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Passion fruit by-product and fructooligosaccharides stimulate the growth and folate production by starter and probiotic cultures in fermented soymilk

Marcela Albuquerque Cavalcanti Albuquerque[a,b], Raquel Bedani[a,b], Jean Guy LeBlanc[c], Susana Marta Isay Saad[a,b,*]

[a] Departamento de Tecnologia Bioquímico-Farmacêutica, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, Av. Prof. Lineu Prestes, 580, 05508-000 São Paulo, SP, Brazil
[b] Food Research Center, University of São Paulo, São Paulo, Brazil
[c] CERELA-CONICET, C.P. T4000ILC, San Miguel de Tucumán, Argentina

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ABSTRACT

Two starter cultures (*Streptococcus* (*St.*) *thermophilus* ST-M6 and TA-40) and five probiotic strains (*St. thermophilus* TH-4, *Lactobacillus* (*Lb.*) *acidophilus* LA-5, *Lb. rhamnosus* LGG, *Lb. fermentum* PCC, and *Lb. reuteri* RC-14) were used to ferment different soymilk formulations supplemented with passion fruit by-product and/or fructooligosaccharides (FOS) with the aim of increasing folate concentrations. Growth and folate production of individual strains were evaluated and the results used to select co-cultures. Both *St. thermophilus* ST-M6 and TH-4 were the best folate producers and were able to increase the folate content of all soymilk formulations when used alone or in co-culture with lactobacilli strains, especially in the presence of both passion fruit by-product and FOS. Thus, passion fruit by-product and FOS could be used as dietary ingredients to stimulate the folate production by selected bacterial strains during the fermentation of soymilk. It was also shown that vitamin production by microorganisms is strain-dependent and may also be influenced by nutritional and environmental conditions.

1. Introduction

Soymilk has been shown to be a good medium for the growth of lactic acid bacteria (LAB) and the ability of some *Lactobacillus* spp. and *Streptococcus thermophilus* strains in metabolizing oligosaccharides during the fermentation of soymilk has been shown in different studies (Bedani et al., 2013; Champagne et al., 2009; Donkor et al., 2007; Lee et al., 2013). The α-galactosidase activity is present in some LAB and this enzyme contributes to the growth of these microorganisms during the fermentation of soy-based products through the hydrolysis of some carbohydrates, such as raffinose and stachyose. This metabolic mechanism results on the production of short chain fatty acids by these microorganisms improving intestinal human's health and reducing non-desirable gastrointestinal side-effects caused by soy products (Fung and Liong, 2010; LeBlanc et al., 2008; LeBlanc et al., 2017). Thus, the α-galactosidase activity is an important physiological characteristic presented by lactobacilli and streptococci strains once humans are not able to metabolize soy oligosaccharides.

Additionally, it is known that the processing of soybeans may cause the loss of some water soluble nutrients such as folate, a soluble B-group vitamin (Arcot et al., 2002; Mo et al., 2013). On the other hand, the ability of some starter and probiotic cultures, belonging to the LAB's group, in producing folate during fermentative processes has been described (Albuquerque et al., 2016; Pacheco da Silva et al., 2016). Probiotics are “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” (Hill et al., 2014).

Previous studies have shown that selected LAB can be used to increase folate content during the fermentation of milks (Gangadharan and Nampoothiri, 2011; Holasová et al., 2005; Laiño et al., 2013; Laiño et al., 2014; Pompei et al., 2007). However, the ability of these microorganisms to produce folate during the fermentation of soymilk supplemented with fruit agro-industrial wastes has not been described yet. Moreover, the use of fermentation as a natural process to bio-enrich soymilks with natural folates produced by food-grade functional microorganisms may be considered as a promising alternative to provide health benefit to consumers and also to increase the economic value of these fermented foods.

Considering that the production of folate by microorganisms is

* Corresponding author at: Departamento de Tecnologia Bioquímico-Farmacêutica, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, Av. Prof. Lineu Prestes, 580, 05508-000 São Paulo, SP, Brazil.
E-mail address: susaad@usp.br (S.M.I. Saad).

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strain-dependent and may depend on different growth conditions, studies have been investigating the impact of different dietary ingredients on folate production by microorganisms (Albuquerque et al., 2016; Espírito-Santo et al., 2015). In this context, passion fruit by-product may be used as fermentable carbohydrates source with prebiotic potential to improve not only the growth but also the production of beneficial metabolites by LAB, including folate, during soymilks fermentation (Corrêa et al., 2016; O'Shea et al., 2015; Albuquerque et al., 2016; Vieira et al., 2017). Prebiotics are defined as "a substrate that is selectively utilized by host microorganisms conferring a health benefit" (Gibson et al., 2017) and, among them, fructo-oligosaccharides (FOS) are important compounds commonly used by food and pharmaceutical industries to modulate positively the human gut microbiota (Valdés-Varela et al., 2017). However, according to Padalino et al. (2012), the presence of FOS did not stimulate the folate production by the microorganisms during the fermentation of milk.

To the best of our knowledge, there is no report about the impact of passion fruit by-product and FOS supplementation on microbial growth and folate synthesis during soymilk fermentation. Therefore, considering the beneficial effect of fruit by-products and prebiotics on growth and beneficial metabolites production by LAB, this study aimed to evaluate the impact of passion fruit by-product and FOS on the growth and folate production by starter and probiotic strains individually and in co-culture to bio-enrich different fermented soymilks.

