International Journal of Food Microbiology 236 (2016) 26–32

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Supplementation with fruit and okara soybean by-products and amaranth flour increases the folate production by starter and probiotic cultures

Marcela Albuquerque Cavalcanti de Albuquerque [a], Raquel Bedani [a], Antônio Diogo Silva Vieira [a], Jean Guy LeBlanc [b], Susana Marta Isay Saad [a,*]

[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] CERELA-CONICET, C.P. T4000ILC, San Miguel de Tucumán, Argentina

ARTICLE INFO

Article history:
Received 2 March 2016
Received in revised form 17 June 2016
Accepted 10 July 2016
Available online 11 July 2016

Keywords:
Folate
Probiotic
Fruit by-products
Okara
Amaranth
Fermentation

ABSTRACT

The ability of two starter cultures (*Streptococcus* (*S.*) *thermophilus* ST-M6 and *St. thermophilus* TA-40) and eleven probiotic cultures (*St. thermophilus* TH-4, *Lactobacillus* (*Lb.*) *acidophilus* LA-5, *Lb. fermentum* PCC, *Lb. reuteri* RC-14, *Lb. paracasei* subsp. *paracasei*, *Lb. casei* 431, *Lb. paracasei* subsp. *paracasei* F19, *Lb. rhamnosus* GR-1, and *Lb. rhamnosus* LGG, *Bifidobacterium* (*B.*) *animalis* subsp. *lactis* BB-12, *B. longum* subsp. *longum* BB-46, and *B. longum* subsp. *infantis* BB-02) to produce folate in a modified MRS broth (mMRS) supplemented with different fruit (passion fruit, acerola, orange, and mango) and okara soybean by-products and amaranth flour was investigated. Initially, the folate content of each vegetable substrate was determined: passion fruit by-product showed the lowest folate content (8 ± 2 ng/mL) and okara the highest (457 ± 22 ng/mL). When the orange by-product and amaranth flour were added to mMRS, all strains were able to increase folate production after 24 h of fermentation. *B. longum* subsp *infantis* BB-02 produced the highest concentrations (1223 ± 116 ng/mL) in amaranth flour. Okara was the substrate that had the lowest impact on the folate production by all strains evaluated. *Lb. acidophilus* LA-5 (297 ± 36 ng/mL) and *B. animalis* subsp. *lactis* BB-12 (237 ± 23 ng/mL) were also able to produce folate after growth in mMRS containing acerola and orange by-products, respectively. The results of this study demonstrate that folate production is not only strain-dependent but also influenced by the addition of different substrates in the growth media.

© 2016 Elsevier B.V. All rights reserved.

1. Introduction

Folate, an essential B-group vitamin, is the generic term for the naturally occurring folates and includes folic acid (FA), which is the fully oxidized synthetic form used in food fortification (Fajardo et al., 2012; Laiño et al., 2013a; LeBlanc et al., 2013; Rossi et al., 2011). This vitamin is involved in important metabolic activities such as DNA replication, repair and methylation and the biosynthesis of nucleic acids and some amino acids. It has also been shown to provide protection against certain types of cancers, and decrease in the risk of cardiovascular disease and is mostly known for its role in the development of the neural tubes of fetuses (Kariluoto et al., 2010; Laiño et al., 2013a).

Since humans are not able to synthesize folates, they need to acquire this vitamin exogenously from foods or dietary supplements (Laiño et al., 2014). Besides having a high cost of production, FA, the chemical form used by many countries for the mandatory fortification of foods, has shown to exert adverse secondary effects when consumed in large quantities, such as masking symptoms of vitamin B_{12} deficiency and possibly promoting certain types of cancer (Bailey and Ayling, 2009; Fajardo et al., 2012). In this sense, the bio-enrichment of foods with natural folates produced by selected microorganisms during the fermentative process has become a promising alternative to mandatory fortification with FA in order to prevent deficiencies that are present in a growing percentage of different populations throughout the world (Gangadharan and Nampoothiri, 2011; Iyer et al., 2009; Laiño et al., 2013a;. Laiño et al., 2013b; Laiño et al., 2014). Some strains of lactic acid bacteria (LAB) and bifidobacteria, mostly from the genus *Streptococcus*, *Lactobacillus*, and *Bifidobacterium*, widely used by the food industry to produce a variety of fermented foods, have been described as folate producers (Crittenden et al., 2003; Padalino et al., 2012; Pompei et al., 2007). In addition to the ability to produce folate, some bacterial strains possess other beneficial properties (such as immunological, neurological, endocrinological effects, can produce bioactive compounds, among others) which make them probiotic which are "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014). The ability of microorganisms to produce folate is a strain specific trait that can be influenced by the growth conditions

* Corresponding author.
E-mail address: susaad@usp.br (S.M.I. Saad).

http://dx.doi.org/10.1016/j.ijfoodmicro.2016.07.008
0168-1605/© 2016 Elsevier B.V. All rights reserved.

including the presence or absence of carbohydrates, proteins or other important nutrients required for the microorganism multiplication (D'Aimmo et al., 2012; Kariluoto et al., 2006; Laiño et al., 2012; Laiño et al., 2013b; Padalino et al., 2012; Pompei et al., 2007; Sybesma et al., 2003). In this context, studies have suggested that different substrates may be used to stimulate folate production by bacteria and in turn increase the natural folate levels in the growth media (Gangadharan and Nampoothiri, 2011; Holasová et al., 2005; Padalino et al., 2012).

In this line, some studies have evaluated the potential of by-products from fruit processing industries (peels, pulps, and seeds) as a source of dietary fibres and other bioactive compounds (Aguedo et al., 2012; López-Vargas et al., 2013; O'Shea et al., 2012; O'Shea et al., 2015). Additionally, there are reports that suggest that okara, a soybean by-product generated from soymilk and tofu (bean curd) industries, is also rich in nutritional and functional compounds (Jiménez-Escrig et al., 2008; Mateos-Aparicio et al., 2010; Stanojevic et al., 2013; Villanueva et al., 2011). The fruit and vegetable by-products generated by the Brazilian industry is either used as animal feed or discarded in the environment, causing environment contamination problems (Ayala-Zavala et al., 2010). A strategy to minimize this problem towards sustainable food processing is the use of these by-products in the development of new value-added products (Bedani et al., 2013; Espírito Santo et al., 2012a; Espírito Santo et al., 2012b). Furthermore, amaranth (*Amaranthus* spp.) is a pseudocereal that has attracted much interest of researchers in recent years, particularly due its excellent nutrient profile, providing good quality protein, dietary fibres, and lipids rich in unsaturated fats (Alvarez-Jubete et al., 2010; Tiengo et al., 2009). Thus, the aim of this study was to evaluate if the supplementation with fruit and okara by-products or amaranth flour affected the ability of two starter cultures (streptococci) and eleven probiotic cultures (streptococci, lactobacilli, and bifidobacteria) to produce folate in culture media.

