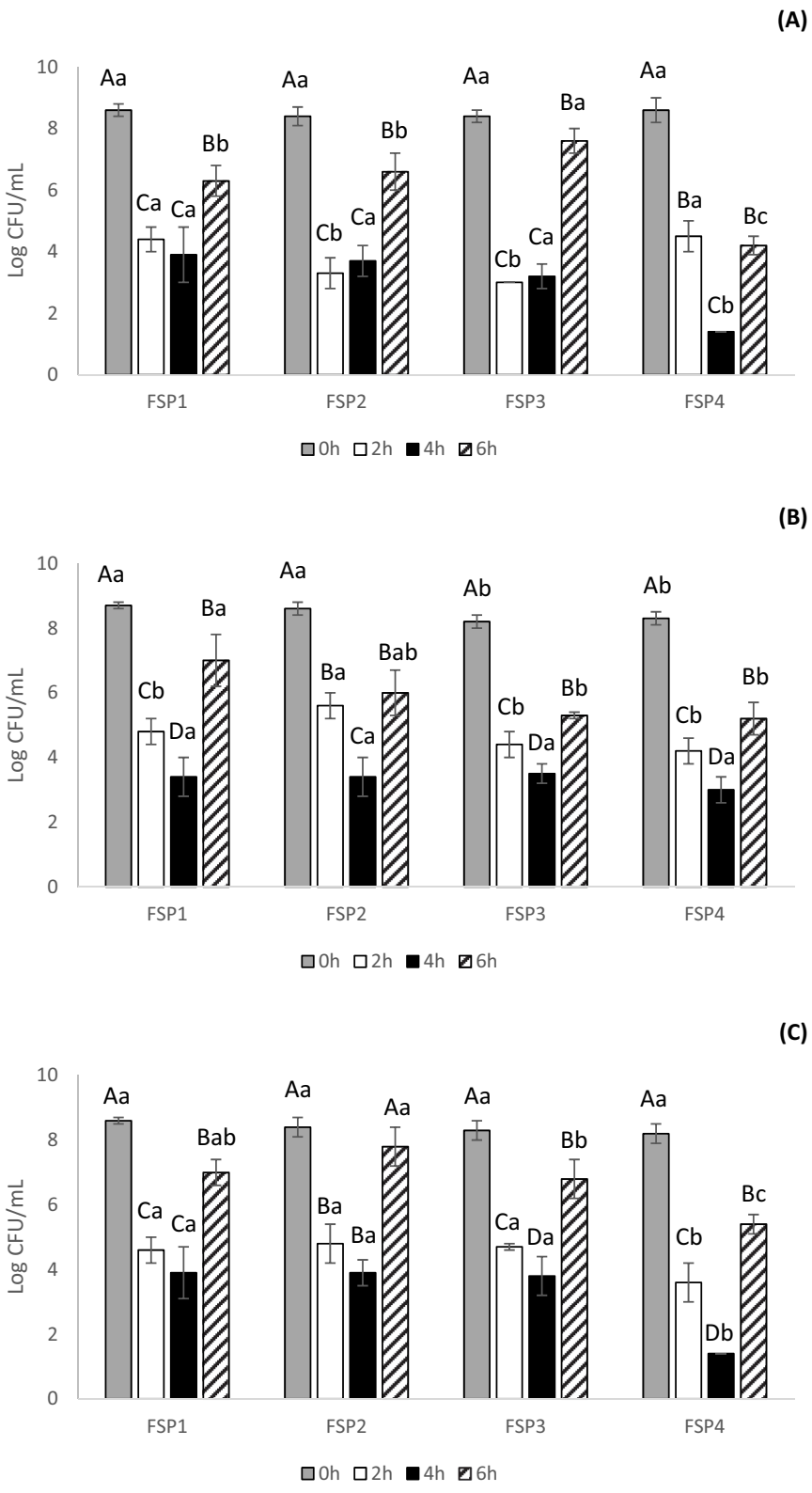

Fig. 2. Survival of *Lactobacillus rhamnosus* LGG (log CFU/mL) in fermented soy products submitted to *in vitro* simulated gastrointestinal conditions during storage at 4 °C for 1, 14, and 28 days (A, B, and C, respectively). FSP1: fermented soy product (FSP, control). F2: fermented soy product supplemented with 1% (w/v) of passion fruit by-product powder. FSP3: fermented soy product supplemented with 1% (w/v) of fructooligosaccharides. FSP4: fermented soy product supplemented with 0.5% (w/v) of passion fruit by-product powder and 0.5% (w/v) of fructooligosaccharides. 0 h: lactobacilli populations before the *in vitro* simulated digestion. 2 h: *in vitro* simulated gastric condition. 4 h: *in vitro* simulated enteric I condition. 6 h: *in vitro* simulated enteric II condition. ^{A,B}Different capital letters denote significant differences between different sampling periods (0, 2, 4, and 6 h) of the *in vitro* assay for the same fermented soy product ($p < 0.05$). ^{a,b}Different small letters denote significant differences between the populations of lactobacilli between different fermented soy products for each gastrointestinal phase ($p < 0.05$).

gastrointestinal conditions increased the bio-accessibility of the vitamin around 3–4 fold considering enteric I and enteric II phases.

4. Discussion

The use of fruit by-products with prebiotic potential to stimulate LAB growth and their beneficial functions, such as vitamin production, has been described previously (Albuquerque et al., 2016; Espírito Santo et al., 2012; Vieira et al., 2017). Supplementation of soy milk with fruit by-products or