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Effect of vegetable by-products on folate production by starter and probiotic microorganisms to develop a bio-enriched fermented soy product

Marcela Albuquerque Cavalcanti de Albuquerque

Thesis presented for the Degree of Doctor in Sciences.

Advisor:
Prof. Dra. Susana Marta Isay Saad

Co-advisor:
Dr. Jean Guy LeBlanc
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Marcela Albuquerque Cavalcanti de Albuquerque

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Comimission of Thesis for the degree of Doctor in Science

Prof. Dra. Susana Marta Isay Saad
Advisor/President

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1st Examiner

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3rd Examiner

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4th Examiner

São Paulo, _____, 2018.

DEDICATION

This thesis is dedicated to my mother Eliane, for everything that she represents to me, for all the efforts that she did for me during my entire life, and for always encouraging me to follow my dreams.

I also dedicate this thesis to my brothers, João Paulo and Pedro Paulo, to my grandfather Pedro Francisco, my grandmothers Dyrce (*in memorian*), Vininha, and Florinda for their help, patience, prayers, and for always encouraging me.

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"*Tu te tornas eternamente responsável por aquilo que cativas.*"
Antoine de Saint-Exupéry, O Pequeno Príncipe.

"You become responsible, forever, for what you have tamed."
Antoine de Saint-Exupéry, The Little Prince.

"O maior inimigo do conhecimento não é a ignorância, mas a ilusão do conhecimento"
Stephen Hawkins

"The greatest enemy of knowledge is not ignorance, it is the illusion of knowledge"
Stephen Hawkins

ABSTRACT

ALBUQUERQUE, M. A. C. **Effect of vegetable by-products on folate production by starter and probiotic microorganisms to develop a bio-enriched fermented soy product**. 2018. 177p. Thesis (PhD) – School of Pharmaceutical Sciences, University of São Paulo, São Paulo, 2018.

This study aimed to evaluate the effect of vegetable by-products, including fruit by-products (passion fruit, orange, acerola, and mango) and soy by-product (okara), on the folate production by starter and probiotic strains for the bio-enrichment of fermented soy products. In the first part of this study, the impact of amaranth flour on folate production by these microorganisms was also evaluated. The effect of vegetable by-products and amaranth flour on the ability of three starters - *Streptococcus thermophilus* (ST-M6, TH-4, and TA-40) and ten probiotic strains (*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 was evaluated, using a modified MRS broth. Most of the microorganisms were able to produce folate. However, folate production was strain-dependent and also dependent on the environmental conditions and on the vegetable substrate used. Passion fruit by-product presented the lowest folate concentration and was selected for the following experiments. Thus, the impact of the supplementation of soymilk with passion fruit by-product and/or commercial prebiotic fructooligosaccharides FOS P95 on the folate production by three *St. thermophilus* strains, as well as four probiotic *Lactobacillus* strains (LA-5, LGG, PCC, and RC-14) were evaluated. *St. thermophilus* ST-M6 and TH-4 produced the highest amounts of folate in all fermented soymilks. The concentration of the vitamin was also high when these strains grew in co-culture with LA-5 and LGG. Soymilk supplemented with both passion fruit by-product and FOS together presented the highest concentration of folate when fermented by the co-culture TH-4+LGG. This co-culture was selected to produce four fermented soy products (FSP). All FSP were bio-enriched with folate produced by the co-culture and the probiotic strain LGG remained always above 8 log CFU/mL until the end of the storage period (28 days at 4ºC). In contrast, the concentration of the vitamin was stable until day 14 then a slight decrease was observed at the end of the storage period. The FSP supplemented with both passion fruit by-product and FOS together may contribute with around 14% of the recommended daily intake for folate if consumed until day 14 of storage. During the *in vitro* simulated gastrointestinal conditions, the folate content of the digested FSP increased from 1.3 to 3.6-fold, especially at the small and large intestinal *in vitro* phases and the strain LGG was recovered. In contrast, *St. thermophilus* TH-4 was not recovered during the assay. Finally, the prebiotic potential of the bioactive compounds present in the fruit by-products was characterized. Fruit by-product water extracts (FWE) containing soluble fibres from fruit by-products were obtained through a hot-water extraction and were associated to phenolic compounds and showed antioxidant activity. The FWE (especially, orange and mango water extracts) presented an anti-inflammatory potential by decreasing the nitric oxide concentration produced *in vitro* by macrophages stimulated with lipopolisaccharides (LPS) from *Salmonella* Thyphymurium. The FWE (especially from mango) were able to stimulate the growth of the strains TH-4 and LGG, as well the folate production by these microorganisms when tested individually and in co-culture. The FWE also increased the adhesion of TH-4 and LGG to Caco-2 cells in an *in vitro* model. These results suggest a prebiotic potential of the fruit by-products evaluated and their potential towards increased folate production by the selected microorganisms. Therefore, the bio-enrichment of fermented soy products with folate produced by beneficial microorganisms is an alternative for the development of functional foods with high folate content. Additionally, fermentable bioactive compounds with functional and/or biological activity, such as soluble fibres associated to phenolic compounds with antioxidant activity, present in the fruit by-products, may act as potential prebiotic ingredients. These bioactive molecules may represent a potential natural alternative to synthetic drugs for the treatment of inflammatory processes.

Keywords: folate, probiotic, fermented soy product, fruit by-product, prebiotic, bioaccessibility, phenolic compounds, anti-inflammatory activity

RESUMO

ALBUQUERQUE, M. A. C. **Efeito de subprodutos vegetais na produção de folatos por microrganismos starter e probióticos para o desenvolvimento de um produto de soja fermentado bioenriquecido**. 2018. 177f. Tese (Doutorado) – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, 2018.

O objetivo deste trabalho foi avaliar o efeito de subprodutos vegetais, incluindo subprodutos do processamento de fruta (maracujá, laranja, acerola e manga) e de soja (okara) na produção de folatos *de novo* por microrganismos *strater* e probióticos para bioenriquecer um produto de soja fermentado. Na primeira etapa deste trabalho, o impacto da farinha de amaranto na produção de folatos pelos microrganismos também foi avaliado. Neste sentido, primeiramente, verificou-se o efeito desses subprodutos vegetais e da farinha de amaranto na capacidade de três cepas *starter* - *Streptococcus thermophilus* (ST-M6, TH-4 e TA-40) e 10 cepas probióticas (*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, e *B. longum* subsp. *infantis* BB-02) em produzir folato utilizando um caldo MRS modificado. A maior parte dos microrganismos testados foi capaz de produzir folato. Entretanto, a produção foi considerada cepa-dependente e, também, dependente das condições ambientais e do tipo de subproduto vegetal empregado. O subproduto de maracujá apresentou a menor concentração de folato e, por isso, foi selecionado para os testes seguintes. Neste sentido, o impacto da suplementação do leite de soja com subproduto de maracujá e/ou com o prebiótico comercial fruto-oligosacarídeo FOS P95 na produção de folato pelas três cepas de *St. thermophilus*, bem como quarto cepas probióticas do gênero *Lactobacillus* (LA-5, LGG, PCC e RC-14), também foi avaliado. Em cultura pura, as cepas de *St. thermophilus* ST-M6 e TH-4 produziram grande quantidade de folato nas formulações de extrato de soja fermentados. A concentração da vitamina foi maior quando tais cepas se desenvolveram em co-cultura com LA-5 e LGG. Observou-se que o extrato de soja suplementado concomitantemente com subproduto de maracujá e FOS apresentou a maior quantidade de folato quando fermentado pela co-cultura TH-4+LGG. Esta co-cultura, portanto, foi selecionada para desenvolver os produtos fermentados de soja (PFS). Todas as formulações foram bioenriquecidas e a cepa LGG manteve-se viável por todo o período de armazenamento (28 dias a 4ºC). Entretanto, a concentração da vitamina manteve-se estável apenas até o dia 14, observando-se uma diminuição da quantidade de folato ao final do período de armazenamento. Constatou-se que o produto fermentado de soja suplementado concomitantemente com subproduto de maracujá e FOS pode contribuir com cerca de 14% da ingestão diária recomendada para folato se consumido até o dia 14 do armazenamento. Além disso, durante a simulação gastrointestinal *in vitro*, observou-se que a digestão aumentou de 1,3 a 3,6 vezes a concentração da vitamina incrementando, consideravelmente, a bioacessibilidade do folato, principalmente nas porções simuladas do intestino delgado e grosso do intestino e a cepa LGG foi recuperada. Entretanto, a cepa *St. thermophilus* TH-4 não foi recuperada durante o ensaio. Por fim, o potencial prebiótico de componentes bioativos presentes nos subprodutos de fruta foi caracterizado. Uma extração *Hot Water* foi conduzida, a fim de obter extratos aquosos de subprodutos de fruta ricos em fibras solúveis associadas a compostos fenólicos com atividade antioxidante. Observou-se, ainda, que tais extratos aquosos de subprodutos de fruta (laranja e manga) apresentaram potencial anti-inflamatório constatado pela diminuição da concentração de óxido nítrico produzido por macrófagos estimulados com lipopolissacarideo (LPS) de *Salmonella* Typhymurium *in vitro*. Além disso, os extratos aquosos de subprodutos de fruta (principalmente o extrato aquoso de subproduto de manga) foram capazes de estimular a multiplicação das cepas TH-4 e LGG, bem como a produção de folatos por estes microrganismos quando avaliados individualmente e em co-cultura. Adicionalmente, esses extratos aquosos de subprodutos de fruta aumentaram a adesao do TH-4 e do LGG a células Caco-2 em modelo *in vitro*. Neste sentido, os resultados sugerem um potencial prebiótico dos subprodutos de fruta testados, de modo a estimular, não somente o desenvolvimento dos microrganismos avaliados mas, principalmente, o potencial destes em produzir folatos na presença dos substratos vegetais testados. O bioenriquecimento dos produtos fermentados de soja com folatos produzidos por microrganismos benéficos emerge como alternativa de alimento potencialmente funcional com alto teor de folato. Adicionalmente, compostos bioativos fermentescíveis e com atividade biológica como, por exemplo, as fibras solúveis associadas a compostos fenólicos com atividade antioxidante, presentes nos subprodutos de fruta testados podem constituir potenciais ingredientes prebióticos, além de representarem uma possível alternativa natural para o tratamento de processos inflamatórios.

Palavras-chave: folato, probióticos, produto fermentado de soja, subprodutos de fruta, prebiótico, compostos fenólicos, atividade anti-inflamatória, bioacessibilidade

LIST OF FIGURES

Chapter 2

Chapter 3

Different capital letters denote significant differences between different fruit water extracts on nitric oxide concentration ($P < 0.05$). PFWE: passion fruit water extract, OWE: Orange water extract, AWE: acerola water extract, MWE: mango water extract. Control: RAW 264.7 macrophages without LPS stimulation.

Figure 2. Folate content after fruit water extracts fermentation. Grey bars present the production of folate by *Streptococcus thermophilus* TH-4. Black bars present the folate production by *Lactobacillus rhamnosus* LGG. White bars present the production of folate by the co-culture (TH-4 + LGG). Textured bars present the folate content of each fruit by-product water extract. Control: Modified MRS broth without fruit by-product water extract ^{A,B} Different capital letters denote significant differences between streptococci folate production among different fruit water extracts fermentation ($P < 0.05$). ^{a,b} Different small letters denote significant differences between lactobacilli folate production among different fruit water extracts fermentation ($P < 0.05$). $^{\alpha,\beta}$ Different Greek letters denote significant differences between co-culture folate production among different fruit water extracts fermentation ($P < 0.05$). **.123**

Figure 3. Effect of fruit by-product water extracts (FWE) on the adhesion ability of *Streptococcus thermophilus* TH-4 and *Lactobacillus rhamnosus* LGG, as pure culture and in co-culture in a model of human colon cell line (Caco-2). (A) Adhesion of *St. thermophilus* TH-4 as pure culture (grey bars) and in co-culture (white bars) with *Lb. rhamnosus* LGG. (B) Adhesion of *Lb. rhamnosus* LGG as pure culture (grey bars) and in co-culture (white bars) with *St. thermophilus* TH-4. ^{A,B} Different capital letters denote significant differences between streptococci or lactobacilli strains adhesion to Caco-2 cells as pure culture ($P < 0.05$). ^{a,b} Different small letters denote significant differences between streptococci or lactobacilli strains adhesion to Caco-2 cells as co-culture ($P < 0.05$). $^{\alpha,\beta}$ Different greek letters denote significant differences between streptococci or lactobacilli strains adhesion to Caco-2 cells as pure culture and co-culture ($P < 0.05$). PFWE: passion fruit water extract, OWE: Orange water extract, AWE: acerola water extract, MWE: mango water extract. Control: without FWE supplementation. **.124**