2. Material and methods

2.1. Amaranth flour and the production of fruit and okara by-products

Passion fruit (*Passiflora edulis* f. *Flavicarpa*), orange (*Citrus sinensis*), acerola (*Malpighia emarginata*), and mango (*Mangifera indica*) by-products were supplied by fruit processing industries (on August, March, July and December 2014, respectively) located in the state of São Paulo (Brazil) and stored at -18 ± 2 °C until use to avoid enzymatic action and microbial contamination. Okara by-product was supplied by UNIVERSOJA (Production and Development Unit for Soybean Derivates) located at the School of Pharmaceutical Sciences of the São Paulo State University (Araraquara, São Paulo, Brazil) and was obtained as a fine powder (<42 mm) as described by Bedani et al. (2013). Commercial amaranth flour (Vida Boa — Produtos Naturais, Limeira, SP, Brazil) was obtained from a local store in the city of São Paulo (São Paulo, Brazil). All fruit by-products were processed according to the method described by Espírito Santo et al. (2012a,b) with some modifications. The fruit by-products were thawed at 4 ± 2 °C for 48 h, washed and bleached using clean water at 100 °C (12 min) followed by ice bath. Then, the fruit by-products were dried in oven under air flow at 60 °C for 24 h until completely dry. Afterwards, the dry material was reduced to fine powder in a blender (Magiclean, Arno, São Paulo, Brazil) and sieves (Granutest, São Paulo, Brazil) were used to standardize the particle size (<42 mm). All powders were stored in polypropylene bags and kept at -18 ± 2 °C until the analysis.

2.2. Irradiation of fruit and okara by-products powders and amaranth flour

Portions of 2.5 g of each powder were weighed in polypropylene bags, sealed and transported to Nuclear and Energy Research Institute (IPEN, São Paulo, Brazil) to perform the irradiation process of the samples using a modification of the method described by Rezende et al. (2014). Briefly, the samples were exposed to radiation (radioactive source ^{60}Co) in a Gammacell 220 irradiator (Atomic Energy of Canada Ltd., Ottawa, Canada) with an activity of 1287.6 Ci using a dose of 25 kGy at a rate of 1.089 kGy/h.

2.3. Microbiological analyses of irradiated samples

After irradiation, each sample (2.5 g) was added to 100 mL of Brain Heart Infusion (BHI) broth (Oxoid, Basignstoke, UK) and incubated at 37 °C for 24 h. After the incubation period, 100 μL of each sample was transferred to 3 sterile plates which were filled with Plate Count agar (Oxoid) or Potato Dextrose agar (Oxoid) supplemented with tartaric acid 10% solution using *pour plate* technique to confirm the absence of any contaminating microorganism.

2.4. Microorganisms, culture media, and growth conditions

The microbial strains employed in this study as well as the culture media and incubation conditions are shown in Table 1.

For the *in vitro* test, a modified MRS medium (mMRS) containing peptone (10 g; Oxoid, Basignstoke, RU), 'LAB-LEMCO' Powder (8 g; Oxoid), yeast extract (4 g; Oxoid), Tween 80 (1 mL; Merck, Hohenbrunn, Germany), ammonium acetate (2 g; Labsynth, São Paulo, Brazil), $MgSO_47H_2O$ (0.18 g; Merck), $MnSO_4H_2O$ (0.05 g; Merck), Na_2SO_4 (2 g; Labsynth), K_2SO_4 (1.25 g; Labsynth), Na_2CO_3 (0.2 g; Labsynth), $CaCl_2 \cdot 2H_2O$ (0.11 g; Labsynth), L(þ)-cysteine HCl (0.5 g; BioChemica, Sigma Aldrich, Switzerland), phenol red (0.18 g; Labsynth) and distilled water (1 L) was used.

2.5. In vitro fermentation assay

The effect of fruit and okara by-products powders and amaranth flour on folate production by different bacteria was evaluated using an *in vitro* model assay adapted from Ryu et al. (2007) and Buriti et al. (2014). Each strain was cultured twice in its respective culture broth and incubation conditions as described in Table 1 for 24 h at 37 °C. An aliquot of 1 mL was taken from the second growth, centrifuged (10,000 *g* for 5 min), washed three times using sterile saline solution (0.85 g NaCl/100 mL), resuspended at the same initial volume (1 mL) using sterile saline and used to inoculate (5 log colony forming units (CFU)/mL) mMRS supplemented with 1% (w/v) of each irradiated

Table 1
Starter and probiotic cultures tested and culture media and incubation procedures employed.

Strains	Code	Type of culture	Culture media	Incubation condition
Streptococcus (*St.*) *thermophilus*				
St. thermophilus	ST-M6*	1	HJ[a]	Aerobic
St. thermophilus	TH-4*	2		
St. thermophiles	TA-40**	1		
Lactobacillus (*Lb.*) spp.				
Lb. acidophilus	LA-5*	2	MRS[b]	Anaerobic[d]
Lb. fermentum	PCC*	2		
Lb. reuteri	RC-14*	2		
Lb. paracasei subsp. *paracasei L. casei*	431*	2		
Lb. paracasei subsp. *paracasei*	F-19*	2		
Lb. rhamnosus	GR-1*	2		
Lb. rhamnosus	LGG*	2		
Bifidobacterium (*B.*) spp.				
B. animalis subsp. *lactis*	BB-12*	2	MRS cysteine (0.05%)[c]	Anaerobic[d]
B. longum	BB-46*	2		
B. longum subsp. *infantis*	BB-02*	2		

*Christian Hansen; **Danisco; 1 — Starter cultures; 2 — Probiotic cultures; [a]Hogg-Jago (HJ) glucose broth (Blomqvist et al., 2006); [b]MRS broth (Oxoid, Basingstoke, UK) with L-cysteine (0.05% w/v, Sigma-Aldrich, St. Louis, USA); [c]MRS broth (Oxoid); [abc]Culture media used to prepare the inoculum. [d]AnaeroGen™ Anaerobic System (Oxoid).

powder. Samples were taken before (0 h) and after 24 h incubation at 37 °C to determine the production of folate by each strain.