2. Material and methods

2.1. Microorganisms

The starters *Streptococcus* (*St.*) *thermophilus* ST-M6 (Christian Hansen, Hørsholm, Denmark) and TA-40 (DuPont Danisco, Dangé, France) and the probiotic strains *St. thermophilus* TH-4, *Lactobacillus (Lb.) acidophilus* LA-5, *Lb. rhamnosus* LGG, *Lb. fermentum* PCC, and *Lb. reuteri* RC-14 (Christian Hansen) were previously selected and used for their ability to produce folate in culture media supplemented with passion fruit by-product (Albuquerque et al., 2016).

2.2. Standardization of passion fruit by-product and fructo-oligosaccharide

Passion fruit (*Passiflora edulis* f. *Flavicarpa*) by-products (PF) were supplied by De Marchi, a processing fruit company located in the state of São Paulo (Brazil), and processed to a fine powder (< 42 μm) according to Albuquerque et al. (2016). FOS P95® (Beneo, Orafti®, Oreye, Belgium) was used as prebiotic ingredient. Both ingredients (PF and FOS) were irradiated to eliminate all contaminating microorganisms, which were verified by the lack of growth on BHI broth, plate count agar and potato dextrose agar plates according to Albuquerque et al. (2016).

2.3. Production of fermented soymilks

Ultra-high temperature (UHT) treated commercial soymilk (Pura Soja, Mais Vita, Yoki) was used to prepare four different formulations: soymilk (SM), SM supplemented with 1% (w/v) of passion fruit by-product (SM + PF), SM supplemented with 1% (w/v) of fructo-oligosaccharides (SM + FOS), and SM supplemented with 0.5% PF and 0.5% FOS (SM + PF + FOS).

An aliquot of each activated strain (grown in Hogg-Jago (HJ) glucose (Blomqvist et al., 2006) or MRS broth for streptococci or lactobacilli, respectively) was washed three times, suspended in sterile saline solution (0.85% NaCl, w/v), and used to inoculate each soymilk formulation (4–5 log CFU/mL). All SM were incubated at 37 °C and viable cell counts and folate content were determined before (0 h) and after 24 h of fermentation.

2.4. Microbiological analysis

Viable *St. thermophilus* strains were plate counted in M17 agar (Oxoid) supplemented with lactose (10%); *Lb. acidophilus* LA-5 on MRS agar containing maltose instead of glucose (Bedani et al., 2013); *Lb. rhamnosus* LGG on MRS agar acidified to pH 5.4 using acetic acid; and *Lb. fermentum* PCC and *Lb. reuteri* RC-14 on MRS agar (Oxoid). All strains were incubated aerobically at 37 °C for 48 h. When in co-culture with *St. thermophilus*, lactobacilli strains were incubated anaerobically in order to be able to differentiate streptococci and lactobacilli colonies.

2.5. Determination of folate

The folate content of all fermented soymilks was determined by a microbiological assay using the indicator strain *Lb. rhamnosus* NCIMB 10463, as described previously (Albuquerque et al., 2016). The advantage of this technique is that all folate forms can be quantified together (expressed as total folate concentrations). The technique has been used by numerous researchers because of this advantage and has been validated by the International Association of Official Analytical Chemists (AOAC, 2007) (AOAC Official Methods 944.12, 992.05, 960.46 and 992.05). Samples must be properly prepared and diluted sufficiently to fall within the linear range of standard curve and special care must be taken when analysing samples that might contain other compounds that could affect the growth of the indicator strain.

Additionally, a tri-enzymatic treatment was applied to all samples as described previously (Laiño et al., 2013). This procedure allows the release of folates bound to carbohydrates and proteins (simulating the digestion of the samples) and cleaves polyglutamyl folates (the main folate forms in foods) to smaller folate forms that can be consumed by the indicator strain *Lb. rhamnosus* NCIMB 10463 during the microbiological assay (Hyun and Tamura, 2005).

2.6. Statistical analysis

Statistical analysis was performed with Minitab 17 Statistical Software® (MINITAB Inc., USA) using one-way ANOVA followed by a Tukey's post hoc test. Student's *t*-test was used to assess differences between two different means. All data represent three analytical repetitions (triplicate) and were expressed as means ± standard deviations (SD). The differences among the samples were considered statistically significant at $p < 0.05$.

3. Results

3.1. Growth of microorganisms in fermented soymilk

All strains were able to grow in the different soymilk formulations (most of them reaching counts above 7 log CFU/mL), except for *Lb. reuteri* RC-14, which only grew when PF was added (Table 1). The growth of *Lb. acidophilus* LA-5 increased in the presence of PF, FOS or PF + FOS. All tested co-cultures used to ferment the different formulations of soymilk also reached counts above 7 log CFU/mL (Table 2).

In both SM + PF and SM + PF + FOS, there was a relevant decrease in pH of the samples fermented by *Lb. acidophilus* LA-5 grown individually or in co-culture with *St. thermophilus* ST-M6 and *St. thermophilus* TH-4 (Table 3). All soymilks fermented by each individual streptococci (ST-M6, TH-4, and TA-40) in the presence of PF and/or FOS presented poor acidification with final pH ranging from 5.9 ± 0.0 to 6.4 ± 0.1. Since *Lb. reuteri* RC-14 only grew in SM + PF, the pH values of the other soymilk formulations did not differ from their initial values (Table 3).