commercial prebiotics provides additional carbohydrates content as energy sources, which may result in an increased metabolic activity of starter cultures, such as *Streptococcus thermophilus* strains, and probiotic microorganisms. Previously, passion fruit by-product and FOS were used to stimulate the growth of different strains

Table 5
Comparison of changes [$\Delta_{LGG\ (logcfu/mL)}$] in the *Lactobacillus rhamnosus* LGG populations of each fermented soy product during 6 h of the *in vitro* simulated gastrointestinal conditions.

Formulations	$\Delta_{LGG\ (logcfu/mL)}$ = T6 $h_{LGG\ (logcfu/mL)}$ − T0 $h_{LGG\ (logcfu/mL)}$		
	Day 1	Day 14	Day 28
FSP1	2.3 ± 0.5^{Ab}	1.2 ± 0.8^{Ab}	1.4 ± 0.4^{Ab}
FSP2	1.7 ± 0.6^{Ab}	2.6 ± 0.8^{Aab}	0.5 ± 0.6^{Bc}
FSP3	0.7 ± 0.4^{Bc}	2.9 ± 0.1^{Aa}	1.5 ± 0.6^{Bbc}
FSP4	4.6 ± 0.3^{Aa}	3.0 ± 0.57^{Ba}	2.9 ± 0.38^{Ba}

FSP1: fermented soy product; FSP2: fermented soy product supplemented with 1% (w/v) of passion fruit by-product; FSP3: fermented soy product supplemented with 1% (w/v) of fructooligosaccharides; FSP4: fermented soy product supplemented with 0.5% (w/v) of passion fruit by-product and 0.5% (w/v) of fructooligosaccharides. Values are expressed as mean ± standard deviation. ^{a,b}Different superscript capital letters in the same column denote significant differences ($p < 0.05$). ^{A,B}Different superscript lowercase letters in the same line denote significant differences ($p < 0.05$).

of *St. thermophilus* and *Lactobacillus* spp. and the folate production by these microorganisms during soy milk fermentation to develop bio-enriched fermented soy-based beverages (Albuquerque et al., 2017).