Figure 4. Principal Component graph of the impact of the physicochemical parameters of fruit by-product water extracts (FWE) on biological and functional effects evaluated. *Streptococcus thermophilus* TH-4; *Lactobacillus rhamnosus* LGG. PFWE: passion fruit by-product water extract; OWE: orange by-product water extract; AWE: acerola by-product water extract; MWE: mango water extract. SF: soluble dietary fibres content; TPC: total phenolic compounds; Folate_TH-4: folate produced by TH-4; Folate_LGG: folate produced by LGG; Folate_TH-4 LGG: folate produced by the co-culture TH-4+LGG; Adhesion_TH-4: adhesion of TH-4 to Caco-2 cells; Adhesion_LGG: adhesion of LGG to Caco-2 cells; Adhesion_TH-4cc: adhesion of in co-culture with LGG to Caco-2 cells; Adhesion_LGGcc: adhesion of LGG in co-culture TH-4 to Caco-2 cells; Growth_TH-4: growth of TH-4 during fermentation of FWE; Growth_LGG: growth of LGG during fermentation of FWE; Growth_TH-4cc: growth of TH-4 in co-culture with LGG during fermentation of FWE; Growth_LGGcc: growth of LGG in co-culture with TH-4 during fermentation of FWE. **.126**

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ABREVIATIONS

ATCC - American type culture collection

AWE – acerola by-product water extract

CFU - colony forming unity

COX-2 - cyclooxygenase-2

DHF - dihydrofolate

DMEM - Dulbecco`s modified Eagle`s medium

DPPH - 1,1-diphenyl-2-picrylhydrazyl radical

ELISA - Enzyme Linked Immuno Sorbent Assay

FA - folic acid

FACM - Folic Acid Casei Medium

FOS – fructooligosaccharides

FSM – fermented soy mixture

FSP – fermented soy product

FWE - fruit by-products water extracts

GIT - gastrointestinal tract

GOS - galacto-oligosaccharides

HLPC - high performance liquid chromatography

HPLC-DAD - High-Performance Liquid Chromatography with Diode-Array Detection

iNOS - nitric oxide synthase

LAB – lactic acid bacteria

LAB – lactic acid bacteria

LPS - lipopolysaccharides

mMRS – modified MRS broth

MWE – mango by-product water extract

NO - nitric oxide

NTDs - neural tube defects

OD - optical density

ORAC - hydrophilic oxygen radical absorbance capacity

OWE – Orange by-product water extract

*p*ABA - *para*-aminobenzoic acid

PF – passion fruit by-product

PFWE - passion fruit by-product water extract

PGA - pteroyl glutamic acid

PSM – pasteurized soy mixture

RDA - recommended daily Allowance

RDI - recommended daily intake

SM – soy milk

T_f - time to reach pH 5.5

THF - tetrahydrofolate

T_{max} - time to reach the V_{max}

TPC – total phenolic content

UHT - ultra high temperature

UHT – ultra high temperature

V_{max} - maximum acidification rate

SUMMARY

Attachment 3. Proof of publication (scientific article): ***Passion fruit by-product and fructooligosaccharides stimulate the growth and folate production by starter and probiotic cultures in fermented soymilk***

Attachment 4. Scientific article under submission: ***Bio-enriched probiotic fermented soy products may improve the survival of Lactobacillus rhamnosus LGG and increase the bio-accessibility of folate under simulated gastrointestinal digestion***

Attachment 5. Scientific article submitted: ***Tropical fruit by-products water extracts as source of soluble fibres associated to phenolic compounds with potential antioxidant, anti-inflammatory, and functional properties***

Attachment 6. Proof of publication (scientific article related to the tesis): ***The impact of fruit and soybean by-products and amaranth on the growth of probiotic and starter microorganisms***

ADDITIONAL FILES

PRESENTATION

This thesis is organized in the form of scientific articles (published, submitted, or to be submitted for publication), and is divided in the following chapters:

Chapter 1: "***Increasing folate content through the use of lactic acid bacteria in novel fermented foods"*** – This chapter presents a literature review addressing the production of vitamin (folate) by microorganisms in food matrices, and resulted in the following book chapter: **Albuquerque, M.A.C.**, Bedani, R., Saad, S.M.I., LeBlanc, J.G. Increasing folate content through the use of lactic acid Bacteria in novel fermented foods.In: Nero, LA Penna, ALB, Todorov, SD eds Latin American fermented foods: from traditional knowledge to innovative applications Boca Raton: CRC Press, 2016. chap 13, p.247-266.

Chapter 2: "***Supplementation with fruit and okara soybean by-products and amaranth flour increases the folate production by starter and probiotic cultures***". – This chapter aimed to evaluate the impact of culture medium supplementation with fruit by-products, okara (soybean by-product) and amaranth flour in the production of folate by probiotic and starter strains. The following published scientific article resulted in this chapter: **Albuquerque, M.A.C.**, Bedani, R., Vieira, A.D.V., LeBlanc, J.G., Saad, S.M.I. (2016). Supplementation with fruit and Okara soybean by-products and amaranth flour increases the folate production by starter and probiotic cultures. *International Journal of Food Microbiology*, v. 236, p. 26-32.

Chapter 3: "***Passion fruit by-product and fructooligosaccharides stimulate the growth and folate production by starter and probiotic cultures in fermented soymilk***" – Based on the results obtained in chapter 2, this section aimed to evaluate the ability of three strains of *St. thermophilus* and four probiotic *Lactobacillus* strains to produce folates during fermentation of different soy milk formulations containing passion fruit by-product and/or fructo-oligosaccharides. This content resulted from the following published scientific article: **Albuquerque, M.A.C.**, Bedani, R., LeBlanc, J.G., Saad, S.M.I. (2017). Passion fruit by-product and fructooligosaccharides stimulate the growth and folate production by starter and probiotic cultures in fermented soymilk. *International Journal of Food Microbiology*, v. 261, p.35-41.

Chapter 4: "***Bio-enriched probiotic fermented soy products may improve the survival of Lactobacillus rhamnosus LGG and increase the bio-accessibility of folate under simulated gastrointestinal digestion***" – Based on the results obtained in chapter 3, this section aimed to evaluate the effect of the supplementation of a fermented soy product with passion fruit by-product and/or FOS on the viability of *Streptococcus thermophilus* TH-4 and *Lactobacillus rhamnosus* LGG in the product and their resistance to *in vitro* simulated gastrointestinal conditions during 28 days of storage (4 °C) of the fermented soy products. In addition, the folate content in the fermented soy products was evaluated during the storage period, as well as the folate bioaccessibility under *in vitro* simulated gastrointestinal digestion. This content resulted in a scientific article, which will be submitted: **Albuquerque, M.A.C.**, Yamacita, D.S., Bedani, R., LeBlanc, J.G., Saad, S.M.I. (2018) Fermented soy products bio-enriched with folates and containing probiotic *Lactobacillus rhamnosus* LGG may improve the bioaccessibility of folate under in vitro simulated gastrointestinal digestion. *International Journal of Food Microbiology*, **to be submitted**.

Chapter 5: ***"Tropical fruit by-products water extracts as source of soluble fibres associated to phenolic compounds with potential antioxidant, anti-inflammatory, and functional properties"*** – This chapter aimed to determine the total dietary and soluble fibre contents, total phenolic content, the phenolic composition, and the antioxidant activity of four different fruit by-products (from passion fruit, orange, acerola, and mango) water extracts (FWE), and to evaluate their anti-inflammatory properties in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. Additionally, we investigated the impact of each FWE on the growth of *Streptococcus thermophilus* TH-4 and *Lactobacillus rhamnosus* LGG, the folate production by both microorganisms, and their *in vitro* adherence abilities to intestinal human epithelial cells. This content resulted in a scientific article which is in submition: **Albuquerque, M.A.C.**, Levit, R., Bedani, R., De Moreno De LeBlanc, A., Saad, S.M.I., LeBlanc, J.G. (2018). Tropical fruit by-products water extracts as source of soluble fibres associated to phenolic compounds with potential antioxidant, anti-inflammatory, and functional properties. *Journal of Functional Foods*, **submitted**.

JUSTIFICATION

Synthetic vitamins, such as folic acid, are widely consumed in many countries due to their mandatory fortification programs, which aim to solve vitamin deficiency problems caused by health and social problems, such as malnutrition and unbalanced diet. Thus, the use of folate producing lactic acid bacteria and probiotic strains emerges as a natural alternative to produce bio-enriched foods with natural folates through fermentation (LAIÑO et al., 2013). Vegetable by-products, especially fruit by-products rich in several bioactive compounds, may exert potential prebiotic effect, stimulating the growth of beneficial microorganisms, and also promote their metabolic activity regarding the production of beneficial compounds during fermentation (ALBUQUERQUE, et al., 2016). Therefore, combining vitamin-producing microorganisms with vegetable by-products seems to be an interesting strategy to develop innovative bio-enriched functional foods (ALBUQUERQUE, et al., 2017). Furthermore, fruit by-product bioactive compounds, such as dietary fibre and phenolic compounds with antioxidant activity may confer biological and functional effects for the improvement of human health.

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ALBUQUERQUE, M.A.C., BEDANI, R., LEBLANC, J.G., SAAD, S.M.I. Passion fruit by-product and fructooligosaccharides stimulate the growth and folate production by starter and probiotic cultures in fermented soymilk. **International Journal of Food Microbiology**, v. 261, p.35-41, 2017.

OBJECTIVES

GENERAL

Evaluate the effect of vegetable by-products over folate production by starter and probiotic microorganisms to develop a bio-enriched fermented soy product.

SPECIFICS

- Evaluate the impact of supplementating of a culture medium with fruit by-products, okara (soybean by-product), and amaranth flour in the production of folate by selected probiotic and starter strains.

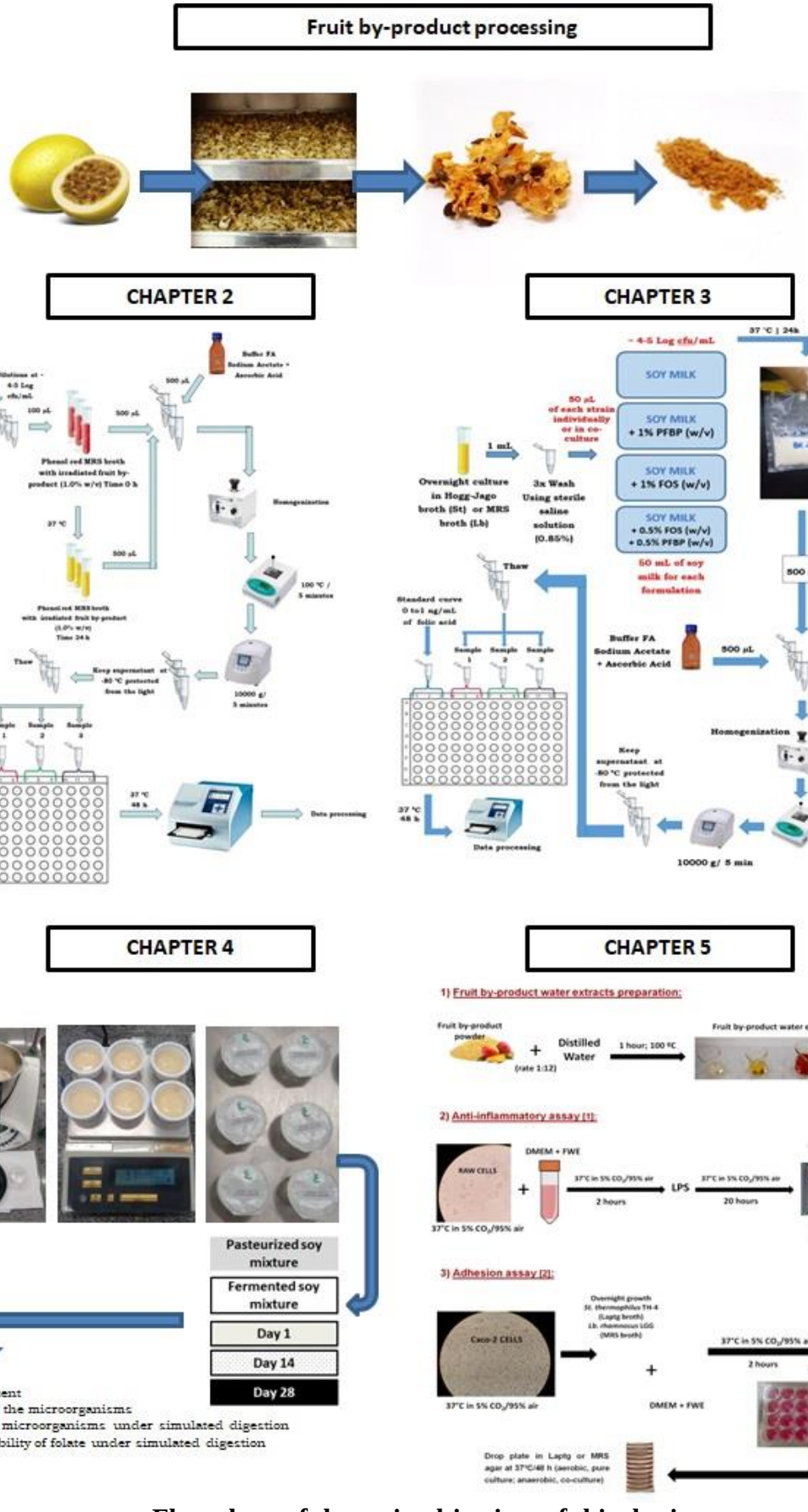
- Evaluate the ability of three strains of *St. thermophilus* and four strains of probiotic Lactobacillus strains (as pure cultures or in co-culture) to produce folates during fermentation of different soy milk formulations containing passion fruit by-product and/or fructo-oligosaccharides.