Table 1
Viable cell counts of *St. thermophilus* and *Lactobacillus* spp. strains (as pure cultures) in different soymilk formulations after 24 h of fermentation.

Strains	Fermented soymilks (log CFU/mL) (A)	(B)	(C)	(D)
Streptococcus thermophilus				
St. thermophilus STM-6	8.1 ± 0.1^{B}	8.7 ± 0.0^{A}	8.5 ± 0.2^{A}	8.7 ± 0.2^{A}
St. thermophilus TH-4	8.7 ± 0.1^{A,B}	8.5 ± 0.0^{B}	8.6 ± 0.12^{A,B}	8.8 ± 0.1^{A}
St. thermophilus TA-40	9.9 ± 0.2^{A}	8.5 ± 0.0^{B}	8.5 ± 0.2^{B}	8.7 ± 0.2^{B}
Lactobacillus spp.				
Lb. acidophilus LA-5	6.6 ± 0.2^{C}	8.4 ± 0.1^{A,B}	8.2 ± 0.2^{B}	8.6 ± 0.3^{A}
Lb. rhamnosus LGG	7.6 ± 0.0^{B}	8.0 ± 0.0^{A}	7.9 ± 0.1^{A}	8.0 ± 0.2^{A}
Lb. fermentum PCC	8.4 ± 0.1^{A}	8.4 ± 0.2^{A}	8.3 ± 0.2^{A}	8.4 ± 0.3^{A}
Lb. reuteri RC-14	2.9 ± 0.2^{C}	7.3 ± 0.2^{A}	2.7 ± 0.3^{C}	5.3 ± 0.1^{B}

(A) Soymilk, (B) Soymilk supplemented with 1% (w/v) of passion fruit by-product, (C) Soymilk supplemented with 1% (w/v) of fructooligosaccharides, (D) Soymilk supplemented with 0.5% (w/v) of passion fruit by-product and 0.5% (w/v) of fructooligosaccharides. ^{A,B}Different capital letters in the same line denote significant differences ($P < 0.05$). Values are expressed as mean ± standard deviation.

3.2. Folate content in the fermented soymilks using folate producing starters and probiotic strains individually and in co-culture

The unfermented formulations (control samples) contained the following folate concentrations: SM (140 ± 1 ng/mL), SM + PF (136 ± 8 ng/mL), SM + FOS (197 ± 14 ng/mL), and SM + PF + FOS (132 ± 7 ng/mL). There were no significant differences between the folate content in these formulations before and after the incubation period (data not shown).

The folate content of all soymilk formulations fermented by the individual cultures is shown in Fig. 1. *St. thermophilus* ST-M6 and TH-4 increased highest amounts of folate in all SM formulations whereas *St. thermophilus* TA-40 consumed the vitamin in these soymilk formulations and *Lb. acidophilus* LA-5 was stimulated to produce folate in SM + PF (Fig. 1). The highest increase in folate concentrations was obtained in the SM + FOS by *St. thermophilus* ST-M6 (1325 ± 77 ng/mL) followed by *St. thermophilus* TH-4 (1250 ± 77 ng/mL) in SM + PF + FOS (Fig. 1).

Regarding the impact of each soymilk formulation on folate production by each strain used as a pure culture, *St. thermophilus* ST-M6 and TH-4 produced the highest amounts of folate in all fermented soymilk formulations, especially in soymilk supplemented with FOS. *St. thermophilus* TA-40 was the only strain that consumed the vitamin in all fermented soymilk samples (Table 4). Regarding lactobacilli strains, in general, they were not able to produce large amounts of folate except for *Lb. rhamnosus* LGG in soymilk, *Lb. acidophilus* LA-5 in SM + PF, and *Lb. fermentum* PCC in SM + PF + FOS (Table 4).

Table 3
pH values of soymilks after 24 h of fermentation by individual starter and probiotics and selected co-cultures.

Individual strains	Fermented soymilks (pH) (A)	(B)	(C)	(D)
Streptococcus thermophilus				
ST-M6	6.4 ± 0.0	5.9 ± 0.0	6.2 ± 0.0	6.0 ± 0.1
TH-4	6.4 ± 0.0	6.2 ± 0.0	6.3 ± 0.0	6.2 ± 0.0
TA-40	6.4 ± 0.1	6.1 ± 0.0	6.1 ± 0.0	6.1 ± 0.0
Lactobacillus spp.				
LA-5	6.1 ± 0.1	4.7 ± 0.1	5.1 ± 0.2	4.6 ± 0.3
LGG	7.6 ± 0.1	7.1 ± 0.1	7.3 ± 0.3	7.1 ± 0.0
PCC	6.1 ± 0.0	5.9 ± 0.0	6.3 ± 0.0	6.0 ± 0.0
RC-14	8.2 ± 0.1	6.8 ± 0.1	8.0 ± 0.1	7.6 ± 0.0
Co-culture				
ST-M6 + LA-5	4.6 ± 0.0	4.4 ± 0.0	4.3 ± 0.0	4.3 ± 0.0
ST-M6 + LGG	5.9 ± 0.2	5.5 ± 0.0	6.3 ± 0.0	6.0 ± 0.0
TH-4 + LA-5	4.5 ± 0.0	4.4 ± 0.0	4.3 ± 0.0	4.3 ± 0.0
TH-4 + LGG	5.9 ± 0.0	5.5 ± 0.0	6.3 ± 0.0	6.1 ± 0.0
Control[a]	8.2 ± 0.1	7.5 ± 0.0	8.0 ± 0.1	7.7 ± 0.2