In the present study, four different fermented soy products, similar to yoghurts, were prepared using the starter *Streptococcus thermophilus* TH-4 and the probiotic *Lactobacillus rhamnosus* LGG in co-culture to produce bio-enriched probiotic fermented soy products with natural folates. This microbial combination was chosen (among several strains and combination of strains previously tested) due to the production of the highest amount of folate in fermented soy-based beverages, especially when supplemented with passion fruit by-product and with the combination of passion fruit by-product and FOS (Albuquerque et al., 2017). Previously, it was observed that different strains of lactic acid bacteria, including *Streptococcus thermophilus* TH-4 and *Lb. rhamnosus* LGG, were able to produce folate during the fermentation of a modified MRS broth supplemented with different fruit by-products (Albuquerque et al., 2016). In addition, it was shown that *Streptococcus thermophilus* strains were also able to produce large amounts of folate during 24 h of fermentation of soy-based beverages supplemented with passion fruit by-product and/or fructooligosaccharides, especially when in co-culture with probiotic lactobacilli strains (Albuquerque et al., 2017). These studies showed that the folate production by the tested strains (individually or when in co-culture), including the *Streptococcus thermophilus* strains, was strain-dependent and also influenced by the kind of substrates used during the fermentation process. In this study, the substrates available in each FSP probably influenced the production of folate by the co-culture TH-4 + LGG, especially when both passion fruit by-product and FOS were employed which confirm the results observed by Albuquerque et al. (2017).

Slight variations in pH values of each FSP were observed during 28 days of storage. This may be due to the metabolic activity of the microorganisms during storage as well as to the low buffering capacity of the soy matrix (Champagne et al., 2009; Farnworth et al., 2007), especially between the end of the fermentation of the pasteurized soy mixture (pH 5.5) and the day 1 of storage (pH 4.6). No significant differences were observed among the maximum acidification rates, the time needed for each FSP to achieve the maximum acidification rate or the time needed to reach pH 5.5 among all FSP. Therefore, the use of passion fruit by-products and/or FOS did not stimulate the growth or the metabolism of *St. thermophilus* TH-4 and *Lb. rhamnosus* LGG during fermentation. According to Farnworth et al. (2007), the fast drop in the pH values during fermentation may lead to a negative impact on the microorganism growth, especially for *St. thermophilus*. Both *St. thermophilus* TH-4 and *Lb. rhamnosus* LGG achieved counts above 8 log CFU/mL at day 1 of storage of all FSP. These counts are very similar to the initial inoculum used to prepare the FSPs. The profile and amounts of soy milk carbohydrates may have influenced the growth and the metabolism of lactic acid bacteria. According to Champagne et al. (2009), soy milk presents a high sucrose content, which may contribute to the *St. thermophilus* strains growth. This is in agreement with the results observed by Albuquerque et al. (2017). Nevertheless, considering that sucrose, glucose, and fructose are widely consumed by *St. thermophilus* and lactobacilli strains, the lack of these carbohydrates in all FSP along storage probably contributed to the decrease in the *St. thermophilus* TH-4 populations. Regarding *Lb. rhamnosus* LGG, this strain presented a great ability to grow in co-culture with *St. thermophilus* strains during the fermentation of different soy-based beverages probably due to a microbial symbiosis (Albuquerque et al., 2017; Champagne et al., 2009). Besides, the folate content produced during fermentation of each FSP probably contributed to keep the viability of *Lb. rhamnosus* LGG during the entire period of storage once lactobacilli strains are known as being folate consumers (Albuquerque et al., 2016).