- Evaluate the effect of the supplementation of a fermented soy product with passion fruit by-product and/or FOS on the viability and resistance of *Streptococcus thermophilus* TH-4 and *Lactobacillus rhamnosus* LGG in the product to *in vitro* simulated gastrointestinal conditions during 28 days of storage (4 °C).

- Evaluate the folate content of the fermented soy products during refrigerated storage (4 ºC) for up to 28 days, as well as the folate bioaccessibility under *in vitro* simulated gastrointestinal digestion.

- Evaluate the total dietary fibre and soluble fibre contents, the total phenolic content, the phenolic composition, and the antioxidant activity of four different fruit by-products water extracts (from passion fruit, orange, acerola, and mango) and their anti-inflammatory properties in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages.

- Evaluate the impact of each fruit water extract on the growth and folate production by *Streptococcus thermophilus* TH-4 and *Lactobacillus rhamnosus* LGG and on the *in vitro* adherence abilities of both microorganisms to intestinal human epithelial cells.



Flow chart of the main objectives of this thesis

ERRATA

In 2017, the Brazilian legislation regarding folic acid fortification was updated. Therefore, along the text the reference to the old legislation may be found in the articles and in the book chapter published prior to this as follows:

“ANVISA. Agência Nacional de Vigilância Sanitária. Resolução RDC n° 344, de 13 de dezembro de 2002. **Aprova o regulamento técnico para a fortificação das farinhas de trigo e farinhas de milho com ferro e ácido fólico,** 2002.”

In the latest publications, the new legislation was cited as follows:

“ANVISA. Agência Nacional de Vigilância Sanitária. Resolução RDC n° 150, de 13 de abril de 2017. **Dispões sobre o enriquecimento das farinhas de trigo e de milho com ferro e ácido fólico,** 2017.”

CHAPTER

.1.

Increasing folate content through the use of lactic acid bacteria in novel fermented foods

Abstract

Folate is an essential B-group vitamin that plays a key role in numerous metabolic reactions such as energy usage and the biosynthesis of DNA, RNA, and some amino acids. Humans cannot synthesize folate so an exogenous supply of this vitamin is necessary to prevent nutritional deficiency. For this reason, many countries possess mandatory folic acid enrichment programs in foods of mass consumption; however, there is evidence that high intakes of folic acid, the synthetic form of folate, but not natural folates, can cause adverse effects in some individuals such as the masking of the hematological manifestations of vitamin B12 deficiency. Currently, many researcher groups are evaluating novel alternatives to increase concentrations of natural folates in foods. Lactic acid bacteria (LAB), widely used as starter cultures for the fermentation of a large variety of foods, can improve the safety, shelf life, nutritional value, flavor, and overall quality of the fermented products. Although most LAB are auxotrophic for several vitamins, it is now known that certain strains have the capability to synthesize some B-group vitamins. In this Chapter, the use of specific strains of folate producing LAB for the production of novel fermented food products will be discussed as will their use as an important strategy to help in the prevention of folate deficiency and as a safer alternative to mandatory folic acid fortification programs.

Keywords: folates, fermented foods, lactic acid bacteria, bio-enrichment, folate quantification, probiotics, prebiotics

1. Introduction

Folic acid, or vitamin B9, is an essential component of the human diet and is involved in many metabolic pathways (LEBLANC et al., 2013; ROSSI et al., 2011). This micronutrient is a water-soluble vitamin and is part of the B-group vitamins. As it is not synthesized by mammals, this vitamin must be obtained through food ingestion (KARILUOTO et al., 2010; LIN & YOUNG, 2000; PADALINNO et al., 2007; SYBESMA et al., 2003).

Folate may be synthesized by various plants and some microorganisms. Dairy and non-dairy products are considered important sources of folate (LAIÑO et al., 2013; PADALINO et al., 2012; SYBESMA et al., 2003). The main forms of folate are tetrahydrofolate (THF), 5-formyltetrahydrofolate (5-FmTHF), and 5-methyltetrahydrofolate (5-MeTHF). According to LIN & YOUNG (2000) and UEHARA & ROSE (2010), the latter is the principal form that is transported and stored in the human body and, therefore, folates derived from the diet needs to be converted into smaller residues called monoglutamates in order to be properly absorbed.

Various activities of our body are related to folate, such as: replication, repair and DNA methylation, biosynthesis of nucleic acids and amino acids protect against certain types of cancers and decreased risk of cardiovascular disease (KARILUOTO et al., 2010).

Folate deficiency is a public health problem (DANTAS et al., 2010) with great impact for pregnant women and consequently on the development of the fetuses (SANTOS et al., 2007). This deficiency, among other factors, may cause defects in neural tube formation, which is a congenital malformation resulting from the failure of the embryonic neural tube closure. This phenomenon may lead to anencephaly and spina bifida (FUJIMORE et al., 2013; LAIÑO et al., 2012). As a result, recommended doses of daily intake were proposed by agencies of various countries in order to reduce the problems caused by folate deficiency in individuals.

To avoid problems caused by folate deficiency, many people consume vitamin supplements. However, these supplements are usually developed from the synthetic folate form (folic acid), which is chemically produced. Besides having a high cost of production, it is known that synthetic folate may cause harm to human health (HUGENSCHIMIDT et al., 2011; WYK et al., 2011). Folic acid may, among other things, mask vitamin B12 deficiency. On the other hand, folate in its natural form (as found in foods or through the production of certain microorganisms) does not cause adverse effects in health (LAIÑO et al., 2013). Thereby, the inclusion of bioenriched foods in the diet, in which folate is produced by non-

synthetic technologies can be an alternative for the lower daily intake of folate (UEHARA & ROSE, 2010).

Certain strains of LAB possess, among other properties, the ability to produce folate, which is dependent on species, strain, growth time, and growth conditions (D'AIMMO et al., 2012; KARILUOTO et al., 2010; LAIÑO et al., 2012; LAIÑO et al., 2013; PADALINO et al., 2012; POMPEI et al., 2007; SAAD, 2006; SYBESMA et al., 2003). The use of vitamin-producing microorganisms in food may represent a more natural supplement alternative with increased acceptability by consumers. The production of foods with higher concentrations of natural vitamins produced by LAB would not cause harm to human health (LEBLANC et al., 2013).

2. Folate

2.1 Chemical structure, bioavailability, and functions

Folate, also known as vitamin B9, is a critical molecule in cellular metabolism. Folate is the generic term of the naturally occurring folates and folic acid (FA), which is the fully oxidized synthetic form of folate added to foods as fortifier and used in dietary supplements (FAJARDO et al., 2015; LAIÑO et al., 2013a). Folic acid or pteroyl glutamic acid (PGA) is comprised of *p*-aminobenzoic acid flanked by a pteridine ring and L-glutamic acid (LAIÑO et al., 2013a; WALKEY et al., 2015). On the other hand, the naturally occurring folates differ in the extent of the reduction state of the pteroyl group, the nature of the substituents on the pteridine ring and the number of glutamyl residues attached to the pteroyl group (LAIÑO et al., 2013a). The natural folates include 5-methyltetrahydrofolate (5-MeTHF), 5-formyltetrahydrofolate (5-FmTHF), 10-formyltetrahydrofolate (10-FmTHF), 5,10-methylenetetrahydrofolate (5,10-methylene-THF), 5,10-methenyltetrahydrofolate (5,10-methenyl-THF), 5-formiminotetrahydrofolate (5-formimino-THF), 5,6,7,8-tetrahydrofolate (THF), and dihydrofolate (DHF). The most naturally occurring folates are pteroylglutamates with two or seven glutamates joined in amide (peptides) linkages to the γ-carboxyl of glutamate. The main intracellular folates are peteroylpentaglutamates and the major extracellular folates are pteroylmonoglutamates. Pteroylpolyglutamates with up to 11 glutamic acid residues occur naturally (LEBLANC et al., 2007).

In general, bioavailability may be defined as the proportion of a nutrient ingested that becomes available to the organism for metabolic process or storage (LEBLANC et al., 2007). It is important to point out that the dietary folate bioavailability may be impaired by the

polyglutamate chain that most natural folate have (MCNULTY & PENTIEVA, 2004, LEBLANC et al., 2007). Natural food folates or pteroylpolyglutamates are hydrolyzed by the enzymes γ-glutamyl hydrolase or human conjugase to pteroylmonoglutamate forms prior to absorption in small intestine, mainly in duodenum and jejunum. Therefore, the dietary polyglutamates are hydrolyzed and then reduced and methylated in the enterocyte before being absorbed (SILVA & DAVIS, 2013). The monoglutamates forms of folate, including FA, are transported across the proximal intestine through a saturable pH-dependent process (IYER & TOMAR, 2009). Higher doses of folic acid are absorbed via a non-saturable passive diffusion process (HENDLER & RORVIK, 2001; IYER & TOMAR, 2009). Folate may be absorbed and transported as monoglutamates into the liver portal vein (LEBLANC et al., 2007). Folate enters the portal circulation as methyl-THF, which is the predominant form of the vitamin in the plasma (SILVA & DAVIS, 2013). The main transporter of folate in plasma is the reduced folate transporter (RFC), which delivers systemic folate to the tissues. The high affinity folate receptors (FRs) are expressed on various epithelia and are another family of folate transporters (ZHAO et al., 2011).

Approximately 0.3% to 0.8% of the body folate pool is excreted daily, in both urine and feces. Renal excretion increases at higher folate intakes (OHRVIK & WITTHOF, 2011, SILVA & DAVIS, 2013), which is normal since the excess of all water soluble vitamins (including the B group vitamins) is excreted.

Evidences suggest that polyglutamate form is 60% to 80% bioavailable compared to the monoglutamate form (GREGORY, 1995, LEBLANC et al., 2007; MELSE-BOONSTRA et al., 2004). Although there are controversies, the absorption efficiency of natural folates is approximately half of that of synthetic FA and the relative bioavailability of dietary folates is estimated to be only 50% in comparison with synthetic folic acid (FORSSEN et al., 2000; GREGORY, 1995; IYER &TOMAR, 2009; MCNULTY & PENTIEVA, 2004; SAUBERLICH et al., 1987). Additionally, folic acid absorption in an empty stomach is twice as available as food folate, and folic acid taken with food is 1.7 times as available as food folate (HENDLER & RORVIK, 2001). In this line, according to SHUABI et al. (2009), the assessment of folate nutritional status is incomplete if content values in the food composition database do not account the differences in bioavailability between naturally occurring folate and synthetic FA as a food fortificant, and folate supplement usage.

Folate-binding proteins from milk may increase the efficiency of folate absorption by protecting dietary folates from uptake by intestinal bacteria, thus leading to increased

absorption in the small intestine (EITENMILLER & LANDEN, 1999). Other dietary factors that may influence the folate bioavailability include: the effects of foods on intestinal pH with potential modification of conjugase activity, presence of folate antagonists, intestinal changes influenced by dietary factors (for example, alcoholism), chelation, and factors that influence the rate of gastric emptying (LEBLANC et al., 2007)

A study developed by POUNIS et al. (2014) assessed the possible differences in folate status in two European Union countries and their possible association with dietary patterns and/or other lifestyles. These researchers reported that both inadequate dietary folate intake and serum levels were observed in Italian participants of their study, whereas in individuals from southwest London, folate status seemed slightly better. According to the authors, differences between country in food group consumption as good sources of folate might explain this result. Additionally, non-smoking habits and physical activity were the two non-dietary, lifestyle characteristics positively associated with folate serum levels.

Evidences suggest that serum folate concentrations express recent folate intake, while red cell folate has a tendency to provide a better reflection of tissue folate status. Thus, serum folate may be considered a good predictor of recent dietary intake (SHUABI et al., 2009; TRUSWELL & KOUNNAVONG, 1997).