(A) Soymilk, (B) Soymilk supplemented with 1% (w/v) of passion fruit by-product, (C) Soymilk supplemented with 1% (w/v) of fructooligosaccharides, (D) Soymilk supplemented with 0.5% (w/v) of passion fruit by-product and 0.5% (w/v) of fructooligosaccharides. ^{A,B} Different capital letters in the same line denote significant differences ($P < 0.05$). Values are expressed as mean ± standard deviation.
[a] Non fermented soymilks.

The folate levels produced by different co-cultures inoculated in the soymilk formulations after 24 h of fermentation are shown in Fig. 2. Since *St. thermophilus* ST-M6 and *St. thermophilus* TH-4 produced the highest amounts of folate after the fermentation of all different soymilks, these microorganisms were selected to be used in co-culture with selected lactobacilli strains. Although *Lb. fermentum* PCC produced the highest amounts of folate and *Lb. reuteri* RC-14 produced or did not consumed the folate present in the soymilks during fermentation, both strains were not selected to be used in co-culture with the selected streptococci strains. They were not chosen because both produced gas during the fermentative process and this would not be a sensory characteristic positively accepted by consumers for an eventual commercial fermented soymilk product. Therefore, *Lb. acidophilus* LA-5 and *Lb. rhamnosus* LGG were selected, not only considering folate production in the presence of passion fruit by-product, but also because they did not produce gas during soymilk fermentation and were able to grow in the presence of passion fruit by-product.

All co-cultures produced high amounts of folate in all soymilk formulations; however, the highest amount of the vitamin was produced in the formulation SM + PF + FOS by the co-culture *St. thermophilus* TH-4 and *Lb. rhamnosus* LGG (1927 ± 49 ng/mL). According to Table 4, the supplementation of soymilks with FOS or PF + FOS had a statistically significant impact on folate production by all co-cultures tested

Table 2
Viable cell counts of *St. thermophilus* (ST) and *Lactobacillus* spp. (LB) strains (as co-cultures) in different soymilk formulations after 24 h of fermentation.

Co-culture	Fermented soymilks (log CFU/mL) (A)		(B)		(C)		(D)	
	ST	LB	ST	LB	ST	LB	ST	LB
ST-M6 + LA-5	8.5 ± 0.3^{A}	8.4 ± 0.1^{b}	8.4 ± 0.3^{A}	8.9 ± 0.2^{a}	8.4 ± 0.3^{A}	8.4 ± 0.2^{b}	8.7 ± 0.1^{A}	8.6 ± 0.2^{b}
ST-M6 + LGG	8.3 ± 0.2^{B}	8.8 ± 0.2^{a}	8.7 ± 0.1^{A}	8.7 ± 0.2^{a}	8.6 ± 0.2^{A}	7.9 ± 0.1^{b}	8.6 ± 0.0^{A}	8.6 ± 0.2^{a}
TH-4 + LA-5	8.5 ± 0.2^{A}	8.3 ± 0.1^{bc}	8.5 ± 0.3^{A}	9.1 ± 0.0^{a}	8.3 ± 0.2^{A}	8.2 ± 0.4^{c}	7.9 ± 0.1^{B}	8.7 ± 0.3^{ab}
TH-5 + LGG	9.1 ± 0.1^{A}	8.6 ± 0.2^{a}	9.0 ± 0.1^{AB}	8.6 ± 0.2^{a}	8.7 ± 0.1^{BC}	8.5 ± 0.3^{a}	8.6 ± 0.4^{C}	8.5 ± 0.2^{a}

(A) Soymilk, (B) Soymilk supplemented with 1% (w/v) of passion fruit by-product, (C) Soymilk supplemented with 1% (w/v) of fructooligosaccharides, (D) Soymilk supplemented with 0.5% (w/v) of passion fruit by-product and 0.5% (w/v) of fructooligosaccharides. ^{A,B} Different capital letters in the same line denote significant differences between streptococci strains growth ($P < 0.05$). ^{a,b}Different small letters in the same line denote significant differences between lactobacilli strains growth ($P < 0.05$). Values are expressed as mean ± standard deviation.