Bio-enrichment of fermented products with natural folate produced by beneficial microorganisms have been described as an alternative to the mandatory fortification programs employed by some countries using synthetic nutrients, such as synthetic vitamins, to fortify foods (Albuquerque et al., 2017; Espírito-Santo et al., 2015; Laiño et al., 2013). According to Albuquerque et al. (2017), *St. thermophilus* TH-4 and *Lb. rhamnosus* LGG were able to produce large amounts of folate during fermentation of soy-based beverages and that passion fruit by-product and FOS stimulated this production, especially when both microorganisms were grown in co-culture. In the present study, the folate contents produced during fermentation of all FSP were widely lower when compared to those produced by the same co-culture in fermented soy-based beverages (Albuquerque et al., 2017). Although the supplementation with passion fruit by-product and FOS has also contributed to increase the folate content of FSP, the lower folate production may be explained by the time of fermentation [no longer than 2 h and 30 min in the present study compared to 24 h described by Albuquerque et al., 2017]. Laiño et al. (2013) observed that the maximum production of folate by different mixes of dairy yoghurt starter cultures occurs between 6 and 8 h of fermentation, the vitamin production was strain-dependent, and that the food matrix may affect folate production. Also, Padalino et al. (2012) observed that, even though the addition of prebiotics may contribute to the growth of beneficial microorganisms resulting in an increase in the bacterial growth rates during milk fermentation, it may result in a reduction in the folate production by these microorganisms. This fact may also be related to the fast drop in the pH values due to the lower buffering capacity of soy proteins during fermentation of soymilks as observed by Farnworth et al. (2007). These authors suggest that the greater and faster production of organic acids by the microorganisms may decrease the growth of *St. thermophilus* when compared to fermented milks due to the fast drop in pH of soy products during fermentation.

Although Padalino et al. (2012) and Oliveira et al. (2009) observed that the use of FOS might increase the acidification rate and stimulate the LAB growth, the same result was not verified in this study once nor FOS or even passion fruit by-product significantly increased the acidification rate or significantly decreased the time of fermentation of each FSP to reach the final pH 5.5 compared to the FSP without supplementation. This fact could be explained by Kaplan and Hutkins (2000). The authors observed that *St. thermophilus* strains and *Lb. rhamnosus* GG were not able to ferment FOS in MRS broth supplemented with this prebiotic, reinforcing the use of soy components during fermentation of FSP with these microorganisms, especially in co-culture. Albuquerque et al. (2017) observed that even after 24 h of soy-based beverages fermentation, when in single cultures, *St. thermophilus* TH-4 and *Lb. rhamnosus* LGG presented a low acidification profile.

The decrease in folate content of each FSP during the storage period of up to 28 days was probably due to the consumption of the vitamin by *Lb. rhamnosus* LGG. This consumption probably kept the lactobacilli