Folate is an essential micronutrient that plays an important role in the human metabolism. It acts as a cofactor in several biosynthetic reactions, serving primarily as a one-carbon donor. It is involved in the methylations and formylations that occur as part of nucleotide biosynthesis; therefore, the folate deficiency may cause defect in DNA synthesis in tissue with rapidly replicating cells (CHIANG et al., 2015; WALKEY et al., 2015). Thereby, the most remarkable consequence of folate deficiency occurs during pregnancy (WALKEY et al., 2015). In general, folates are involved in a wide number of key metabolic functions including DNA replication, repair, and methylation and biosynthesis of nucleic acid, some amino acids, pantothenate, and other vitamins (LAIÑO et al., 2013).

Owing to the role of folate in nucleotide biosynthesis, its privation impairs DNA synthesis in embryonic tissue, resulting in a reduction in the rate of cellular division and congenital malformations of the brain and spinal cord, such as neural tube defects (NTDs). Chronic alcoholism may lead to folate deficiency that may result in megaloblastic anemia. Additionally, the folate deficiency leads to elevated plasma homocysteine levels, a risk factor for cardiovascular diseases (CRAVO et al., 2000).

2.2 Food rich in folate and folate requirements

As human beings do not synthesize folates, it is necessary to assimilate this vitamin from exogenous sources. Folate occurs in most foods, with at least 50% being in the polyglutamate form. Folic acid is thermolabile and thus may be destroyed by cooking (SILVA & DAVIS, 2013).

In general, folates are present in most foods such as legumes (beans, nuts, peas, and others), leafy greens (spinach, asparagus, and Brussels sprouts), citrus, some fruits, grains, vegetables (broccoli, cauliflower), liver, and dairy products (milk and fermented dairy products) (EITENMILLER & LANDEN, 1999, WALKEY et al., 2015). Fermented milk products, for example yogurt, may contain higher concentration of folates (around 100 μg/L) compared to non-fermented milk (around 20 μg/L) (IYER & TOMAR, 2009; LIN & YOUNG, 2000). Scientific evidences suggest that certain strains of LAB may synthesize natural folates (ARYANA, 2003; LAIÑO et al., 2014; LIN & YOUNG, 2000). In this sense, the use of LAB in fermentative process may represent an interesting biotechnological approach to increase folate levels in milk (LAIÑO et al., 2013b, LAIÑO et al., 2014). It is noteworthy that the ability of microorganisms to produce folate is considered strain-dependent (D'AIMMO et al., 2012; LAIÑO et al., 2014; LEBLANC et al., 2013). Nevertheless, these folate sources are unstable and large losses occur as a result of heat exposure, typical of many food and cooking procedures (LIU et al., 2012). This phenomenon may hamper to estimate the intake of total dietary folates by consumers (WALKEY et al., 2015).

The lack of folates in the diet is one of the most common nutritional deficiencies in the world and has severe consequences on health (HERBISON et al., 2012). Traditionally, folate deficiency in humans has been related to macrocytic or megaloblastic anemia. However, this deficiency is also associated with health disorders such as cancer, cardiovascular diseases, and neural tube defects in newborns (OHRVIK and WITTHOFT, 2011; WANG et al., 2007). In this line, to reduce the risk of neural tube defects in newborns, the increased folate consumption for woman in the periconceptional period is crucial to keep an optimal folate status by considering the relationship between folate intake and blood folate concentration (STAMM & HOUGHTON, 2013).

Regarding the mean dietary reference intakes, epidemiological evidences indicate that a suboptimal folate intake may be widespread in the population in both developing and developed countries (FAJARDO et al., 2015; HERMANN & OBEID, 2011). The increase in folate intake in the population may be achieved by the consumption of foods naturally rich in

folates; use of FA supplements; and consumption of foods fortified with synthetic FA or natural folates (LÓPEZ-NICOLÁS et al., 2014). It is worth mentioning that the potential adverse effects of synthetic FA, such as masking symptoms of vitamin B_{12} deficiency and promoting certain types of cancer, have inhibited mandatory fortification in some countries (FAJARDO & VARELA-MOREIRAS, 2012). According to FAJARDO et al. (2015), the promotion of folate intake from natural food sources continues to be a health strategy to reach a safe and adequate nutritional status.

Regarding the promoting of certain kind of cancers, a case-control design study with 408 volunteers developed by CHIANG et al. (2014) evaluated the association between serum folate and the risk of colorectal cancer (CRC) in subjects with CRC or colorectal adenomatous polyps (AP, a precursor of CRC), and healthy subjects. The authors concluded that higher serum folate concentration (≥ 13.55 ng/mL) appeared to be associated with increased risk in subjects with AP while serum folate had no effect on CRC risk in healthy controls. Additionally, these researchers speculate that serum folate might play a dual role regarding CRC risk.

The daily recommended intake (DRI) of folate in the European Union (EU) is 200 and 600 μg/day for adults and women in periconceptional period, respectively (IOM, 2006). The recommended dietary allowance (RDA) of folate in adults is also 200–400 mg/day (FAO/WHO, 2002) and the body stores around 10-20 mg which is usually sufficient for only about 4 months. Pregnancy significantly increases the folate requirement, particularly throughout periods of rapid fetal growth (for example, in the second and third trimester). In addition, during the lactation, losses of folate in milk also increase the folate requirement of mothers (FAO/WHO, 2002; MCPARTLIN et al., 1993). According to the Brazilian legislation, the daily recommended intake of folate is also 0.4 mg/day for adults, 0.6 mg/day for pregnant, and 0.5 mg/day for women who are breastfeeding (ANVISA, 2005).

2.3 Fortification programs

Several countries have attempted to ensure adequate folate intake and prevent the disorders related to folate deficiency by mandatory FA fortification of cereal products. Procedures for mandatory fortification of wheat flour with FA have been in place in several countries; however, in many cases, these regulations have not been implemented or lack independent controls (CRIDER et al., 2011). As a result, in 2006, the World Health Organization and the Food and Agricultural Organization of the United Nations issued

guidelines to help countries to set the Target Fortification Level, the Minimum Fortification Level, the Maximum Fortification Level and the Legal Minimum Level of folic acid to be used to fortify flour with FA (FAO/WHO, 2006). In the United States and Canada, the implantation of mandatory fortification of cereal grain products with FA occurred in 1998. The United States of America program adds 140 μg of FA per 100 g of enriched cereal grain product and has been estimated to provide 100-200 μg of folic acid per day to women of childbearing age (CRIDER et al., 2011; QUINLIVAN & GREGORY, 2007; RADER et al., 2000; YANG et al., 2007,). In countries such as Argentina and Brazil, the flour fortification with FA became obligatory in 2002 and 2004, respectively. The Brazilian legislation recommends the addition of 150 μg of folic acid per 100 g of wheat and maize flours (ANVISA, 2002). Other countries with mandatory FA fortification programs include Canada (150 μg/100 g), Costa Rica (180 μg/100 g), Chile (220 μg/100 g), and South Africa (150 μg/100 g) (CRIDER et al., 2011). Evidences suggest that NTDs has declined (19% to 55%) in Canada, South Africa, Costa Rica, Chile, Argentina, and Brazil since the introduction of folic acid fortification practices (CRIDER et al., 2011).

On the other hand, many countries have not adopted a National Fortification Program with FA due to its potential undesirable adverse effects (LAIÑO et al., 2013a). In several European countries, the fortification is not obligatory mainly due to a concern that FA fortification may damage individuals with undiagnosed vitamin B12 deficiency (SMITH et al., 2008). In Italy, for example, FA fortification is not mandatory and the supplementation of women of childbearing age or health promotion strategies aim at increasing intake of dietary sources (POUNIS et al., 2014). Similarly, Finland does not allow mandatory fortification of staple foods with FA. In this country, a balanced diet, rich in folate, is recommended for all women planning a pregnancy or in early pregnancy, to obtain at least 400 μg of folate daily. Additionally, a daily supplement of 400 μg of FA is recommended for all women planning a pregnancy or in early pregnancy and voluntary fortification of certain food products is allowed (KARILUOTO et al., 2014; SAMANIEGO-VAESKEN et al., 2012).

Due to the potential risks of the fortification with FA, there has been growing interest in the fortification of foodstuffs with natural form folates (IYER & TOMAR, 2009; LEBLANC et al., 2007; SCOTT, 1999). In such cases, natural folates, such as 5-MeTHF, that are usually found in foods and produced by microorganisms do not mask B12 deficiency; therefore they might be considered a promising alternative for fortification with FA (KARILUOTO et al., 2014; LAIÑO et al., 2014; SCOTT, 1999).

3. Folate production by microorganisms

Nowadays, there is an increased demand by consumers to acquire healthy diets through the intake of natural foods without or at least with lower amounts of chemical preservatives. Folate deficiency occurs in several countries and the main reasons are the insufficient intake of food, restricted diets, and low purchasing power to obtain fortified foods. In order to prevent this deficiency problem, the governments of some countries adopted mandatory FA fortification programs as discussed previously. However, the supplementation of foods with synthetic FA is considered by some researchers as potentially dangerous for human health because people who keep normal or elevated folate ingestion from normal diets would be exposed to a higher FA intake which could mask hematological manifestations of B12 vitamin, as also mentioned previously (LEBLANC et al., 2007). For these reasons, it is necessary to develop alternatives to the use of synthetic FA. In some counties, mandatory folate fortification is not allowed and, in these cases, natural folate enhancement of foods appears as a promising alternative.

Lactic acid bacteria are a very important group of microorganisms for the food industry since they are used to ferment a large variety of foods, such as dairy, vegetables, and types of breads (CAPOZZI et al., 2012). This bacterial group can improve the safety, shelf-life, nutritional value, flavor, and overall quality of fermented products through the production of many beneficial compounds in foods. Among these compounds, some strains have the ability of producing, releasing and/or increasing B-group vitamins. It is known that some strains used as starter cultures in the fermentation process are able to synthesize folate as reported by many studies (CRITTENDEN et al., 2003; IYER et al., 2010; LAIÑO et al., 2012; LIN & YOUNG, 2000). Folates produced by microorganisms are natural (especially 5-MeTHF are produced) and do not cause adverse effects to the human body. Therefore, studying and selecting folate-producing strains and using them to develop folate bio-enriched food would be beneficial to the food industry since it is a very cheap process that provides high value-added to their products and to consumers who demand more natural foods.

Some studies have focused on the screening of several strains of LAB for their ability to produce folate, which can be produced intracellularly and/or extracellularly by selected microorganisms (Table 1). SYBESMA et al. (2003) evaluated the effects of cultivation conditions on folate production by LAB strains and they observed that the intracellular and extracellular folate produced by *Streptococcus* (*S.*) *thermophillus* was influenced by the medium pH values. At lower pH, this microorganism produced more extracellular folate than

those that grew in higher pH values. A possible explanation is that at low intracellular pH, folate is protonated and become electrically neutral. Thus, folate would enhance transport across the membrane, increasing the amount of this vitamin in the extracellular medium. However, for *Lactococcus* (*Lc.*) *lactis*, these authors identified no difference in the intra- and extracellular folate distribution when the microorganism grew in low or high pH. Kariluoto et al. (2010) identified some folate producer microorganisms which presented higher intracellular folate content when they grew in high pH values.

Lc. lactis and *S. thermophillus* are two industrially important microorganisms that have the ability to produce folate but other LAB such *Lactobacillus* (*L.*) *delbrueckii* subsp. *bulgaricus*, *L. acidophilus*, *L. plantarum*, *L. reuteri*, *Leuconostoc lactis*, *Propionibacterium* spp., and *Bifidobacterium* spp. also have this ability (CRITTENDEN et al., 2003; D'AIMMO et al., 2012; HUGENSCHMIDT at al., 2011; LAIÑO et al., 2014; LIN & YOUNG, 2000; POMPEI et al., 2007; SANTOS et al., 2008). These species are normally involved in the fermentation of dairy products. However, natural microbiota from raw materials used by the food industry may also affect the folate level of some cereal products (KARILUOTO et al., 2010).

Lactic acid bacteria starter cultures isolated from artisanal Argentinean yogurts were tested by LAIÑO et al. (2012). In this study certain strains of *S. thermophillus* and *L. delbrueckii* subsp. *bulgaricus* were reported to be able to increase folate in folate-free culture medium. It is important to mention that not all lactobacilli strains are able to produce folates and it has historically been believed that, in yogurt production, *S. thermophilus* produces folates whereas *L. bulgaricus* consumes this vitamin in a synbiotic relationship. It is now known that some strains of *L. bulgaricus* can in fact produce folates (LAIÑO et al., 2012). Therefore, the screening of different lactobacilli strains within different species should be conducted since the production of folate by microorganism is strain-dependent (Table 1). SYBESMA et al. (2003) identified a *L. plantarum* strain that was able to produce folate. In this context, NOR et al. (2010) verified that the use of *L. plantarum* I-UL4 led to an increase in folate content from 36.36 to 60.39 µg/L using an optimized medium formulation compared to Man Rogosa Sharp (MRS) broth.