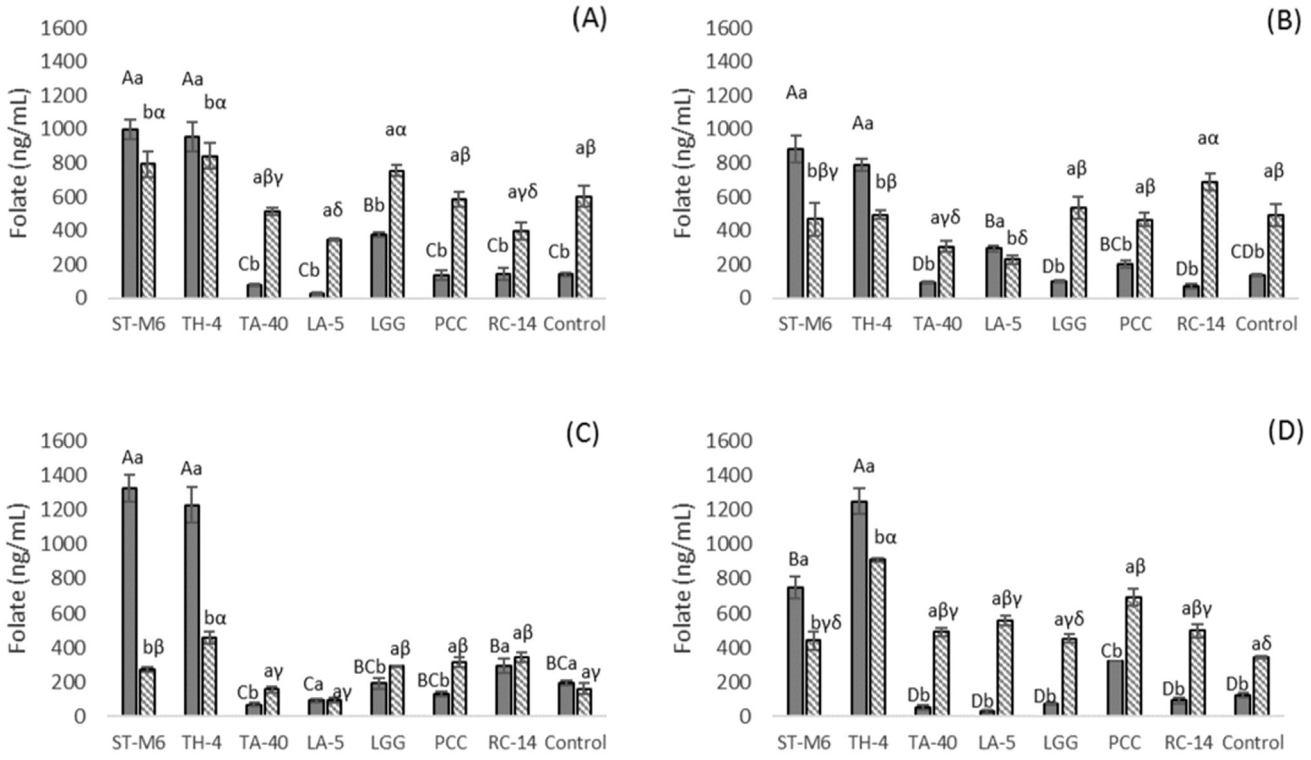

Fig. 1. Total folate content of different soymilk formulations after 24 h of fermentation by starter and probiotic strains as pure cultures. Traditional microbiological assay (grey bars); Tri-enzymatic treatment (textured bars). ^{A,B} Different capital letters denote significant differences between traditional microbiological assay results ($P < 0.05$). $^{\alpha,\beta}$ Different Greek letters denote significant differences between tri-enzymatic extraction results ($P < 0.05$). ^{a,b}Different small letters denote significant differences between traditional microbiological assay and tri-enzymatic extraction results ($P < 0.05$). (A) Soymilk, (B) Soymilk supplemented with 1% (w/v) of passion fruit by-product, (C) Soymilk supplemented with 1% (w/v) of fructooligosaccharides, (D) Soymilk supplemented with 0.5% (w/v) of passion fruit by-product and 0.5% (w/v) of fructooligosaccharides. See item 2.1 for description of strains.

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Table 4
Comparison of changes (from 0 h to 24 h) in the folate content produced by strains of *St. thermophilus* and *Lactobacillus* spp. inoculated as pure culture and co-culture in different soymilk formulations using traditional microbiological assay.

Strains	Δ Folate (ng/mL)			
	SM	SM + PF	SM + FOS	SM + FOS + PF
St. thermophilus				
ST-M6	837 ± 56^{B}	755 ± 81^{BC}	1161 ± 77^{A}	614 ± 65^{C}
TH-4	773 ± 89^{B}	657 ± 37^{B}	1085 ± 103^{A}	1097 ± 77^{A}
TA-40	−59 ± 6^{AB}	−40 ± 7^{A}	−125 ± 8^{C}	−77 ± 13^{B}
Lactobacillus spp.				
Lb. acidophilus LA-5	−112 ± 7^{B}	154 ± 18^{A}	−98 ± 11^{B}	−91 ± 9^{B}
Lb. rhamnosus LGG	227 ± 16^{A}	−39 ± 6^{B}	−4 ± 32^{B}	−51 ± 5^{B}
Lb. fermentum PCC	0 ± 32^{C}	61 ± 20^{B}	−60 ± 14^{D}	197 ± 1^{A}
Lb. reuteri RC-14	1 ± 38^{B}	−71 ± 14^{B}	97 ± 42^{A}	−30 ± 16^{B}
Co-culture				
ST-M6 + LA-5	600 ± 10^{C}	390 ± 25^{C}	1143 ± 149^{A}	957 ± 50^{B}
ST-M6 + LGG	726 ± 35^{C}	710 ± 24^{C}	1017 ± 23^{B}	1466 ± 37^{A}
TH-4 + LA-5	939 ± 42^{C}	893 ± 28^{C}	1235 ± 24^{A}	1120 ± 26^{B}
TH-4 + LGG	544 ± 25^{D}	1053 ± 93^{C}	1227 ± 1^{B}	1795 ± 49^{A}

*ΔFolate = Folate T24 (ng/mL) – Folate T0 (ng/mL); T0 = initial concentration of folate (0 h); T24 = final concentration of folate after 24 h. SM: soymilk (control); SM + PF: soymilk supplemented with 1% (w/v) of passion fruit by-product; SM + FOS: soymilk supplemented with 1% (w/v) of fructooligosaccharides; SM + FOS + PF: soymilk supplemented with 0.5% (w/v) of passion fruit by-product and 0.5% (w/v) of fructooligosaccharide. ^{A,B}Different capital letters in the same line denote significant differences ($P < 0.05$). Values are expressed as mean ± standard deviation.