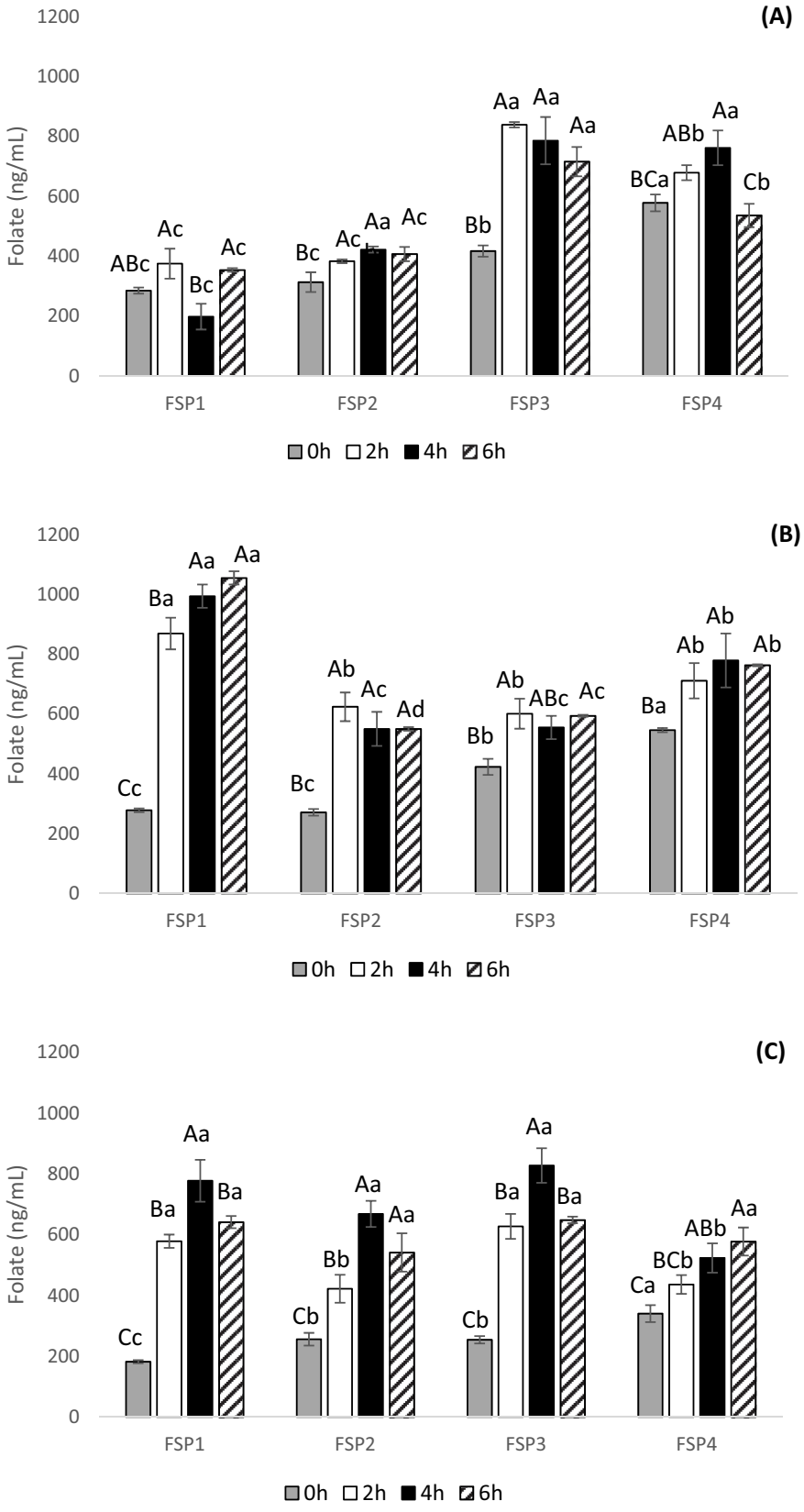

Fig. 3. Folate concentration in fermented soy products submitted to *in vitro* simulated gastrointestinal conditions during storage at 4 °C for 1, 14, and 28 days (A, B, and C, respectively). FSP1: fermented soy product. FSP2: fermented soy product supplemented with 1% (w/v) of passion fruit by-product powder. FSP3: fermented soy product supplemented with 1% (w/v) of fructooligosaccharides. FSP4: fermented soy product supplemented with 0.5% (w/v) of passion fruit by-product powder and 0.5% (w/v) of fructooligosaccharides. 0 h: folate content before the *in vitro* simulated digestion. 2 h: folate content at the end of the *in vitro* simulated gastric condition. 4 h: folate content at the end of the *in vitro* simulated enteric I condition. 6 h: folate content at the end of the *in vitro* simulated enteric II condition. ^{A,B}Different capital letters denote significant differences between the folate content among the different gastrointestinal condition by each fermented soy product ($p < 0.05$). ^{a,b}Different small letters denote significant differences between the folate content between different fermented soy products for each gastrointestinal phase ($p < 0.05$).

LGG viable and presenting counts above 8 log CFU/mL until the end of the storage period. This finding is in agreement with the current opinion of official organizations and agencies like the Health Canada and the Italian Ministry of Health, that suggest a level of 10^9 CFU of probiotic per serving food (daily portion) to consider the general claim of supporting a healthy gut microbiota (Hill et al., 2014). In addition, Ouwehand et al. (2018) stated that the effect of probiotics, such as *Lb. rhamnosus* LGG, is dose dependent and that the use of single or multi strains may also impact on the probiotics health benefits.