MASUDA et al. (2012) isolated 180 LAB strains from a Japanese food named *nukazuke*, a traditional Japanese pickle made of salt and vegetables in a fermented rice bran bed. From these 180 isolated strains, only 96 grew in a free-folate medium. Since 58.4% of the strains belonged to the *Lactobacillus* genus, a significant number of strains did not grow

in the folate-free medium clearly demonstrating that not all lactobacilli strains produce this important vitamin. However, three lactobacilli strains (*L. sakei* CN-3, *L. sakei* CN-28 and *L. plantarum* CN-49) were shown to produce extracellulary high levels of folate 101±10 µg/L, 106±6 µg/L, and 108±9 µg/L, respectively.

Table 1. Reports on folate production by microorganisms in folate-free medium.

Microbial species	Extracellular content	Intracellular content	Total content	Reference
Lactococcus species				
Lc. lactis subsp. *cremoris* MG1363	46 µg/L	69 µg/L	115 µg/L	Sybesma et al. (2003)
Lc. lactis subsp. *lactis* NZ9000	11 µg/L	245 µg/L	256 µg/L	Sybesma et al. (2003)
Lactobacillus species				
L. amylovoros CRL887	68.3 ± 3.4 µg/L	12.9 ± 1.3 µg/L	81.2 ± 5.4 µg/L	Laiño et al. (2014)
L. plantarum CRL103	16.7 ± 3.4 µg/L	40.5 ± 4.2 µg/L	57.2 ± 5.2 µg/L	Laiño et al. (2014)
L. acidophilus Crl1064	21.9 ± 2.3 µg/L	15.3 ± 1.4 µg/L	37.2 ± 3.1 µg/L	Laiño et al. (2014)
L. delbrueckii subsp. *bulgaricus* CRL8711	86.2 ± 0.3 µg/L	8.6 ± 0.1 µg/L		Laiño et al. (2012)
L. helveticus	1 ng/mL	90 ng/mL	89 µg/L	Sybesma et al. (2003)
Streptococcus species				
S. thermophilus CRL803	76.6 ± 7.0 µg/L	15.9 ± 0.2 µg/L		Laiño et al. (2012)
Bifidobacterium species				
B. adolescentes ATCC 15703			8865 ± 355 µg/100g DM[1]	D'Aimmo et al. (2012)
B. catenulatum ATCC 27539			9295 ± 750 µg/100g DM[1]	D'Aimmo et al. (2012)
Propionibacterium species				
P. freudenreichii	25 ± 3 ng/mL			Hugenschimidt et al (2011)

[1]Dry matter

Different studies have evaluated the effect of *p*ABA (*para*-aminobenzoic acid), a precursor of folate, on folate production by LAB. Not all strains possess the genes necessary for *p*ABA biosynthesis and this could be a limiting factor for folate production in some microorganisms (ROSSI et al., 2011). In a review about production of folate by probiotic bacteria, ROSSI et al. (2011) showed the presence or absence of genes and enzymes necessary for the biosynthesis of DHPPP (6-hydroxymethyl-7,8-dihydropterin pyrophosphate), tetrahydrofolate-polyglutamate, chorismate, and *p*ABA from the sequenced genomes of some *Lactobacillus* spp., *Bifidobacterium* spp., and other LAB. According to these authors, *L. plantarum* is able to produce folate only in the presence of *p*ABA, since the ability to synthesize *p*ABA *de novo* does not appear in several members of the genus *Lactobacillus*. This could explain why many lactobacilli do not produce this vitamin.

Other strains of lactobacilli, such as *L. amylovorus*, *L. acidophilus*, *L. casei*, *L. fermentum*, *L. paracasei*, and *L. plantarum*, isolated from a wide range of artisanal Argentinean dairy products, were tested for the ability to produce folate in a folate-free synthetic medium. Folate amounts were found in the supernatant of some strains belonging to each of these bacterial species (LAIÑO et al., 2014).

In addition to *Lactobacillus*, another important probiotic genus is *Bifidobacterium*. This group has a relevant impact on human health, due to its association to beneficial effects by the gut microbiota. D'AIMMO et al. (2012) investigated a total of 19 strains of *Bifidobacterium* for their capacity to produce folate in free-folate medium. The results showed that the highest value of folate was found for *Bifidobacterium* (*B.*) *catenulatum* ATCC 27539 (9,295 µg per 100 g of dry matter). On the other hand, the lowest value was found for *Bifidobacterium animalis* subsp. *animalis* ATCC 25527 (220 µg per 100 g of dry matter).

POMPEI et al. (2007) administered three bifidobacteria strains (*B. adolescentis* MB 227, *B. adolescentis* MB 239, and *B. pseudocatenulatum* MB 116) to folate-depleted Wistar rats. These deficient rats were positively affected by the administration of bifidobacteria strains, since these bacteria produced folate *in vivo* and could thus be consider probiotic microorganisms.

Folate-producing probiotic strain could be used to develop new functional foods without the need of recurring to fermentation, since these microorganisms could produce vitamins directly in the GIT. Probiotics are "live microorganisms which when administered in adequate amounts confer a health benefits on the host" (FAO/WHO, 2002). The most

commonly studied probiotics have been associated with strains from *Lactobacillus* and *Bifidobacterium*.

Folates are very sensitive to heat treatments and the amount of this vitamin in vegetables, for example, could decrease after the cooking process (DELCHIER at al., 2014). Pasteurization and ultra high temperature (UHT) processes of raw milk also reduce folate levels (LIN & YOUNG, 2000). Since lower pH levels have been shown to protect folates from heat-destruction, fermentation can be used to produce folate bio-enriched foods by lowering pH values and preventing vitamin losses and, in this way, avoiding the need to supplement/fortify foods with synthetic FA.

Besides LAB, other microorganisms are also able to produce folate. KARILUOTO et al. (2010) isolated 20 strains of bacteria from three commercial oat bran products and tested them for their ability to produce folate. *Bacillus subtilis* ON5, *Chryseobacterium* sp. NR7, *Curtobacterium* sp. ON7, *Enterococcus durans* ON9, *Janthinobacterium* sp. RB4, *Paenibacillus* sp. ON11, *Propionibacterium* sp. RB9 and *Staphylococcus kloosii* RB7 were the best folate producers in culture medium. KARILUOTO et al. (2006) also evaluated the potential of three sourdough yeasts, *Candida milleri* CBS 8195, *Saccharomyces cerevisiae* TS 146, and *Torulaspora delbrueckii* TS 207 to produce folate. A baker's yeast *S. cerevisiae* ALKO 743 and four *Lactobacillus* strains from rye sourdough were also examined. The strains did not produce significant amounts of extracellular folates in yeast extract-peptone-D glucose medium but others should be tested in order to identify new folate producing strains that could be useful in the production of bakery products.

4. Bio-enriched foods with folate produced by microorganisms

As previously mentioned, an alternative to FA supplementation is the development of new food products bio-enriched with natural folates produced by microorganisms, using fermentative process. This strategy might be an innovative and cheap way to increase this vitamin in different products. However, it is very important to identify more microorganisms as folate producers, especially LAB, since these bacteria are widely used in fermentative process of dairy products. As discussed above, this group of bacteria is extensively used by the food industry, mainly in dairy products like fermented milk or yogurts. It is known that the folate content in milk is not high, especially after the application of pasteurization or UHT processes (LIN & YOUNG, 2000), and thus the fermentation of this product by folate-producing microorganisms could increase the levels of this vitamin. Natural folates produced

by microorganisms, such as 5-methyltetrahydrofolate, are usually found in foods (LAIÑO et al., 2014).

Several studies have evaluated the production of folate by different LAB strains in milk environments. In this line, LAIÑO et al. (2013a) tested starter cultures, including *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*, for folate production in milk. In the study, the authors observed that the strains used in co-culture increased folate levels significantly (180 ± 10 µg/L of folate) compared to unfermented milk (250% increase) and to commercial yogurts (125% increase). Also, the folate amount showed no significant changes during the product shelf-life (28 days of storage at 4 ºC) making this product interesting from a technological point of view.

GANGADHARAN & NAMPOOTHIRI (2011) evaluated a fermented skimmed milk using a strain of *Lc. lactis* subsp. *cremoris*. The authors obtained 187 ng/mL of folate using a 5 L bioreactor. The effect of sorbitol and mannitol on folate content was evaluated. Mannitol promoted an increased folate production compared to sorbitol. This strain also enriched cucumber (10 ± 0.2 to 60 ± 1.9 ng/mL) and melon juice (18 ± 0.9 to 26 ± 1.6 ng/mL) in folates. Folate binding proteins from milk scavenger the vitamin from blood plasma, protecting it, thus preventing folate losses as well as improving its bioavailability and stability when consuming dairy products (NYGREN-BABOL & JÄGERSTAD, 2012).

HOLASOVÁ et al. (2005) investigated folate increase in fermented milk by the fermentation process and through the addition of fruit components, such as pineapple, sour cherry, kiwi, apricot, peach, apple, strawberry, blueberry, and raspberry. After 12 h at 37 ºC, the researchers observed that milk sample inoculated with butter starter cultures of *S. thermophilus* and *Bifidobacterium longum* achieved 3.39 µg/100 g while the milk sample inoculated with butter starter cultures of *S. thermophilus* and *Propionibacterium* spp. achieved 4.23 µg/100g of 5-methyltetrahydrofolate. Moreover, the incorporation of strawberry led to the highest amount of folate. The authors concluded that the addition of this fruit component to the fermented milks may increase the product's natural folate content.

Kefir grains are a kind of natural immobilized culture and the beverage fermented by these grains is recognized as a probiotic dairy product. In this way, the milk fermentation process may increase vitamin content using kefir and a *Propionibacterium* culture. WYK et al. (2011) included *Propionibacterium freudenreichii* strains into kefir grains and observed that the best treatment delivered 19% Recommended Dietary Allowance of folate per 200 mL of product.

The applicability of *L. amylovorus* strain in co-culture with yogurt starter cultures (*L. bulgaricus* CRL871, *S. thermophilus* CRL803 and CRL415) to produce folate bio-enriched fermented milk was evaluated by LAIÑO et al. (2014). In this study, a yogurt containing high folate content (263.1 ± 2.4 µg/L) was obtained. DIVYA & NAMPOOTHIRII (2015) identified two strains of *Lc. lactis* (CM22 and CM28) isolated from cow milk and checked if these strains, when encapsulated, might be used to fortify milk and ice cream with natural folate through the products fermentation. The resulting fermented products showed an enhancement of the folate content; however, the vitamin production by *L. lactis* CM22 was higher than by *L. lactis* CM28 demonstrating once again that folate production is a strain-dependent trait.

An interesting study conducted by IYER & TOMAR (2011) assessed the effect of folate-rich fermented milk produced by two strains of *S. thermophilus* (RD 102 and RD 104) on hemoglobin level using mice model. Four groups of eight mice 30 ± 10 days old each were fed with four formulations (group 1, a basal diet with a synthetic anemic diet; group 2, a basal diet with skim milk; group 3, a basal diet with fermented skim milk produced by RD 102 and group 4, a basal diet with fermented skim milk produced by RD 104). The groups of animals that received the milks fermented with the folate producing strains (group 3 and 4) showed significant increases in mice hemoglobin level compared with the control groups.

Several studies have shown that some LAB strains may exert, beyond their sensorial attributes to the food, beneficial properties to the host. Certain strains of LAB can produce significant amounts of folate and are able to survive to the gastrointestinal tract (GIT) passage. Therefore, the identification and selection of possible probiotic folate producers would be very important to the development of probiotic foods with increased nutritional value (LAIÑO et al., 2013b). In this context, CRITTENDEN et al. (2003) investigated the potential of probiotic cultures regarding the synthesis and utilization of folate in milk. The authors concluded that the combination of strains of *S. thermophilus* and *B. animalis* increased more than six fold (72 ng/g) the folate content of milk. The researchers also showed that *S. thermophilus* and all probiotic bifidobacteria strains were the best folate producers in the study, whereas *Lactobacillus* strains depleted folate in the skimmed milk. In addition, milk fermentation by *Enterococcus faecium* also increased folate content.

In another study, the production of natural folates was evaluated using *Lc. lactis* subsp. *cremoris* to fortify skimmed milk and fruit juices (GANGDHARAN & NAMPOOTHIRI, 2014). The results showed that this microorganism was able to produce folate and enhance the

vitamin content in skimmed milk and in cucumber and water melon juice. To test different food matrices, not only milk, in order to enrich food with natural folate, it is important to develop new bio-enriched products since, due to lactose intolerance or milk proteins allergy, not everybody can consume dairy products. Fermented folate enriched fruit juices could be an alternative for this kind of consumers, as well as for vegetarians.