2.6. Microbiological assay for folate measurement

2.6.1. Samples processing

The samples preparation for folate determination was carried out according to Laiño et al. (2013a), with some modifications. Samples of inoculated mMRS broth supplemented with the different substrates (500 μL) were aseptically withdrawn before (0 h) and after (24 h) the fermentation assay. In each sample, 500 μL of protection buffer (0.82 g/100 mL of sodium acetate with 1 g/100 mL of ascorbic acid) was added. The resulting mixture (1 mL) was homogenized and boiled (100 °C) for 5 min. This step was performed to precipitate proteins and release folate from binding proteins present in the culture media and also to sterilize the samples. The samples were then centrifuged (10,000 *g* for 5 min) and the supernatant was collected aseptically and stored at −80 °C for total folates determination. Non-inoculated samples were used as controls and analysed simultaneously in all assays.

2.6.2. Folate determination

The measurement of the total folate was performed using a microbiological assay with *Lb. casei* subsp. *rhamnosus* NCIMB 10463 (a folate consumer with natural resistance to chloramphenicol) as the indicator strain as described previously (Pacheco Da Silva et al., 2016).

The indicator strain, stored at −80 °C in MRS broth with 20% of glycerol, was inoculated twice in fresh MRS broth and incubated at 37 °C for 24 h before use. After growth, an aliquot of 1 mL was taken and washed 3 times with sterile saline solution, resuspended in the original volume and an aliquot of 120 μL was inoculated in 3 mL of fresh Folic Acid Casei Medium (FACM, Difco, Becton, Dickinson, and Co., Sparks, Maryland) and incubated at 37 °C for 24 h. This last step was repeated to deplete folate reserves in the indicator strain and the second culture was used to perform the folate determination. An aliquot of 1 mL of the second culture in FACM was taken and the washing procedure repeated 3 times, and then 480 μL of the inoculum (representing approximately 2×10^9 CFU/mL) was inoculated in 12 mL of FACM (double concentration) containing 20 mg/mL chloramphenicol (to decrease the potential of microbial contaminants) and 100 μL of this inoculum was added to each well of a 96 well sterile microplate (Corning, NY, USA).

All frozen samples were thawed at room temperature (25 °C) in the absence of light and processed in light reduced conditions since folate is light sensitive. The samples were diluted using phosphate buffer 0.1 M and 100 μL of each diluted sample was added into one well of the sterile microplate containing the indicator strain. In each microplate, a standard curve was prepared using HPLC grade folic acid (BioChemica, Sigma Aldrich, Switzerland) diluted in the phosphate buffer 0.1 M at different concentrations (between 0 and 1.0 mg/L). Samples were diluted (normally in a 1/40 until 1/700 relation using phosphate buffer 0.1 M), in order to obtain values within the range of the standard curve. Sterile plate covers were placed on the microtiter plates that were then incubated for 48 h statically at 37 °C protected from the light. After this optimized incubation period, the optical density (OD) was read at 595 nm using a microplate reader (Multiscan™ FC Microplate Photometer, Thermo Scientific, USA). The folate concentration of each sample was determined in triplicate. To obtain the final folate concentrations, the values obtained from the standard curve were multiplied by the dilution factor and expressed as ng/mL.

2.7. Statistical analysis

The experiment was performed in triplicate and all values were expressed as means ± standard deviations (SD). Statistical analyses were performed with Minitab 15 Statistical Software® (MINITAB Inc., USA) using one way ANOVA followed by a Tukey's post hoc test, and differences were considered statistically significant at $p < 0.05$.

3. Results

After the irradiation process, no contaminants were detected in the fruit by-products, okara, and amaranth flour (data not shown). The folate values presented in Fig. 1 represent the initial folate concentrations for each substrate before the fermentation process (0 h). In general, okara was the substrate that showed the highest initial concentration of folate (457 ± 22 ng/mL) and passion fruit by-product showed the lowest concentration of this vitamin (8 ± 2 ng/mL) (Fig. 1). Additionally, there was no significant change between the initial and the end levels of folate for each tested substrate without any addition of strain (controls) after 24 h of fermentation (data not shown).

The effect of each tested fruit by-product, okara, and amaranth flour on folate production by the different tested strains evaluated was determined after 24 h of fermentation in a modified MRS medium containing 1% (w/v) of each individual substrate (Table 2). All folate values presented were considered as net production values since the folate concentration of the mMRS broth (27 ± 3 ng/mL) was subtracted in these results.

Bifidobacterium longum subsp. *infantis* BB-02 showed the highest folate production (633 ± 36 ng/mL), followed by *Lb. reuteri* RC-14 (575 ± 28 ng/mL) after 24 h of fermentation in the mMRS broth supplemented with passion fruit by-product and the other strains produced varying amounts of the vitamin except for *St. thermophilus* TH-4 and *Lb. paracasei* subsp. *paracasei* F-19 that consumed it (Fig. 2).

All strains were able to produce folate after the fermentation of the mMRS broth supplemented with orange by-product. Once again, *Lb. reuteri* RC-14 and *B. longum* subsp. *infantis* BB-02 showed the highest increase in the concentration of folate (782 ± 7 and 773 ± 36 ng/mL, respectively). Regarding the addition of acerola by-product, it caused *Lb. fermentum* PCC to produce the largest amount of folate (516 ± 68 ng/mL) compared to the other strains. *Lb. reuteri* RC-14 also produced large amounts of folate with this substrate (381 ± 40 ng/mL), which were not significantly different compared to *Lb. acidophilus* LA-5 (310 ± 36 ng/mL) and *B. longum* subsp. *infantis* BB-02 (299 ± 8 ng/mL). *Lb. rhamnosus* GR-1, *Lb. paracasei* subsp. *paracasei* F-19, *L. casei* 431, and *Lb. rhamnosus* LGG consumed the folate present in the medium supplemented with acerola by-product. Similar to what was observed in passion fruit and orange by-products, *B. longum* subsp. *infantis* BB-02 (214 ± 3 ng/mL) and *Lb. reuteri* RC-14 (168 ± 5 ng/mL) were the main folate producers after the fermentation of mango by-products. From the total of 13 strains tested, five of them (*Lb. acidophilus* LA-5, *Lb. rhamnosus* LGG, *Lb. casei* 431, *Lb. paracasei* subsp. *paracasei* F-19, and *Lb. rhamnosus* GR-1) consumed the folate present in the medium supplemented with mango by-product. As previously mentioned, okara showed the highest initial folate concentration (457 ± 22 ng/