Another possibility to explain the decrease in the folate content of

Table 6
Comparison of changes in the folate content of each fermented soy product at the *in vitro* simulated small and large intestine conditions (enteric I and enteric II phases from the *in vitro* simulated gastrointestinal assay) at days 1, 14, and 28 of storage.

Time	FSP1	FSP2	FSP3	FSP4
ΔFolate (ng/mL) after 2 h (enteric I phase)*				
Day 1	−108 ± 47^{Bc}	109 ± 10^{Bb}	386 ± 79^{Ba}	144 ± 58^{Ab}
Day 14	716 ± 39^{Aa}	311 ± 57^{Ab}	119 ± 39^{Cc}	235 ± 90^{Abc}
Day 28	595 ± 69^{Aa}	413 ± 43^{Ab}	571 ± 57^{Aab}	149 ± 48^{Ac}
*Δ Folate (ng/mL)** after 4 h (Enteric II phase)*				
Day 1	−198 ± 7^{Cd}	104 ± 14^{Bb}	281 ± 49^{Ba}	−61 ± 39^{Bc}
Day 14	780 ± 22^{Aa}	281 ± 6^{Ab}	172 ± 3^{Cd}	219 ± 3^{Ac}
Day 28	457 ± 20^{Ba}	318 ± 63^{Abc}	386 ± 11^{Aab}	238 ± 46^{Ac}

T0: fermented soy product at days 1, 14, or 28, before the *in vitro* simulated gastrointestinal assay. FSP1: fermented soy product. FSP2: fermented soy product supplemented with 1% (w/v) of passion fruit by-product powder. FSP3: fermented soy product supplemented with 1% (w/v) of fructooligosaccharides. FSP4: fermented soy product supplemented with 0.5% (w/v) of passion fruit by-product powder and 0.5% (w/v) of fructooligosaccharides. Values are expressed as mean ± standard deviation. ^{A,B}Within a column, different superscript capital letters denote significant differences between the folate content of different days of storage for each fermented soy product at the same enteric phase ($p < 0.05$). ^{a,b}Within a row, different superscript lowercase letters denote significant differences between different fermented soy products for each day of storage at the same enteric phase ($p < 0.05$).

* ΔFolate (ng/mL) = Folate Enteric I (ng/mL) − Folate T0 (ng/mL).

** ΔFolate (ng/mL) = Folate Enteric II (ng/mL) − Folate T0 (ng/mL).

FSP during storage may be attributed to the negative relationship between low pH of the FSP and the microbial production of folate as described previously (Albuquerque et al., 2017; Padalino et al., 2012; Sybesma et al., 2003). Even considering the decrease in the folate content during storage, the folate levels of FSP2 and FSP4 at day 28 were higher than those presented by their respective pasteurized unfermented soy mixtures (PSM). Additionally, the period of storage could be reduced to 14 days, since all FSP would present higher folate amounts. Therefore, the folate content of all FSP (especially at days 1 and 14) confers a *status* of bio-enriched fermented soy products to these food products by increasing > 10% of the vitamin compared to the initial concentration of the vitamin (before fermentation) for each FSP. At day 14, FSP4 presented an increase of approximately 3.5-fold compared to the initial vitamin content presented by its respective pasteurized soy mixture. In addition to the beneficial health effects promoted by the intake of FSP (Bedani et al., 2014) and by the consumption of the probiotic strain *Lb. rhamnosus* LGG, all formulations of FSP produced in the present study would be able to deliver natural folates to the consumers, leading to a new and innovative functional product, source of natural folates and other beneficial nutrients which could improve nutritional and human health effects.

Lb. rhamnosus LGG is known for its health benefits by relieving and preventing antibiotic-associated diarrhoea, childhood infections and allergies (Fong et al., 2015). The use of *in vitro* simulated gastrointestinal conditions to evaluate the survival of probiotic strains is considered as an important trait to investigate the potential of these microorganisms to achieve the intestinal environment alive and lead to beneficial health effects (Bedani et al., 2013; Matias et al., 2016). In addition, the survival of microorganisms during simulated gastrointestinal conditions may be strongly influenced by the food matrix (Costa et al., 2017).