POMPEI et al. (2007) evaluated the production of folate by some *Bifidobacterium* strains with potentially probiotic properties. According to the results obtained, the best folate producers were *B. adolescentis* MB 115 (65 ng/mL) and *B. pseudocatenulatum* MB 116 (82 ng/mL). Even though the strains were cultivated in folate-free synthetic medium, their use as folate producers ought to be evaluated since probiotic bifidobacteria strains are commonly used in different food matrices and these might affect folate production. All this information is very significant since it has been suggested that the microbiota in the small and large intestine, which contains LAB and bifidobacteria, is able to produce folate that can be assimilated by the host (CAMILO et al., 1996, D'AIMMO et al., 2014). Prebiotics are defined as "selectively fermentable ingredients that allow specific changes in the composition and/or activity of gastrointestinal microbiota that allow benefits to the host" (GIBSON et al., 2004; GIBSON et al., 2010). PADALINO et al. (2012) studied the effect of galactooligosaccharides (GOS) and fructooligosaccharides (FOS) on folate production by some bifidobacteria, lactobacilli, and streptococci strains in milk. The authors observed that the milk containing fructooligosaccharides (FOS) and fermented by *B. catenulatum* (23.5 µg/100 mL) and *L. plantarum* (11.21 µg/100 mL), after 10 h of fermentation, showed the highest folate levels. On the other hand, when milk was supplemented with galactooligosaccharides (GOS), *S. thermophilus, B. adolescentis*, and *L. delbrueckii* were able to produce higher concentrations of folate than in milk supplemented with FOS. According to these results, the production of folate depends on the strain and the growth media, as mentioned previously. These results suggest that the consumption of prebiotics could selectively be used to increase folate production in the GIT.

Besides LAB, other microorganisms may produce folate and increase this vitamin content in foods. The fermentative process of rye dough may promote an increase in folate content. *Candida milleri* CBS8195, *Saccharomuces cerevisiae* TS 146, and *Torulaspora delbrueckii* TS 207 are sourdough yeasts evaluated by KARILUOTO et al. (2006) for their abilities to produce or consume folate. The researchers verified that folate content was increased by yeasts after sterilized rye flour fermentation. Since most studies using food-grade

microorganisms involve milk fermentation, the development of new products using different food matrices fortified with natural folate is an additional challenge in this area. In this sense, the applicability of yeast strain for bio-fortification of folates in white wheat bread was investigated by HJORTMO et al. (2008). White wheat bread is usually produced with commercial baker's yeast that is able to produce natural folate (27-43 µg/100g). However, when *Sacchacaromyces cerevisiae* CBS7764 was used by the authors, folate levels in white wheat bread were 3 to 5-fold higher. In this way, according to KARILUOTO et al. (2006) and HJORTMO et al. (2008) it is possible increase folate content in yeast fermented foods using specific yeast strains. However, it is also important to determine an efficient cultivation procedure to allow the maximum development and activity of the selected strain.

5. Folate analysis in food

5.1 Microbiological assay and tri-enzyme treatment

Folate quantification in food may be conducted using several methods. Nevertheless, the microbiological assay seems to be the only official method according to American Association of Analytical Chemists (AOAC). In this method (AOAC, 2006), the strain *L. rhamnosus* ATCC 7469 is used as the indicator strain to estimate total folate in food. For this purpose, bacterial growth in a 96-well microtiter plate is compared through turbidity given by optical density values of different samples after incubation. This technique is able to detect most of the folate natural forms. However, the response decreases when the number of glutamyl residues linked to the pteroyl group increases and the measurement of folates is complicated since there are many different forms of the vitamin. In order to measure all the polyglutamated forms it is important to enzymatically deconjugate them prior to analysis.

In this sense, the use of tri-enzyme treatment before folates measurement is essential for obtaining the maximum values of food folate since this vitamin, in food, is possibly trapped by carbohydrate and protein matrices (AISO & TAMURA, 1998; CHEW et al., 2012; IYER et al., 2009; TOMAR et al., 2009). The treatment includes the use of α-amylase and protease, besides the traditional treatment that uses pteroylpoly-γ-glutamyl hydrolase (AACC, 2000; CHEW et al., 2012).

After the use of tri-enzyme treatment, the amount of folate usually increases when compared to the traditional microbiological assay. IYER et al. (2009) evaluated the use of tri-enzyme method followed by the microbiological assay to determine the folate content of different Indian milk species. Buffalo milk showed the highest amount of folate (60 µg/L)

when compared to goat, cow, and sheep milk (10, 44, and 56 µg/L, respectively). According to TAMURA et al. (1997), the use of tri-enzyme treatment seems to show an essential rule to determine food folate content and the food folate tables should be updated after using this tri-enzyme methodology to accurately establish the dietary folate requirements in human. The instability of several folate forms (for example, tetrahydrofolate) promotes underestimated folate values in databanks and antioxidants like ascorbic acid are important as folate protectors during analysis (STRANDLER et al., 2015). Composition of foods and analytical procedures are difficulties faced by researchers to perform international folate content comparisons and to estimate the real intake of this vitamin (FAJARDO et al., 2012).

The use of commercial enzymes still shows an important barrier: their very high cost. Alternatives have been developed to make these assays cheaper. In this way, an in-house folate conjugase from chicken pancreas was prepared and tested to quantify the folate content present in several foods (SOONGSONGKIAT et al., 2010). The authors observed that single-enzyme treatment, using folate conjugase from chicken pancreas, may be used to deconjugate folate in some food matrices (i.e. soybean and asparagus); however, the tri-enzyme treatment was necessary to quantify total folate content in egg and whole milk powder. Total folate may be 20-30% higher after tri-enzyme extraction than after treatment with conjugase alone (RADER et al., 1998). The researchers also quantified the folate values after cooking and observed that cooked (boiled) soybean and asparagus retained about 75% and 82% of total folate. In this line, MAHARAJ et al. (2015) investigated the effect of boiling and frying on the retention of folate in some Fijian vegetables using microbiological assay and tri-enzyme treatment. The authors concluded that the boiling process promoted higher folate loss (10-64%) and that this fact might have been favored by water solubility of this vitamin.

As folate values may be underestimated due to the methods employed, YON & HYUN (2003) measured the folate content in several foods consumed by Koreans by microbiological assay, comparing the two extraction methods (single and tri-enzymatic). The values obtained by the authors are presented in Table 2. Folate contents obtained after tri-enzymatic treatment are apparently higher than those which did not receive this treatment. This observation shows the importance of using amylase and protease plus conjugase to recover higher values of the vitamin.

DIVYA & NAMPOOTHIRI (2015) used *Lc. lactis* CM28 as probiotic strain to ferment and fortify skimmed milk with natural folate. The addition of folate precursors, prebiotics, and reducing agents was performed to optimize the medium and, thus, the

extracellular folate was increased four folds. After deconjugation, the total folate value achieved 129.53 ± 1.2 µL. After 15 days of cold storage of fermented milk, about 90% of the folate produced was retained in the active form.

The folate content of six common food samples of Bangladesh (lentil, Bengal gram, spinach, basil, milk, and topa boro rice) was measured by microbiological assay using tri-enzyme extraction method (protease, α-amylase, and chicken pancreases as deconjugase). The highest folate contents were recorded for spinach (195 µg/100 g) and the lowest for milk (10 µg/100 g) (RAHMAN et al., 2015). IWATANI et al. (2003) also evaluated the folate content of vegetables commonly consumed in Australia. However, tri-enzyme treatment was not as efficient as single-enzyme extraction in the study since the vegetables samples investigated contain low amount of starch and protein and the highest reported folate level was 425 µg/100 g.

Table 2. Measurement of folate content in food after conjugase (CT) and tri-enzyme treatment (TT).

Food	Folate (µg/100g)		%increase[1]
	CT[2]	TT[2]	
Corn	100 ± 10	129 ± 5	29
Rice	5 ± 1	18 ± 3	260
Rice (cooked)	3 ± 1	8 ± 0	167
Wheat flour	6 ± 1	16 ± 2	167
Soybean	176 ± 31	318 ± 62	81
Soybean milk	16 ± 7	34 ±7	113
Potatoes	14 ± 2	27 ± 3	93
Cabbage	71 ± 20	135 ± 68	90
Carrot	29 ± 19	31 ± 19	7
Lettuce	46 ± 21	57 ± 19	24
Tomato	34 ± 4	52 ± 8	53
Apple (red)	5 ± 3	7 ± 6	40
Banana	16 ± 14	16 ± 7	0
Orange	47 ± 9	51 ± 4	9
Orange juice	31 ± 3	58 ± 6	87
Chicken's egg	36 ± 9	115 ± 18	219
Milk	6 ± 0	13 ± 1	117
Yogurt (curd type)	13 ± 4	24 ± 1	85
Yogurt (liquid type)	12 ± 5	32 ± 14	167

[1] % increase = (TT folate values – CT folate values)/ CT folate values x 100.

[2] Folate values expressed by mean of three different samples (duplicate) and respective standard deviations. Adapted from YON & HYUN (2003).

Studies of folate measurements in food usually showed folate values from raw vegetables, fruits, milk, and cereal-grain products (AISO & TAMURA, 1998; CHEW et al., 2012; YON and HYUN, 2003; RADER et al., 2000). Nevertheless, it is relevant to investigate the total amount of folate or the most important forms of this vitamin, as 5-methyltetrahydrofolate, present in other food products in order to determine real folate values for official nutritional tables.

5.2 Other analysis methods

As previously mentioned, microbiological assay is the method mostly used to determine folate content in foods, especially because this technique is the only one recognized as official (AOAC, 2006). Another method also usually employed for folate quantification is the high performance liquid chromatography (HLPC). In both cases, the use of tri-enzyme extraction, based on the use of amylase, protease, and folate conjugase, is necessary to determine the real total folate and/or folate forms food contents.

IYER & TOMAR (2013) compared the folate values obtained from three methods employed for quantification of this vitamin. Microbiological assay, Enzyme Linked Immuno Sorbent Assay (ELISA), and HPLC were used. According to the authors of the study, HPLC was the most sensitive method for folic acid determination while microbiological assay was highly efficient, sensitive, and reproducible, able to estimate total folate, which has supported the potential use of microbiological assay for dietary folate estimation. ELISA showed lower response for some folate derivates except for folic acid and dihydro folic acid. HPLC and liquid chromatography coupled with mass spectrometry is another method currently applied (ARAYA-FARIAS et al., 2014; PHILLIPS et al., 2011; TYAGI et al., 2015; VISHNUMOHAN et al., 2011). These methods can distinguish several forms of folate present in food samples while microbiological assay determines only the total folate content. Besides the methods discussed above, novel photosynthetic proteins-based devices - biosensors - have been developed for application in food analysis for folate measurement (INDYK, 2011).

6. Conclusions

In this chapter, foods bio-enriched with natural folates produced by microorganisms were discussed as promising alternatives for the low intake of this vitamin and might be considered with more attention by the food industry. In general, the development of novel fermented foods with increased folate content due to a fermentative process would raise the commercial and nutritional value of these products and could replace food fortifications using chemically synthesized vitamin, which would therefore become unattractive targets. The production of folate by microorganisms, such as LAB, is strain-dependent and can be affected by environment conditions, such as pH. Therefore, the proper selection of folate-producing strains might be useful for the development of new functional foods. Although most of the studies have assessed the production of folate in fermented milk, other food matrices might also be bio-enriched with natural folate, such as bread, kefir, vegetables, and fruit juices, since all these foods are produced through a fermentation process. Additionally, the folate content determination in foods using more sensitive methods, such as the tri-enzymatic treatment, would be stimulated since they may provide more accurate values of this vitamin in different food matrices.

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CHAPTER

.2.

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

Abstract

The ability of two starter cultures (*Streptococcus* (*S.*) *thermophilus* ST-M6 and *S. thermophilus* TA-40) and eleven probiotic cultures (*S. 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.

Keywords: Folate, probiotic, fruit by-products, okara, amaranth, fermentation

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 & 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 & NAMPOOTHIRI, 2011; IYER et al., 2009; LAIÑO et al., 2013a, 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, amongst 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 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., 2010, 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 & NAMPOOTHIRI, 2011; HOLASOVÁ et al., 2005; PALADINO 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; STANOJECIV 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 ºC 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 (less than 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, 2012b) with some modifications. The fruit by-products were thawed at 4 ± 2 ºC for 48 h, washed and bleached using clean water at 100 ºC (12 min) followed by ice bath. Then, the fruit by-products were dried in oven under air flow at 60 ºC 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 (less than 42 µm). All powders were stored in polypropylene bags and kept at -18 ± 2 ºC 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 ºC 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_4$7H2O (0.18 g; Merck), $MnSO_4$H2O (0.05 g; Merck), Na_2SO_4 (2 g;

Labsynth), K_2SO_4 (1.25 g; Labsynth), Na_2CO_3 (0.2 g; Labsynth), $CaCl_2.2H_2O$ (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.