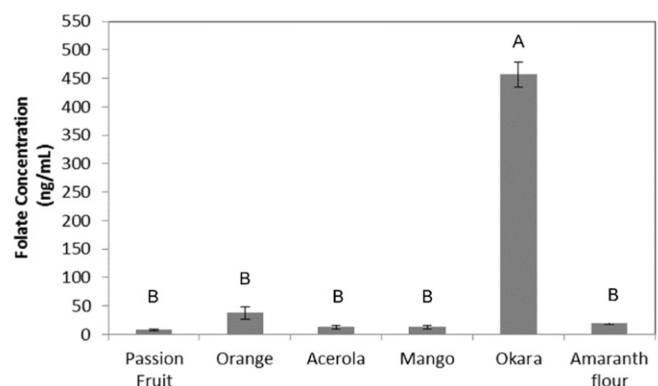

Fig. 1. Folate concentration in vegetable by-products and amaranth flour. [A,B]Different capital letters denote significant differences between the tested by-products and/or amaranth flour.

Table 2
Comparison of changes (from 0 h to 24 h) in the folate content after the fermentation with each strain of mMRS containing the different substrates.

	ΔFolate (ng/mL)*					
	Fruit by-product					
Strains	Passion fruit	Orange	Acerola	Mango	Okara soybean by-product	Amaranth flour
St. thermophilus						
ST-M6	34 ± 1[b]	99 ± 17[a]	43 ± 4[b]	45 ± 5[b]	−248 ± 30[c]	2 ± 14[b]
TH-4	−2 ± 1[c]	83 ± 1[a]	14 ± 2[bc]	59 ± 1[ab]	−99 ± 35[d]	3 ± 8[c]
TA-40	4 ± 9[b]	275 ± 12[a]	14 ± 5[b]	6 ± 7[b]	−93 ± 11[c]	19 ± 4[b]
Lactobacillus spp.						
LA-5	106 ± 13[b]	32 ± 6[cd]	297 ± 36[a]	−26 ± 3[d]	−244 ± 15[e]	30 ± 17[cd]
LGG	68 ± 10[c]	151 ± 45[b]	−26 ± 1[d]	−24 ± 7[d]	261 ± 29[a]	157 ± 12[b]
431	7 ± 0[b]	119 ± 55[a]	−18 ± 5[b]	−33 ± 4[b]	−21 ± 9[b]	80 ± 18[a]
F-19	−24 ± 0[bc]	8 ± 7[ab]	29 ± 9[a]	−29 ± 1[c]	−48 ± 17[c]	21 ± 4[a]
PCC	276 ± 2[b]	127 ± 7[cd]	504 ± 68[a]	26 ± 1[cd]	−106 ± 1[d]	258 ± 41[b]
RC-14	566 ± 30[b]	748 ± 12[a]	365 ± 41[c]	154 ± 7[d]	29 ± 2[e]	679 ± 42[ab]
GR-1	7 ± 0[c]	236 ± 29[a]	−8 ± 2[c]	−25 ± 2[c]	−22 ± 6[c]	177 ± 22[b]
Bifidobacterium spp.						
BB-12	55 ± 12[c]	237 ± 23[a]	117 ± 18[b]	4 ± 8[cd]	−28 ± 11[d]	227 ± 1[a]
BB-02	601 ± 34[bc]	738 ± 32[b]	284 ± 11[c]	201 ± 4[c]	293 ± 1[c]	1223 ± 116[a]
BB-46	305 ± 33[a]	58 ± 0[c]	121 ± 30[b]	64 ± 10[bc]	255 ± 4[a]	144 ± 13[b]

Within a row, different superscript letters denote significant differences between the tested by-products and/or amaranth flour for each strain.
* ΔFolate = Folate T24 (ng/mL) − Folate T0 (ng/mL); T0 = initial concentration of folate; T24 = final concentration of folate.

mL) and, among the 13 strains tested, only 3 strains excelled in the production of this vitamin: *B. longum* subsp. *infantis* BB-02 (710 ± 25 ng/mL), *Lb. rhamnosus* LGG (706 ± 7 ng/mL), and *B. longum* subsp. *longum* BB-46 (693 ± 26 ng/mL). The folate present in the okara by-product was consumed by the majority of strains tested when compared with the other by-products or amaranth flour. The addition of amaranth flour in the growth media promoted the production of folate by all the evaluated strains with *B. longum* subsp. *infantis* BB-02 producing the highest amount of the vitamin (1241 ± 117 ng/mL) followed by *Lb. reuteri* RC-14 (697 ± 44 ng/mL).

In order to identify which substrate (fruit and okara by-products or amaranth flour) had the highest impact on the production of folate, a comparison of the folate production of each strain after their addition in the growth media was performed considering the difference between the folate values obtained before (0 h) and after (24 h) fermentation at 37 °C (Table 2). In general, orange by-product was the substrate that showed the highest impact on folate production by the different strains tested and the opposite was observed for mango by-product that showed the lowest production. The three strains of *St. thermophilus* (STM-6, TH-4, and TA-40), *Lb. reuteri* RC-14 and *Lb. rhamnosus* GR-1 showed the highest production of folate in the presence of orange by-product and *Lb. acidophilus* LA-5 and *Lb. fermentum* PCC produced more folate after the fermentation of acerola by-product. Okara promoted an increased production of folate by *Lb. rhamnosus* LGG. The folate concentrations produced by *Lb. paracasei* subsp. *paracasei* F-19, *L. casei* 431, and *B. animalis* subsp. *lactis* BB-12 during fermentation of orange by-product did not differ significantly from fermentation of amaranth flour ($p > 0.05$). Compared to the other substrates tested, *B. longum* subsp. *longum* BB-46 produced a higher concentration of folate after the fermentation of passion fruit by-product and okara, while the highest production of this vitamin by *Lb. paracasei* subsp. *paracasei* F-19 was achieved after the fermentation of acerola by-product and amaranth flour.

There was no correlation between the growth of strains with folate production. All strains grew in all the substrates and although some minor differences in the final bacterial counts exist in some strains using some substrates, these differences are not associated with differences in folate concentrations. Folate production is strain specific and substrate specific and independent of the growth of the cells in this study (data not shown).