Bedani et al. (2014) investigated the impact of fermented soy products supplemented with okara (a soybean by-product) and the prebiotic fibre inulin on probiotics survival during similar *in vitro* simulated gastrointestinal conditions. The authors observed that, although okara and inulin did not contribute to the microorganism's survival during storage, the fermented soy product could be important to protect probiotics against gastrointestinal juices during *in vitro* digestion. In our study, no protective effect of passion fruit by-product or FOS on the microorganism's survival was observed during simulated gastrointestinal conditions. The only exception was FSP supplemented with both passion fruit by-product and FOS (FSP4), which promoted a higher decrease in the survival of *Lb. rhamnosus* LGG during simulated gastrointestinal conditions assay compared to the control (FSP1). Therefore, it is possible to suggest that the FSP produced in this study may contribute to keep the survival of *Lb. rhamnosus* LGG during the *in vitro* digestion, which is in agreement with Bedani et al. (2014).

In this study, the survival of *Lactobacillus rhamnosus* LGG as pure culture without being incorporated in a food matrix was not evaluated; however, this was performed previously (Costa et al., 2017). In this previous study, it was shown that the surviving population of LGG as pure culture after the simulated gastrointestinal assay was of 2.86 log CFU/mL. This meant a reduction of 6.3 log CFU/mL, considering the initial population of 9.2 log CFU/mL of the microorganism in the culture media employed. In this current study, a reduction of LGG populations that ranged from 0.5 ± 0.6 log CFU/mL (FSP2 at day 28) to 4.58 ± 0.3 log CFU/mL (FSP4 at day 1) was observed. In this case, the bio-enriched FSPs tested were able to improve the survival of the LGG strain during the *in vitro* simulated gastrointestinal conditions. These results are in line with Bedani et al. (2013), who also observed that fermented soy-based food matrix could improve the survival of lactobacilli strains when compared to their fresh cultures.

Regarding the survival of *St. thermophilus* TH-4, no viable cell was recovered in any gastrointestinal phase, which might be related to the lysis of cells during the gastrointestinal simulation or due to the technique used to determine its viability. Concomitant to the determination of *St. thermophilus* TH-4 and *Lb. rhamnosus* LGG survival, the bio-accessibility of folate during *in vitro* digestion was evaluated. Considering that some *St. thermophilus* strains are great folate producers (Albuquerque et al., 2016; Laiño et al., 2013), and that soy-based products stimulate folate production by *St. thermophilus* TH-4 during fermentation (Albuquerque et al., 2017), the intracellular folate content of *St. thermophilus* TH-4 probably contributed to increase the folate concentration and its bio-accessibility during the *in vitro* digestion of all FSP. Additionally, the production of intracellular folate by *St. thermophilus* strains was observed by Laiño et al. (2012) and Pacheco da Silva et al. (2016) which supports the hypothesis of release of intracellular folate by the lysis of *St. thermophilus* cells.

In general, all FSP increased the bio-accessibility of folate during the *in vitro* gastrointestinal conditions. According to Mo et al. (2013), *in vitro* simulated gastrointestinal models are an efficient alternative to measure bio-accessibility of nutrients and are therefore representative for *in vivo* bioavailability in humans. Although there it a lack of information regarding the bio-accessibility of folate, this study contributes to the knowledge regarding the use of the bio-enriched fermented soy products as potential functional foods aiming to improve the folate status in vitamin deficient individuals.