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		
Lb. fermentum	PCC*	2		
Lb. reuteri	RC-14*	2	MRS[b]	Anaerobic[d]
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	Anaerobic[d]
B. longum	BB-46*	2	cysteine	
B. longum subsp. *infantis*	BB-02*	2	(0.05%)[c]	

*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).

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 37ºC. An aliquot of 1 mL was taken from the second growth, centrifuged (10000 *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% (m/v) of each irradiated powder. Samples were taken before (0 h) and after 24 h incubation at 37ºC 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 uL) 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 ºC) 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 ºC 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 ºC in MRS broth with 20% of glycerol, was inoculated twice in fresh MRS broth and incubated at 37 ºC 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 ºC 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 x 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 ºC) 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 ºC 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® (MINITABInc., USA) using one way ANOVA followed by a Tukey's posthoc 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 Figure 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) (Figure 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).

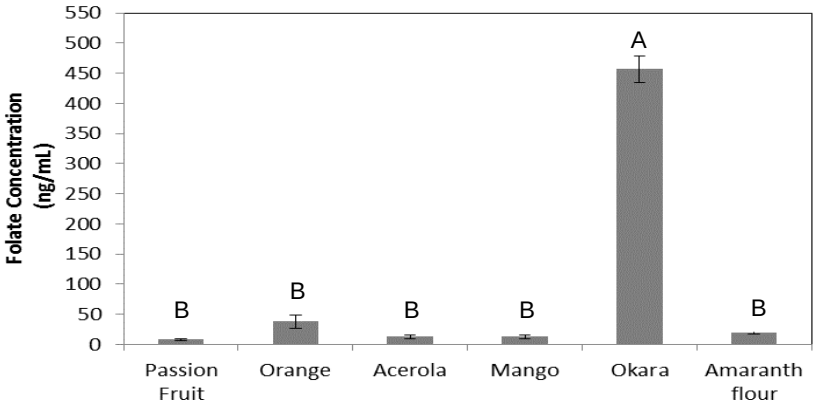

Figure 1. Folate concentration in vegetable by-products and amaranth flour. [A,B] Different capital letters denote significant differences between the tested by-products and/or amaranth flour.

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% (m/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 *S. thermophilus* TH-4 and *Lb. paracasei* subsp. *paracasei* F-19 that consumed it (Figure 2).

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.

Strains	Δ Folate (ng/mL)*					
	Fruit by-product				Okara soybean by-product	Amaranth Flour
	Passion Fruit	Orange	Acerola	Mango		
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}

*ΔFolate = Folate T24 (ng/mL) – Folate T0 (ng/mL);

T0= initial concentration of folate; T24= final concentration of folate.

[a,b] Within a row, different superscript letters denote significant differences between the tested by-products and/or amaranth flour for each strain.

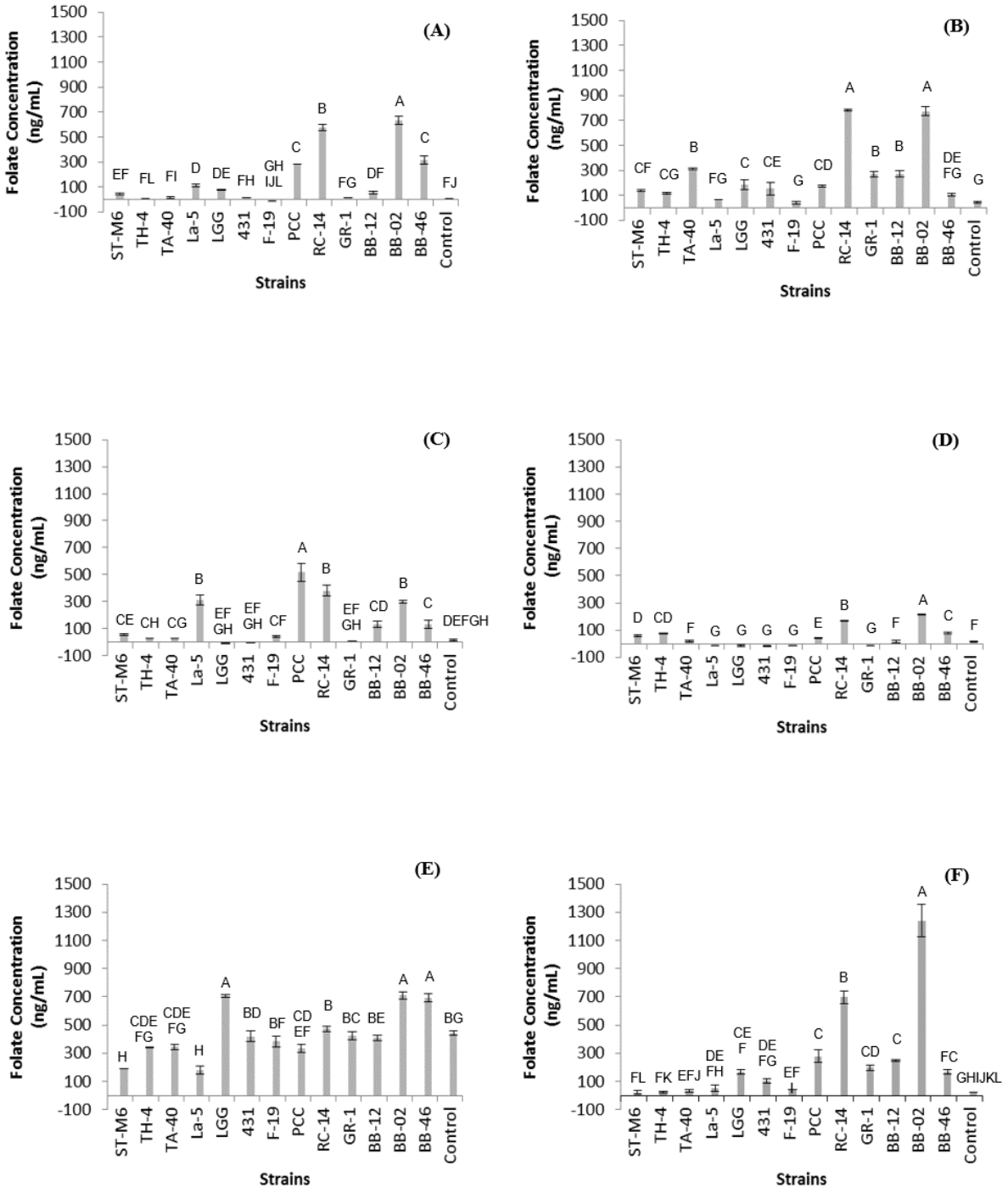

Figure 2. Folate production by starter and probiotic strains* after 24 h of fermentation of a Modified MRS Phenol Red Broth supplemented with fruit by-products, soybean okara by-product and amaranth flour. (A) Modified MRS Phenol Red Broth supplemented with 1% of passion fruit by-product, (B) Modified 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.

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/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 37ºC (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 *S. thermophilus*, none of the three strains tested produced folate after fermentation of okara (Figure 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 (Figure 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 *S. 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 vitamin in different fermented milk products. The orange by-product showed the best impact on folate production by the *S. 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 & 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., 2010; LAIÑO et al., 2012; LAIÑO et al., 2013a; PADALINO et al., 2012; POMPEI et al., 2007; SYBESMA et al., 2003). LIN & 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). *Lb. 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., 2016).

PADALINO et al. (2012) evaluated the effect of two prebiotic fibres (fructooligosaccharide and galactooligosaccharide) on folate production by strains of *S. 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 & 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. bifidum* 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.

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CHAPTER

.3.

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

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 fructo-oligosaccharides (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.

***Keywords*:** Folate, probiotic, passion by-product, FOS, fermented soymilk

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 & 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 & 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 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 ºC 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 ºC 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) (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 & TAMURA, 2013).

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).

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	$8.7 \pm 0.1^{A\,B}$	8.5 ± 0.0^{B}	$8.6 \pm 0.12^{A\,B}$	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}	$8.4 \pm 0.1^{A\,B}$	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.

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.

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 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*	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. * Non fermented soymilks

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 Figure 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 (Figure 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 (Figure 1).

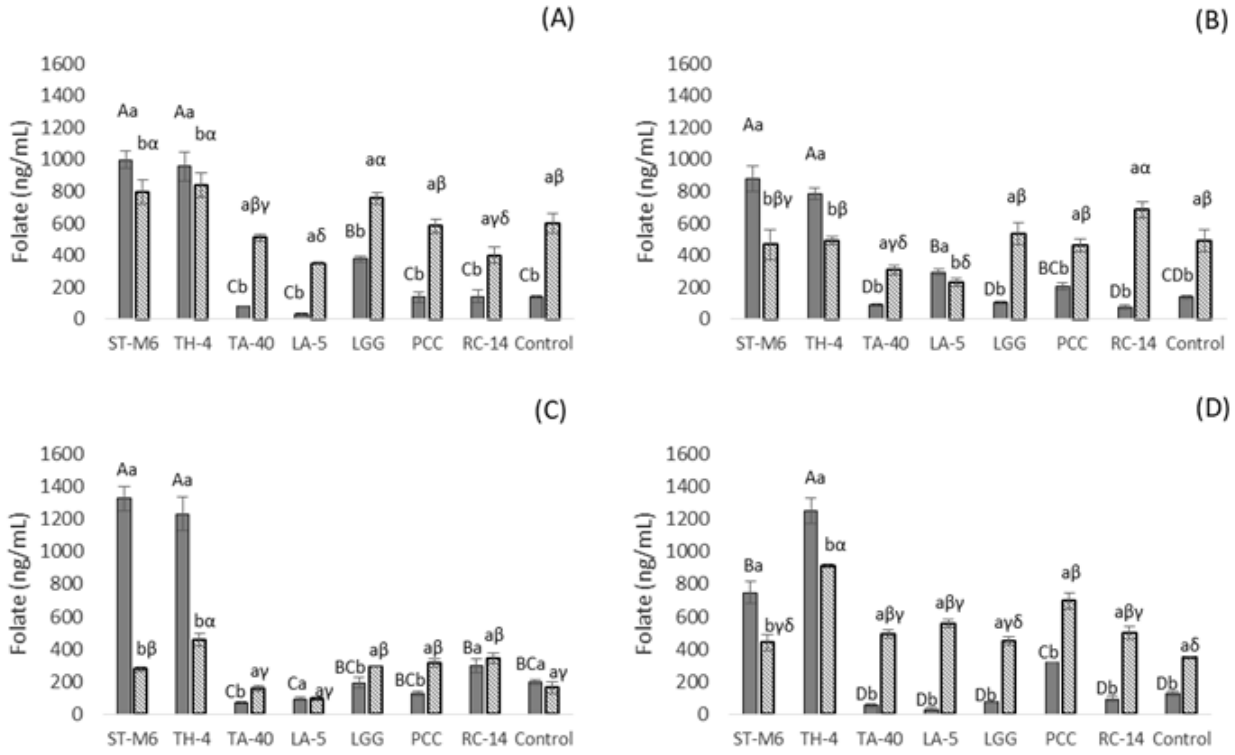

Figure 1. Total folate content of different soymilk formulations after 24 h of fermentation by starter and probiotic strains as pure cultures. Traditional microbiological assay (grey bars); Tri-enzymatic treatment (textured bars). ^{A,B} Different capital letters denote significant differences between 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.

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 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.

The folate levels produced by different co-cultures inoculated in the soymilk formulations after 24 h of fermentation are shown in Figure 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.