($P < 0.05$).

3.3. Influence of tri-enzymatic treatment for folate extraction from the different fermented soymilks

The folate concentrations in the different soymilks fermented using pure cultures or co-cultures after performing the tri-enzymatic treatment are shown in Fig. 1 and Fig. 2, respectively. Although the concentration of folate increased for most of samples after the tri-enzymatic treatment, the folate content of all four soymilk formulations fermented by *St. thermophilus* ST-M6 and by *St. thermophilus* TH-4 as pure cultures decreased after the enzymatic treatment. Additionally, the folate content of soymilk supplemented with passion fruit by-product fermented by *Lb. acidophilus* LA-5 also decreased after the enzymatic treatment. All soymilk formulations fermented by each co-culture showed a decrease in the folate content.

4. Discussion

Increased attention has been given to soy-based foods because they are a good source of nutrients, can promote beneficial health effects to the host, and can be used as dairy substitutes. According to Farnworth et al. (2007), *St. thermophilus* can grow in soy-based products due to its ability to ferment sucrose, although it was shown that fruit by-products may cause negative effects on growth and viability of some starter and probiotic cultures due to their acidity and the presence of several antimicrobial compounds (Espírito Santo et al., 2012). In the present study, the opposite effect is observed since the presence of passion fruit by-product stimulated all streptococci and lactobacilli strains used as pure cultures and in co-culture. When in co-culture with *Lb. rhamnosus* LGG, *St. thermophilus* TH-4 counts increased in both soymilk and soymilk supplemented with passion fruit by-product, probably because of a