Therefore, in our study, although FSP4 presented an important increase on the folate content during the gastrointestinal phases contributing to increase the vitamin bio-accessibility (day 1), the folate released during the *in vitro* digestion of FSP1 at day 14 was higher. This fact could be explained by the use of carbohydrate sources, such as passion fruit by-product and FOS, to supplement FSP, which could act as folate binders entrapping free forms of folate. In contrast, FSP without carbohydrate supplementation (FSP1) might have contributed to increase the folate content during the *in vitro* digestion once its carbohydrate content is probably lower when compared to the other supplemented FSP and probably decreases the entrapping effect of these ingredients (Arkbåge et al., 2003).

Arkbåge et al. (2003) investigated the supplementation of yoghurt and pasteurized milk with folate-binding proteins and observed that the addition of these components decreased the bio-accessibility of folic acid and (6*S*)-5-methyl-tetrahydrofolate by entrapping the vitamin

during the *in vitro* digestion. Previously, a tri-enzymatic treatment was used to release folates from carbohydrates and proteins present in fermented soy-based beverages supplemented with passion fruit by-products and/or FOS (Albuquerque et al., 2017). Some forms of folate were reported as nonstable to the tri-enzyme method, probably because of the pH of the solutions of enzymes, to the heat treatment, and/or the food matrix (Albuquerque et al., 2017; Patring et al., 2005). In the current study, the simulated digestion assay was modified to confer a more realistic metabolic condition and was shown to improve the folate bio-accessibility of the fermented soy products.

In our study, it is important to highlight the increase in the folate bio-accessibility during the enteric I phase, which simulates the small intestine where the folate absorption occurs (Visentin et al., 2014). Although the folate content of all FSP was observed to decrease during storage, probably by the vitamin consumption by *Lb. rhamnosus* LGG, during the *in vitro* digestion the folate content increased significantly from the initial folate content (0 h) observed at day 1, 14, and 28 of storage in the *in vitro* assay. Therefore, if the concentration of folate of each FSP before digestion is considered, an increase of at least 3.5-fold was observed, depending on the FSP tested regarding the folate content for day 14 of storage. In case the bio-accessibility of folate is considered, depending on the digested FSP, an increase of 1.3 to 3.6-fold in the folate content at the enteric I phase was observed, which could improve the folate content to be absorbed in the small intestine. Comparing the enteric I phase of FSP1 and FSP4 with the folate content of their unfermented soy mixtures, the increase in the folate content was approximately 5.9 and 4.9-fold higher for both FSP, respectively.

Considering the enteric II phase of the *in vitro* simulated gastrointestinal assay, that simulates the human large intestine, the increase in the folate bio-accessibility in this gastrointestinal phase may contribute to supply the microbiota with natural folates once this nutrient is required for the metabolism of many microorganisms (LeBlanc et al., 2017). Similar results were observed by Mo et al. (2013), who determined the bio-accessibility of folate in *tempe* (a fermented soy product). The folate content of this fermented product was observed to increase during the *in vitro* digestion, which increased the bio-accessibility and was important to establish *tempe* as a good source of folate.

5. Conclusions

The bio-enriched FSP evaluated in our study may be considered a good source of folate, especially if supplemented with passion fruit by-product and FOS; a single dose of this probiotic containing product could provide 14% of the recommended daily folate intake. The functional characteristics of the FSPs should be economically explored since there is a lack of bio-enriched fermented products in the global markets. Additionally, FSP protected *Lb. rhamnosus* LGG during the *in vitro* simulated gastrointestinal conditions, during which folate bio-accessibility even increased. However, it is important to point out that folate bioavailability must be further assayed using appropriate double-blind placebo-controlled *in vivo* clinical trials in order to confirm that the FSPs developed in this study may indeed contribute for the human folate requirements and if it could be considered as a probiotic food regarding beneficial health effects to humans.

Abbreviations

LAB	lactic acid bacteria
FOS	fructooligosaccharides
FSM	fermented soy mixture
FSP	fermented soy product
PSM	pasteurized soy mixture
RDI	recommended daily intake
T_{max}	time to achieve the maximum acidification rate
T_f	time to reach pH 5.5
V_{max}	maximum acidification rate

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Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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