4. Discussion

Two starter cultures and eleven probiotic strains of considerable importance for the food industry were evaluated regarding their capacity to produce folate after 24 h fermentation of modified MRS medium supplemented with fruit by-products, okara and amaranth flour. Studies have shown that vegetable products, in particular those obtained from the processing of fruits (peel, skin, seeds), are important sources of dietary fibre and other bioactive compounds (Aguedo et al., 2012; López-Vargas et al., 2013; O'Shea et al., 2012; O'Shea et al., 2015). Nutritional and functional properties may also be considered for okara and amaranth flour (Alvarez-Jubete et al., 2010; Tiengo et al., 2009). Several studies have employed high-performance liquid chromatography (HPLC) to measure folate content; however, this technique has limitations in that there is not one condition available that can separate and quantify all the different folate derivatives that exist in nature (D'Aimmo et al., 2012; Padalino et al., 2012). In this sense, the microbiological assay was adopted in the present study as the technique for quantifying folate, since it allows the determination of total folate without the use of standards for each chemical form of this vitamin. Moreover, this technique is the only official method for quantifying folate in food proposed by the American Association of Analytical Chemistry (AOAC) (Tomar et al., 2009).

Regarding *St. thermophilus*, none of the three strains tested produced folate after fermentation of okara (Fig. 2). This fact may be related to the elevated initial concentration of folate available in the culture medium supplemented with this by-product. Pompei et al. (2007) observed that high concentrations of folate reduced the production of this vitamin by some strains of bifidobacteria. Since okara was the substrate with the highest initial concentrations of folate (Fig. 1), the presence of high concentrations of this vitamin in the medium might have inhibited the activation of the metabolic pathway for folate biosynthesis. It is noteworthy that, although some species have the potential to produce folate, this characteristic is strain-dependent and the proper selection of folate producing bacteria is essential when the objective is to work with microorganisms which produce increased amounts of this vitamin (Laiño et al., 2012). Sybesma et al. (2003) and Crittenden et al. (2003) demonstrated that strains of *St. thermophilus* were able to produce high concentrations of folate compared to other LAB and bifidobacteria and were probably responsible for the increase in the content of this

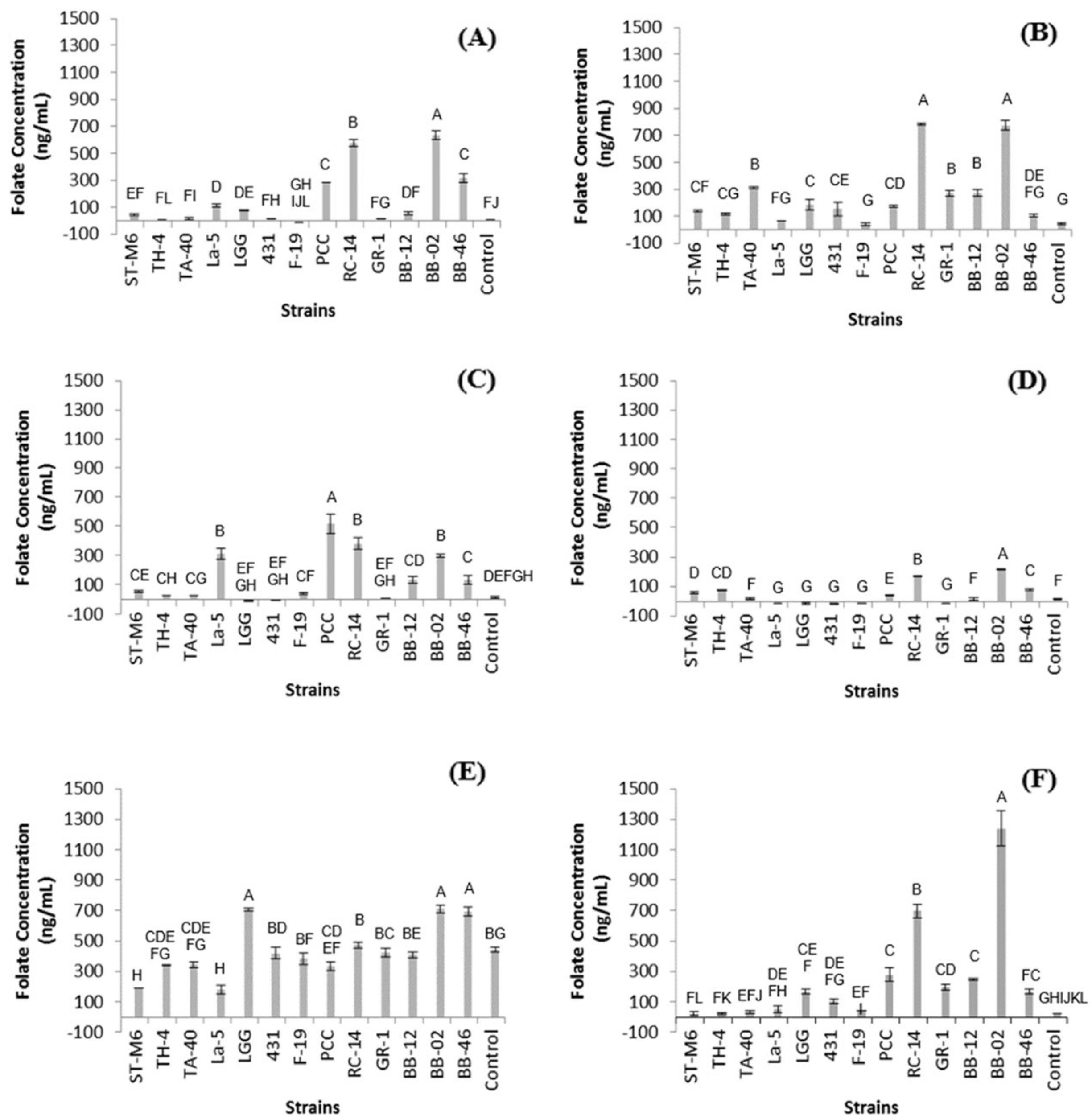

Fig. 2. Folate production by starter and probiotic strains* after 24 h of fermentation of a Modified MRS Phenol Red Broth supplemented with fruit by-products, soybean okara by-product or amaranth flour. (A) Modified MRS Phenol Red Broth supplemented with 1% of passion fruit by-product, (B) modified MRS Phenol Red Broth supplemented with 1% of orange by-product, (C) modified MRS Phenol Red Broth supplemented with 1% of acerola by-product, (D) modified MRS Phenol Red Broth supplemented with 1% of mango by-product, (E) modified MRS Phenol Red Broth supplemented with 1% of soybean okara by-product, and (F) Modified MRS Phenol Red Broth supplemented with 1% of amaranth flour; [A,B]Different capital letters denote significant differences between the tested strains ($p < 0.05$). *See Table 1 for description of strains.

vitamin in different fermented milk products. The orange by-product showed the best impact on folate production by the *St. thermophilus* strains tested in this study. Thus, we hypothesized that the nutrients and/or bioactive compounds present in the orange by-product (for example, dietary fibres, especially the soluble portion) could be stimulating the folate production by these streptococci strains. Nevertheless, further studies are needed to demonstrate this hypothesis. Tomar et al. (2009) tested the influence of para-aminobenzoic acid (*pABA*, a precursor of folate) and lactose in the production of the vitamin by *St. thermophilus*. These authors showed that the presence of these substances increased the folate production; however, high concentrations of lactose and *pABA* did not promote an additional increase in the production of the vitamin. In contrast, Padalino et al. (2012) observed that the presence of prebiotic ingredients in the culture medium did not stimulate folate synthesis by the strains tested.