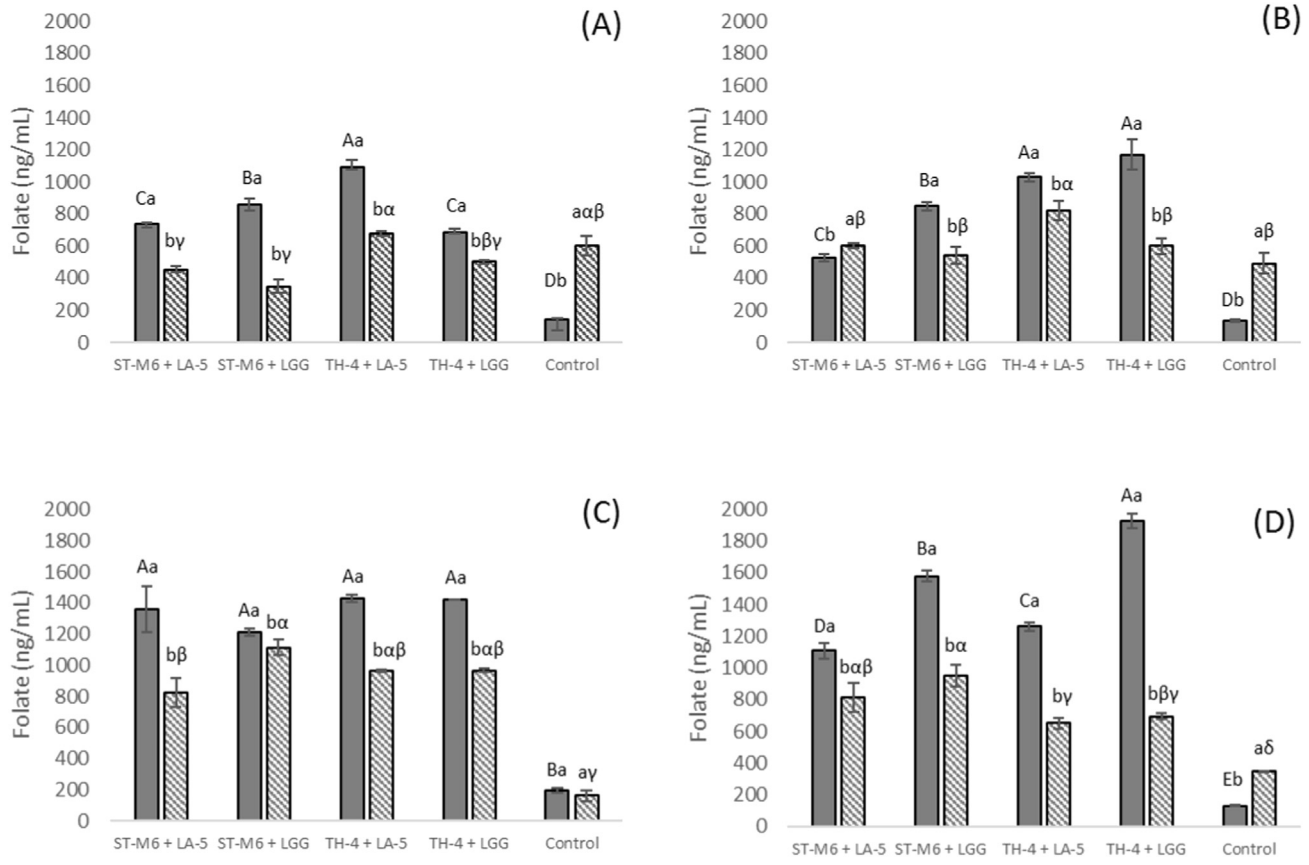

Fig. 2. . Total folate content of different soymilk formulations after 24 h of fermentation by *Lactobacillus* spp. strains with *Streptococcus thermophilus* strains. Traditional microbiological assay (grey bars); Tri-enzymatic treatment (textured bars). ^{A,B} Different capital letters denote significant differences between traditional microbiological assay results ($P < 0.05$). $^{\alpha,\beta}$ Different Greek letters denote significant differences between tri-enzymatic extraction results ($P < 0.05$). ^{a,b}Different small letters denote significant differences between traditional microbiological assay and tri-enzymatic extraction results ($P < 0.05$). (A) Soymilk, (B) Soymilk supplemented with 1% (w/v) of passion fruit by-product, (C) Soymilk supplemented with 1% (w/v) of fructooligosaccharides, (D) Soymilk supplemented with 0.5% (w/v) of passion fruit by-product and 0.5% (w/v) of fructooligosaccharides. See item 2.1 for description of strains.

symbiosis with *Lb. rhamnosus* LGG. Farnworth et al. (2007) observed that adding sugar cane to soy beverages enhanced the growth speed of *St. thermophilus*, which lead to a faster drop of pH and to the production of nutritional compounds contributing to further lactobacilli growth.

Considering the low buffering capacity of soy beverages, one would expect that streptococci and lactobacilli counts would not be as high as in fermented milks (Champagne et al., 2009; Espírito Santo et al., 2012). However, in both pure and co-cultures, all streptococci strains and most of lactobacilli strains were able to grow, and this growth was stimulated by the presence of passion fruit by-product and FOS. This is in agreement with Espírito Santo et al. (2012) and Padalino et al. (2012). The only exception was *Lb. reuteri* RC-14, which was only stimulated in the presence of passion fruit by-product, probably due to the carbohydrates and other bioactive compounds of this ingredient. We observed that this strain was not able to ferment FOS when we used a modified MRS broth supplemented with this prebiotic instead of glucose (data not shown). These results are in accordance with Saminathan et al. (2011), who tested three different *Lb. reuteri* strains and all of them showed poor growth in the presence of FOS. It is important to state that pure culture models may not reflect the environmental behaviour of bacteria in human intestinal tract which is why the determination of the best probiotic/prebiotic combination to achieve optimized results is essential (Watson et al., 2012).

Regarding pH values, *Lb. acidophilus* LA-5 probably produced the higher amounts of organic acids when compared to the other microorganisms given the notable decrease in the pH of all soymilks, especially in soymilk supplemented with passion fruit by-product with or without FOS. It is known that *Lb. acidophilus* are homofermentative strains producing large amounts of lactic acid and that the carbon source (in our study, passion fruit by-product and FOS) may affect the growth and production of organic acid by these strains (Yeo and Liong, 2010). We observed that the presence of passion fruit by-product probably led to a higher production of lactic acid by *Lb. acidophilus* LA-5 leading to the lower pH in soymilks and also when this bacterium was in co-culture with *St. thermophilus* (ST-M6 and TH-4).

It has been shown that several beneficial compounds such as short-chain fatty acids, amino acids and vitamins are produced by LAB during fermentation processes (Watson et al., 2012). While testing the effect of FOS and GOS (galacto-oligosaccharides) in the growth and folate production by some folate-producing bacteria in milk and cultured media, Padalino et al. (2012) concluded that the addition of both prebiotics contributed to increase the bacteria growth rates resulting in a reduction in the production of folate by the microorganisms used. This was confirmed by Sybesma et al. (2003), who demonstrated that folate production is further stimulated when bacterial growth is inhibited by the presence of growth-inhibiting substances, such as antibiotics and salts and by Padalino et al. (2012). These authors postulated that there may exist a negative relationship between the low pH of the medium (resulting from organic acid synthesis during the fermentation by microorganisms) and the microbial production of folate including that some labile forms of folate may be affected and degraded by the low environmental pH. In our work, we observed that both *St. thermophilus* ST-M6 and TH-4, as pure cultures, maintained their folate synthesis ability during bacterial growth and these LAB were the best folate producers in all fermented soymilks (reaching concentrations above 700 ng/mL of folate). This fact is in accordance to the literature that

describes *St. thermophilus* strains as being good folate producers (Iyer et al., 2010; Laiño et al., 2012; Laiño et al., 2013). In a previous study, *St. thermophilus* ST-M6 produced a discreet amount of folate in a modified MRS broth supplemented with passion fruit by-product while *St. thermophilus* TH-4 consumed this vitamin in the same supplemented culture media (Albuquerque et al., 2016). In the present study, we hypothesized that some soymilk components may have contributed to increase, not only the growth of both streptococci (ST-M6 and TH-4), but also enhanced the folate concentration of all soymilk formulations during their fermentation. Both streptococci (ST-M6 and TH-4) produced very high amounts of folate in soymilk supplemented with FOS. When *St. thermophilus* TH-4 fermented soymilk supplemented with both passion fruit by-product and FOS, it produced even more of the vitamin when in co-culture with *Lb. rhamnosus* LGG. Padalino et al. (2012) observed that the use of FOS did not stimulate the production of folate in culture medium and milk by most of the tested strains. Nevertheless, in our study, this prebiotic seemed to be important to the synthesis of folate by all co-cultures tested.

The presence of passion fruit by-product in soymilk also contributed for the production of folate by *Lb. acidophilus* LA-5 and *Lb. fermentum* PCC. This is in agreement with Albuquerque et al. (2016), who showed that *Lb. acidophilus* LA-5 was able to produce folate in a modified MRS broth supplemented, not only with passion fruit by-product, but also with other fruit by-products. This was also in agreement with Espírito-Santo et al. (2015), who showed that *Lb. rhamnosus* LGG ATCC 53103 was able to produce folate during fermentation of apple juice.

Considering that both *St. thermophilus* ST-M6 and TH-4 were the best streptococci folate producers, they were selected to be used in co-culture with *Lb. acidophilus* LA-5 and *Lb. rhamnosus* LGG.

A tri-enzymatic treatment was used to release folates bound to carbohydrates and proteins present in the tested fermented soymilk samples and to cleave the polyglutamyl chains into small forms of folate. Although several studies describe the application of this tri-enzyme methodology (Aiso and Tamura, 1998; Laiño et al., 2013; Pacheco da Silva et al., 2016), the method is not uniform and many researchers report difficulties in selecting the most suitable protocols to use, since some food samples may react differently to this enzymatic treatment (Hyun and Tamura, 2005). In our study, we observed that the tri-enzymatic treatment increased folate content in most of samples. However, for both *St. thermophilus* ST-M6 and TH-4 (that showed the highest productions of the vitamin when the traditional microbiological assay method was used to measure the folate content), after the tri-enzymatic treatment, the vitamin levels of all soymilks fermented by these both strains decreased. These results were not expected, since the tri-enzymatic method aims to liberate bound folate and thus increase its quantification in the samples (Yon and Hyun, 2003). The same folate content decrease was observed for all soymilk samples fermented by all selected co-cultures, when either *St. thermophilus* ST-M6 and TH-4 were used. Considering that folate is not a stable compound, especially the tetrahydrofolates, it is possible that some label forms of folate that were produced by *St. thermophilus* ST-M6 and TH-4, and also by each co-culture, during the fermentation of soymilk formulations in this study were affected by the steps of the tri-enzymatic treatment used. According to Patring et al. (2005), the food matrix, the pH of the enzyme solutions, the long period of incubation and the boil interventions to inactivate each enzyme solution can degrade folates. Further studies are necessary to elucidate which labile forms of folate are produced by both *St. thermophilus* ST-M6 and TH-4 and how these labile folates are lost during the tri-enzyme treatment of fermented soymilks tested in this work. Nevertheless, although the exact quantity of folate might vary using the tri-enzymatic treatment, it is clear that not only the passion fruit substrate, but especially FOS (with or without PF), were able to increase strain growth and folate concentrations in fermented soymilk preparations using selected strains. Although it was shown that FOS and PF can stimulate the growth of some strains, we do not consider that these compounds would affect the growth of the folate indicator strain used for quantification because the samples are highly diluted and the residual amount of prebiotics would not affect the growth of this strain.

5. Conclusions

All starters and most probiotic microorganisms used in this study were able to ferment different soymilk formulations. The presence of passion fruit by-product stimulated folate production by *Lb. acidophilus* LA-5 and *Lb. fermentum* PCC; however, when FOS and PF were added together, only *Lb. fermentum* PCC increased folate levels. In the presence of FOS alone, *St. thermophilus* ST-M6, *St. thermophilus* TH-4 and *Lb. reuteri* RC-14 increased folate concentrations in soymilk. Folate production was thus strain dependent and sometimes influenced by the addition of PF or FOS in soymilk. *St. thermophilus* ST-M6 and TH-4 were the best folate producers in all fermented soymilks when used alone or in co-culture with lactobacilli strains. In this latter case, folate production cannot be ascribed to the action of the lactobacilli strains but rather to the total action of the co-culture used.

This work represents a promising and cheaper technological process to produce new folate bio-enriched non-dairy fermented foods. According to The World Health Organization (FAO/WHO, 2002), the daily recommended intake of folates is 400 μg for a normal adult. One portion (100 mL) of the fermented soymilk supplemented with PF + FOS prepared with the co-culture TH-4 + LGG would contribute to approximately 45% RDA for adults, being not only an innovative functional folate bio-enrich product but also an alternative to the consumption of fermented dairy products. The use of B vitamin-producing LAB is a more economical and sustainable than the use of chemical synthesized vitamins (Capozzi et al., 2012) and this study confirms that novel non-dairy foods can be obtained using these beneficial microorganisms. The use of passion fruit by-product and other important prebiotics not only could serve as a growth stimulating factor but also increase natural folate levels. Further studies are required in other substrates and with other starter cultures in order to optimize the use of these fruit by-products on folate concentrations of novel food preparations and other methods (such as HPLC) must be used to elucidate which folate forms are being produced by the folate-producing strains.

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