For the synthesis of folate *de novo* the presence of 6-hydroxymethyl-7,8-diidropterin pyrophosphate (DHPPP) and *pABA* are required (LeBlanc et al., 2013). According to Rossi et al. (2011), most *Lactobacillus* spp. strains seem to be unable to produce folate. Nevertheless, the results presented by Laiño et al. (2012), Lin and Young (2000) and the results obtained in our study refute this claim, since strains such as *Lb. reuteri* RC-14, *Lb. fermentum* PCC, *Lb. acidophilus* LA-5, and *Lb. rhamnosus* LGG produced folate after the fermentation of the substrates tested even though all the genes for folate biosynthesis have not been identified in these strains. It is important to mention that the production of folate by microorganisms depends on the species and growth conditions

(D'Aimmo et al., 2012; Kariluoto et al., 2006; Laiño et al., 2012; Laiño et al., 2013a; Padalino et al., 2012; Pompei et al., 2007; Sybesma et al., 2003). Lin and Young (2000) also observed the production of folate by *Lb. acidophilus* strains in a culture medium and in milk; however, the authors could not explain these results. In contrast, Sybesma et al. (2003) and Crittenden et al. (2003) reported that the *Lb. acidophilus* strains consumed the folate available in the medium. The use of fruit juice as a substrate for folate production by lactobacilli was investigated by Espírito-Santo et al. (2015), who found that *Lb. plantarum* and *Lb. fermentum* were able to increase the concentration of folate in apple juice. In our study, *Lb. fermentum* PCC should be highlighted for the increased folate production after the fermentation of acerola by-product.

Bifidobacteria strains have also been tested for the production of folate previously. D'Aimmo et al. (2012) tested 19 strains of bifidobacteria for the production of the main forms of folate using a folate-free culture medium. These researchers found that *B. catenulatum* ATCC 27539 produced the highest amount of this vitamin (9295 μg per 100 g), while *B. animalis* subsp. *animalis* ATCC 25527 produced lower folate levels (220 μg per 100 g). Considering the information available in databases on the genomic sequence of bifidobacteria (Kyoto Encyclopedia of Genes and Genomes, KEEG), Rossi et al. (2011) found that *B. dentium*, *B. adolescentis*, *B. longum* subsp. *longum*, and *B. longum* subsp. *infantis* have the genes responsible for the biosynthesis of 6-hydroxymethyl-7,8-dihydropterin pyrophosphate (DHPPP), a folate precursor. In contrast, the authors pointed out that these genes were not identified in *B. animalis* subsp. *lactis* (strains AD011, Bl-04 and DSMZ 10140) thus these strains could probably not produce folate, being auxotrophic microorganisms for this vitamin. In our study, *B. longum* subsp. *infantis* BB-02 synthesized high levels of folate after the fermentation of all substrates tested, particularly after the addition of amaranth flour (Table 2). *Bifidobacterium longum* subsp. *longum* BB-46 also produced folate during fermentation of all substrates tested; however, okara was the by-product that promoted the greatest effect on the production of this vitamin by this strain (Table 2). According to Pompei et al. (2007), *B. longum* subsp *infantis* strains are able to produce large amounts of folate whereas *B. longum* strains usually produce low concentrations of this vitamin. Contrary to what has been found in the scientific literature, our results shows that the strain *B. animalis* subsp *lactis* BB-12 was able to produce folate, especially from the fermentation of orange and acerola by-products and amaranth flour (Table 2). *Lactobacillus acidophilus* LA-5 and *B. animalis* subsp. *lactis* BB-12 have no genes for folate production in their genomes, thus these strains may use an unknown pathway to produce folate such as was the case for the production of thiamine (vitamin B1) for *L. plantarum* WCFS1 (Magnúsdóttir et al., 2015).

Padalino et al. (2012) evaluated the effect of two prebiotic fibres (fructooligosaccharide and galactooligosaccharide) on folate production by strains of *St. thermophilus*, *Bifidobacterium* spp., and *Lactobacillus* spp. in culture medium and milk. In this previous study it was shown that the presence of prebiotics did not stimulate the production of folate by the strains evaluated, even though these prebiotic fibres increased the growth rate of each bacterium. These authors suggested that some of the prebiotic compounds may have been fermented by the microorganisms, promoting an increase in acetic and lactic acid levels and lowering the pH of the medium. According to Paine-Wilson and Chen (1979), low pH values could inactivate some sensitive forms of folate. Sybesma et al. (2003) also observed this event and found that when the pH of the medium was kept constant (non-acidified), the folate production by LAB increased. These results are consistent with our findings that folate production is not always associated with microbial growth.

To the best of our knowledge, this is the first study where the effect of different fruit by-products, okara and amaranth flour were evaluated on folate production by some strains of bifidobacteria and LAB. Additionally, this study is an initiative to stimulate the use of the waste generated by soy and fruit industries in order to reduce the accumulation of these residues in nature and add value to these underused substrates. Therefore, the results of the present study reinforce that the folate production is strain-dependent and may be influenced by different growth conditions, such as the presence of different substrates. Our results also suggest that the initial folate content in okara may have inhibited the production of this vitamin by different strains. Also, orange by-product was the best substrate to promote folate production by all strains tested. In general, from 13 strains evaluated regarding folate production, 8 strains produced the highest amounts of total folate after 24 h of orange by-product fermentation. Amaranth flour also influenced positively on the production of folate of all tested strains. This is the first study that has shown that *Lb. acidophilus* La-5 and *B. animalis* subsp. *lactis* BB-12 were able to produce folates, a surprising result since the folate biosynthesis genes were not found in their published genomes. All of the strains used in this study were able to produce folates as shown by increased concentrations which varied depending on the substrates added to the growth media. Further studies are required to understand how these strains are able to increase folate concentrations using the by-products tested in this study and if they are able to grow and produce the vitamin in folate-free conditions.

Acknowledgments

The study was sponsored by Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP — Project #2013/50506-8), within the frame of the international thematic project "Strategic Research Collaboration in Food Science in the State of São Paulo, Brazil and Denmark — 2013" FAPESP/DCSR (project "Bioactive components from by-products of food processing used in a synbiotic approach for improving human health and well-being (BioSyn)"). M.A.C. Albuquerque and R. Bedani gratefully acknowledge their PhD and Post-Doc fellowships from CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior). The authors wish to thank Chr Hansen and Danisco Brasil Ltda for kindly providing the microorganisms, to De Marchi (Jundiaí, São Paulo, Brazil) for the donation of passion fruit by-product, Prof. Susy Sabato, Elizabeth Somessari and Carlos Gaia from IPEN (Instituto de Pesquisas Energéticas e Nucleares) for the irradiation of the vegetables samples used in this study, Prof. Bernadette Franco, head of the Food Microbiology Laboratory at the School of Pharmaceutical Sciences of University of São Paulo, for providing some of the required equipments to perform this work, Kátia Silva for her technical assistance, and Prof. Sérgio Fracallanza (IMPG/UFRJ) for his time and words of encouragement.

References

Aguedo, M., Kohnen, S., Rabetafika, N., Bossche, S.V., Sterckx, J., Blecker, C., Beauve, C., Paquot, M., 2012. Composition of by-products from cooked fruit processing and potential use in food products. J. Food Compos. Anal. 27, 61–69.

Alvarez-Jubete, L., Wijngaard, H., Arendt, Gallagher, E., 2010. Polyphenol composition and *in vitro* antioxidant activity of amaranth, quinoa buckwheat and wheat as affected by sprouting and baking. Food Chem. 119, 770–778.

Ayala-Zavala, J.F., Rosas-Dominguez, C., Vega-Vega, V., Gonzalez-Aguilar, G.A., 2010. Antioxidant enrichment and antimicrobial protection of fresh-cut fruits using their own by-products: looking for integral exploitation. J. Food Sci. 25, 175–181.

Bailey, S.W., Ayling, J.E., 2009. The extremely slow and variable activity of dihydrofolate reductase in human liver and its implications for high folic acid intake. Proc. Natl. Acad. Sci. U. S. A. 106, 15424–15429.

Bedani, R., Rossi, E.A., Saad, S.M.I., 2013. Impact of inulin and okara on *Lactobacillus acidophilus* La-5 and *Bifidobacterium animalis* Bb-12 viability in a fermented soy product and probiotic survival under *in vitro* simulated gastrointestinal conditions. Food Microbiol. 34, 382–389.

Blomqvist, T., Steinmoen, H., Håvarstein, L.S., 2006. Natural genetic transformation: a novel tool for efficient genetic engineering of the dairy bacterium *Streptococcus thermophilus*. Appl. Environ. Microbiol. 72, 6751–6756.

Buriti, F.C.C., Santos, K.M.O., Sombra, V.G., Maciel, J.S., Sá, D.M.A.T., Salles, H.O., Paula, R.C.M., Feitosa, J.P.A., Moreira, A.C.O.M., Moreira, R.A., Egito, A.S., 2014. Characterisation of partially hydrolysed galactomannan from *Caesalpinia pulcherrima* seeds as a potential dietary fibre. Food Hydrocoll. 35, 512–521.

Crittenden, R.G., Martinez, N.R., Playne, M.J., 2003. Synthesis and utilization of folate by yoghurt starter cultures and probiotic bacteria. Int. J. Food Microbiol. 80, 217–222.

D'Aimmo, M.R., Mattarelli, P., Biavati, B., Carlsson, N.G., Andlid, T., 2012. The potential of bifidobacteria as a source of natural folate. J. Appl. Microbiol. 112, 975–984.

Espírito Santo, A.P., Perego, P., Converti, A., Oliveira, M.N., 2012a. Influence of milk type and addition of passion fruit peel powder on fermentation kinetics, texture profile and bacterial viability in probiotic yogurts. LWT Food Sci. Technol. 47, 393–399.

Espírito Santo, A.P., Cartolano, N.S., Silva, T.F., Soares, F.A.S.M., Gioielli, L.A., Perego, P., Converti, A., Oliveira, M.N., 2012b. Fibres from fruit by-products enhance probiotic viability and fatty acid profile and increase CLA content in yogurts. Int. J. Food Microbiol. 154, 135–144.

Espírito-Santo, A.P., Carlin, F., Renard, C.M.G.C., 2015. Apple, grape or orange juice: which one offers the best substrate for lactobacilli growth? — a screening study on bacteria viability, superoxide dismutase activity, folates production and hedonic characteristics. Food Res. Int. 78, 352–360.

Fajardo, V., Alonso-Aperte, E., Varela-Moreiras, G., 2012. Lack of data on folate in convenience foods: should ready-to-eat products considered relevant for folate intake? The European challenge. J. Food Compos. Anal. 28, 155–163.

Gangadharan, D., Nampoothiri, K.M., 2011. Folate production using *Lactococcus lactis* spp. *cremoris* with implications for fortification of skim milk and fruit juices. LWT Food Sci. Technol. 44, 1859–1864.

Hill, C., Guarner, F., Reid, G., Gibson, G.R., Merenstein, D.J., Pot, B., Morelli, L., Canani, R.B., Flint, H.J., Salminen, S., Calder, P.C., Sanders, M.E., 2014. Expert consensus document: The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat. Rev. Gastroenterol. Hepatol. 11, 506–514.

Holasová, M., Fiedlerová, V., Roubal, P., Pechacová, 2005. Possibility of increasing natural folate contente in fermented milk products by fermentation and fruit component addition. Czech J. Food Sci. 23, 196–201.

Iyer, R., Tomar, S.K., Singh, R., Sharma, R., 2009. Estimation of folate in milk by microbiological assay using tri-enzyme extraction method. Milchwissenschaft 64, 125–127.

Jiménez-Escrig, A., Tenorio, M.D., Espinosa-Martos, I., Ruperez, P., 2008. Health-promoting effects of a dietary fibre from the soybean by-products okara in rats. J. Agric. Food Chem. 56, 7495–7501.

Kariluoto, S., Aittamaa, M., Korhola, M., Salovaara, H., Vahteristo, L., Piironen, V., 2006. Effects of yeasts and bacteria on the levels of folates in rye sourdoughs. Int. J. Food Microbiol. 106, 137–143.

Kariluoto, S., Edelmann, M., Herranen, M., Lampi, A.M., Shmelev, A., Salovaara, H., Korhola, M., Piironen, V., 2010. Production of folate by bacteria isolated from oat bran. Int. J. Food Microbiol. 143, 41–47.

Laiño, J.E., LeBlanc, J.G., De Giori, G.S., 2012. Production of natural folates by lactic acid bacteria starter cultures isolated from artisanal Argentinean yogurts. Can. J. Microbiol. 58, 581–588.

Laiño, J.E., Juarez del Valle, M., Savoy de Giori, G., LeBlanc, J.G.L., 2013a. Development of a high concentration yogurt naturally bio-enriched using selected lactic acid bacteria. LWT Food Sci. Technol. 54, 1–5.

Laiño, J.E., Savoy de Giori, G., LeBlanc, J.G., 2013b. Folate production by lactic acid bacteria. In: Watson, R.R., Preedy, V.R. (Eds.), Bioactive Food as Dietary Interventions for Liver and Gastrointestinal Disease. Academic Press, San Diego, pp. 251–270.

Laiño, J.E., Juarez de Valle, M., Savoy de Giori, G., LeBlanc, J.G.J., 2014. Applicability of a *Lactobacillus amylovorus* strain as co-culture for natural folate bio-enrichment of fermented milk. Int. J. Food Microbiol. 191, 10–16.

LeBlanc, J.G., Milani, C., Savoy de Giori, G., Sesma, F., Van Sinderen, D., Ventura, M., 2013. Bacteria as vitamin suppliers to their host: a gut microbiota perspective. Curr. Opin. Biotechnol. 24, 160–168.

Lin, M.Y., Young, C.M., 2000. Folate levels in cultures of lactic acid bacteria. Int. Dairy J. 10, 409–413.

López-Vargas, J.H., Fernández-López, J., Pérez-Álvarez, J.A., Viuda-Martos, M., 2013. Chemical, physico-chemical, technological, antibacterial and antioxidant properties of dietary fibre powder obtained from yellow passion fruit (*Passiflora edulis* var. *flavicarpa*) co-products. Food Res. Int. 51, 756–763.

Magnúsdóttir, S., Ravcheev, D., de Crécy-Lagard, V., Thiele, I., 2015. Systematic genome assessment of B-vitamin biosynthesis suggests co-operation among gut microbes. Front. Genet. 6, 1–18.

Mateos-Aparicio, I., Redondo-Cuenca, A., Villanueva-Suárez, M.J., Zapata-Revilla, M.A., Tenorio-Sanz, M.D., 2010. Pea pod, broad bean and okara, potential sources of functional compounds. LWT Food Sci. Technol. 43, 1467–1470.

O'Shea, N., Arendt, E.K., Gallagher, E., 2012. Dietary fibre and phytochemical characteristics of fruit and vegetable by-products and their recent applications as novel ingredients in food products. Innovative Food Sci. Emerg. Technol. 16, 1–10.

O'Shea, N., Ktenioudaki, A., Smyth, T.P., McLoughlin, P., Doran, L., Auty, M.A.E., Arendt, E., Gallagher, E., 2015. Physicochemical assessment of two fruit by-products as functional ingredients: apple and orange pomace. J. Food Eng. 153, 89–95.

Pacheco da Silva, F.F., Biscola, V., LeBlanc, J.G., Gombossy de Melo Franco, B.D., 2016. Effect of indigenous lactic acid bacteria isolated from goat milk and cheeses on folate and riboflavin content of fermented goat milk. LWT Food Sci. Technol. 71, 155–161.

Padalino, M., Perez-Conesa, D., López-Nicolás, R., Prontela-Saseta, C., 2012. Effect of fructoologosaccharides and galactooligosaccharides on the folate production of some folate-producing bacteria in media cultures or milk. Int. Dairy J. 27, 27–33.

Paine-Wilson, B., Chen, T.S., 1979. Thermal destruction of folacin: effect of pH and buffer ions. J. Food Sci. 44, 717–722.

Pompei, A., Cordisco, L., Amaretti, A., Zanoni, S., Matteuzzi, D., Rossi, M., 2007. Folate production by bifidobacteria as potential probiotic property. Appl. Environ. Microbiol. 73, 179–185.

Rezende, A.C.B., Igarashi, M.C., Destro, M.T., Franco, B.D.G., Landgraf, M., 2014. Effect of gamma radiation of *Salmonella* strains, *Listeria monocytogenes*, and Shiga toxin-producing *Escherichia coli* and sensory evaluation of minimally processed spinach (*Tetragonia expansa*). J. Food Prot. 10, 1656–1833.

Rossi, M., Ameratti, A., Raimoundi, S., 2011. Folate production by probiotic bacteria. Nutrients 3, 114–118.

Ryu, S.I., Kim, B.G., Park, M.S., Lee, Y.B., Lee, S.B., 2007. Evaluation of enhanced hygroscopicity, bifidogenicity, and anticariogenicity of enzymatically synthesized β-galactosyl-trehalose oligosaccharides. J. Agric. Food Chem. 55, 4184–4188.

Stanojevic, S.P., Baract, M.B., Pesic, M.B., Jankovic, V.S., Vucelic-Radovic, B.V., 2013. Bioactive proteins and energy value of okara as a by-product in hydrothermal processing of soymilk. J. Agric. Food Chem. 61, 9210–9219.

Sybesma, W., Starrenburg, M., Tijsseling, L., Hoefnagel, M.H.N., Hugenholts, J., 2003. Effects of cultivation conditions on folate production by lactic acid bacteria. Appl. Environ. Microbiol. 69, 4542–4548.

Tiengo, A., Faria, M., Netto, F.M., 2009. Characterization and ACE-inhibitory activity of amaranth proteins. J. Food Sci. 74, H121–H126.

Tomar, S.K., Srivatsa, N., Iyer, R., Singh, R., 2009. Estimation of folate production by *Streptococcus thermophilus* using modified microbiological assay. Milchwissenschaft 64, 260–263.

Villanueva, M.J., Yokoyama, W.H., Hong, Y.J., Barttley, G.E., Rupérez, P., 2011. Effect of high-fat diets supplemented with okara soybean by-product on lipid profiles of plasma, liver and faeces in Syrian hamsters. Food Chem. 124, 72–79.