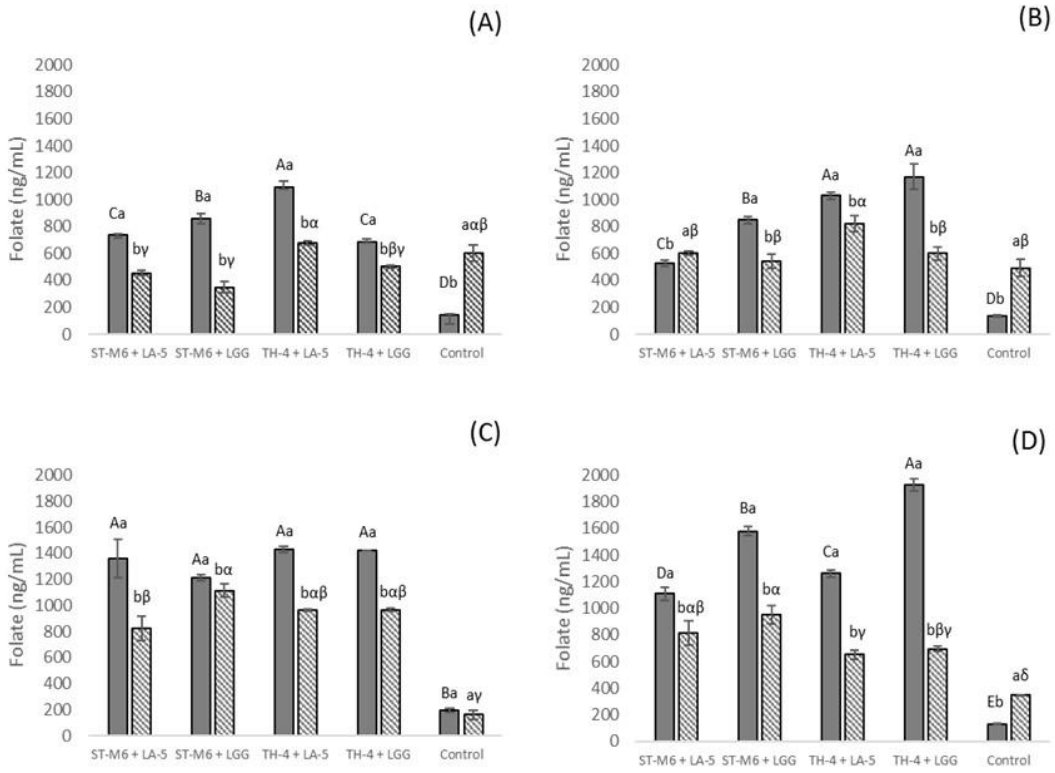

Figure 2. Total folate content of different soymilk formulations after 24 h of fermentation by *Lactobacillus* spp. strains with *Streptococcus thermophilus* strains. Traditional microbiological assay (grey bars); Tri-enzymatic treatment (textured bars). ^{A,B} Different capital letters denote significant 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.

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 ($P < 0.05$).

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 Figure 1 and Figure 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 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 & 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. 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 & 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 & 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 & 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 400µg for a normal adult. One portion (100mL) 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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CHAPTER

.4.

Fermented soy products bio-enriched with folates and containing probiotic Lactobacillus rhamnosus LGG may improve the bioaccessibility of folate under in vitro simulated gastrointestinal digestion

Abstract

This study aimed to evaluate the effect of supplementation of a fermented soy product (FSP) with passion fruit by-product (PFBP) and/or fructooligosaccharides (FOS) on the viability and resistance of *Streptococcus thermophilus* TH-4 and *Lactobacillus rhamnosus* LGG to *in vitro* simulated gastrointestinal conditions during storage. The folate content and bioaccessibility under simulated gastrointestinal digestion during FSP storage was also evaluated. Four FSP containing or not PFBP and/or FOS were studied and had their kinetic parameters determined during fermentation, as well as pH, viability, and folate contents before and after fermentation and during 28 days of storage at 4ºC. Survival of *Lb. rhamnosus* LGG and folate bioaccessibility during simulated gastrointestinal conditions were also evaluated. No differences (p<0.05) between 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 viability (> 8 log CFU/mL) during storage, whereas *St. thermophilus* TH-4 populations decreased by day 14. 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 bioaccessibility. 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 bioaccessibility in the gut.

Keywords: probiotic, folate, passion fruit by-product, fructooligossaccharides, fermented soy product, bioaccessibility

1. Introduction

The consumption of soy products has increased not only as an alternative to dairy products but 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 also 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* 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, not only for the improvement of soy unsavoury and the decrease in the anti-nutritional substrates present, but also leads 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. According to HILL et al. (2014), probiotics are "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host".

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 fructooligossacharides (FOS) and other potential prebiotics, such as 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 also, 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 hematological manifestations of vitamin B12 deficiency (BAILEY & 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 soymilks 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. Bioaccessibility 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 & RYCHLIK, 2017). Therefore, this study aimed to evaluate the effect of the supplementation of a fermented soy product with passion fruit by-product and/or FOS on the viability and resistance of *Streptococcus thermophilus* TH-4 and *Lactobacillus rhamnosus* LGG in the product to simulated gastrointestinal conditions during 28 days of storage (4 °C). Additionally, the folate content of the fermented soy products was evaluated during the storage period, as well as the folate bioaccessibility under *in vitro* simulated gastrointestinal digestion.

2. Material and Methods

2.1 Microorganisms and growth conditions

Streptococcus (St.) thermophilus TH-4 and *Lactobacillus (Lb.) rhamnosus* LGG (Chr. Hansen, Copenhagen, Denmark) stored at -80 ºC, 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 37º C for 24h. Next, each microbial culture was mixed using a vortex, transferred to 250 mL of their respective fresh broths, and incubated at 37 ºC for 24h. Each final microbial culture (262.5 mL) was centrifuged (22,000g 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 (item 2.2).

2.2 Production of the fermented soy products

Three different batches of four different formulations of fermented soy products (FSP) described in Table 1 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 (PSM), (2) fermentation of 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 (< 42μm), 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 semi-industrial mixer Thermomix® (model TM31-127V, Vorwerk, Wollerau, Switzerland).

A volume of 1 L of a ultra-high temperature (UHT) treated commercial soymilk (Pura Soja, Mais Vita, Yoki®, Pouso Alegre, MG, Brasil) was heated until 50 ºC 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 ºC, 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 ºC, 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 ºC 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 item 2.1) were added to each pasteurized soy mixture. Then, each PSM was transferred to two sterile flasks (500 mL) for fermentation at 37 ºC using a water-bath equipment coupled to a CINAC system (*Cynetique d'acidification*, Ysebaert, Frépillon, France). The fermentation was performed until each PSM achieved pH 5.5. Next, the fermented soy mixture (FSM) was stored at 4 ºC for 18h approximately. Concentrated passion fruit juice (12.5% w/w) was added to the FSM and mixed using the semi-industrial mixer Thermomix® (model TM31-127V, Vorwerk). Aliquots of 35 g of each FSP were packaged using appropriated polypropylene plastic pots for food products (Tries Aditivos Plásticos, São Paulo, Brazil) and sealed with metallic covers with varnish in a sealer (Delgo Nr. 1968, Delgo Metalúrgica, Cotia, Brazil). The pots containing the different FSP were stored at 4 ºC for up to 28 days.

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

Ingredients	**Soy mixture formulations****			
(g/100 mL of commercial soy milk*)	**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

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

2.3 Kinetic parameters

Considering the fermentation of each FSP, maximum acidification rate (V_{max}) was established as the time variation of pH (dpH/dT) and expressed as 10^{-3} pH units/min. Additionally, 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 4ºC. The folate concentration of each FSP was determined at days 1, 14, and 28 of storage. The survival of *St. thermophilus* TH-4 and *Lb. rhamnosus* LGG and the bioaccessibility of folate during the *in vitro* simulated gastrointestinal conditions for each FSP were determined at days 1, 14, and 28 of storage.

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 was plate counted in M17 agar (Oxoid) supplemented with sterile lactose solution 10% (w/v) and *Lb. rhamnosus* LGG in MRS agar (Oxoid). All strains were incubated at 37 ºC for 48 h. Considering that both strains grew in co-culture, lactobacilli strains were incubated anaerobically (Anaerobic System Anaerogen, Oxoid) in order to be able to differentiate streptococci and lactobacilli colonies.

2.6 Determination of pH values and folate content

The pH values of the FSP were determined in triplicates (three different pots of the same batch, a total of 6 pots for each formulation) with a pHmeter Orion, Three Stars model (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). Briefly, 500 µL from each FSP or each gastrointestinal phase (item 2.5.1) was transferred to a sterile microtube and completed with 500 µL of protection buffer (0.82 g/100

mL of sodium acetate with 1 g/100 mL of ascorbic acid). The resulting mixture (1 mL) was mixed and boiled (100 ºC) for 5 min. Then, the mixture was cooled and centrifuged (10,000 *g* for 5 min). The supernatant was collected aseptically and stored at -80 ºC for total folate determination.

The indicator strain was cultivated in MRS broth (37ºC for 24h). A volume (1mL) of the grown indicator strain was washed three times using sterile saline solution 0.85% (NaCl, w/v). An aliquot of 120 µL of the washed indicator strain was inoculated in 3 mL of Folic Acid Casei Medium (FACM) and incubated at 37 ºC for 24h. This step was repeated twice and, from the second FACM, an aliquot of 1 mL containing the indicator strain was washed three times with sterile saline solution and 480 µL of the washed indicator strain was added to 12 mL of fresh two-fold FACM. Next, 100 µL of this inoculum was plated into each well of a 96-well microplate. A standard curve was prepared using HPLC grade folic acid (BioChemica, Sigma Aldrich, Switzerland) and phosphate buffer 0.1 M (pH 6.6-6.8). Samples stored at -80 ºC were thawed and diluted using the same phosphate buffer. The microplate was incubated at 37 ºC for 48h and then 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 *In vitro* simulated gastrointestinal conditions

2.7.1 Survival of microorganisms and evaluation of folate bioaccessibility

The survival of *St. thermophilus* TH-4 and *Lb. rhamnosus* LGG during simulated gastrointestinal conditions *in vitro* was performed according to LISERRE et al. (2007) with modifications suggested by BURITI et al. (2010) and BEDANI et al. (2013). Additionally, to evaluate the bioaccessibility of folate in the final product (0 hours of assay) and from each gastrointestinal phase (gastric, enteric I, and enteric II, respectively, after 2, 4, and 6 hours 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 item 2.4) released from each FSP in each phase of the *in vitro* simulated gastrointestinal conditions 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).

Briefly, 25 g of each FSP were mixed with 225 mL of sterile saline solution 0.85% (w/v) in triplicates. From each mixture, 10 mL were transferred to 9 sterile flasks (3 flasks per replicate). Next, the pH was adjusted to 1.5-2.5 using 1N HCl (Merck) and a solution containing pepsin (from porcine stomach mucosa, Sigma Aldrich) and lipase (Amano lipase G, from *Penicilium camemberti*, Sigma Aldrich) in sufficient amounts to reach a concentration of 3 g/L and 0.9 mg/L, respectively, per flask. All 9 flasks were incubated at 37 ºC, with agitation of 150 rpm (Metabolic Water Bath Dubnoff MA-095, Marconi, Piracicaba, Brazil) during 2 h, characterizing the simulated gastric phase. After this period, 3 flasks were removed from incubation to proceed with the analysis (microorganisms survival and folate bioaccessibility).

In the next step, to increase the pH (5.0-5.5) of the 6 remaining flasks, an alkaline solution [6g of micro-pearl NaOH (Synth, Diadema, Brazil) and 14 g of $PO_4H_2Na.2H_2O$ (Synth) and distilled water up to 1 L] containing sufficient amounts of bile (bovine bile, Sigma Aldrich) and pancreatin (pancreatin from porcine pancreas, Sigma Aldrich) were added to each flask to reach a final concentration of 10 g/L and of 1 g/L, respectively. Samples were incubated again at 37 ºC for more 2 h under agitation (total of 4 h of incubation), leading to simulated enteric I phase. After this period, 3 flasks more were removed from incubation to proceed with the analysis.

In the last step, the 3 remaining flasks were removed and the pH was increased to 6.5-7.5 using the same alkaline solution containing bile and pancreatin to maintain the final concentration of 10 g/L and 1 g/L, respectively. The 3 flasks with samples were incubated again at 37 ºC for more 2 h under agitation, leading to simulated enteric II phase and reaching 6 h of assay. After this period, the last 3 flasks were removed from incubation to proceed with the analysis.

Viable cell counts of *St. thermophilus* TH-4 and *Lb. rhamnosus* LGG were performed to determine the survival of the microorganisms. For this, aliquots (1 mL) were collected from triplicate samples from each gastrointestinal phase, after 2 h, 4 h, and 6 h (three different flasks of the same trial for each time) and were pour-plated in specific culture media for each microorganism as described previously (item 2.4). The adjustment of the dilutions was done properly and the results were presented as log CFU/g of FSP.

2.8 Statistical analyses

Three independent batches for each FSP were produced. The experiments were carried out in triplicates and all values were expressed as means ± standard deviations (SD). Statistical analyses were performed with Minitab 17 Statistical Software® (MINITABInc., USA) using one way ANOVA followed by a Tukey's posthoc test. Comparison between two different means were assessed by *Student's t-test*. Differences were considered statistically significant at $p < 0.05$.

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 the four FSM ($p < 0.05$) at the end of the fermentation period. Additionally, no significant difference ($p < 0.05$) was observed between the time that each PSM achieved V_{max} (T_{max}) or the time to reach the pH 5.5 (T_f) and complete fermentation. 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).

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 ± 0.17^{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 ± 0.14^{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 ($P < 0.05$).

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

At the end of the storage period, all FSP presented a slight pH decrease that was statistically different from the initial pH at day 1 ($p < 0.05$) (data not shown). Regarding the pH behavior along storage, significant differences were observed for the same FSP during 28 days of storage. The pH values ranged from 4.6 to 4.4 except for FSP4, which pH ranged from 4.6 to 4.5 being slightly higher and significant different ($p < 0.05$) from the other FSP samples at day 28. Apparently, a slight post-acidification was observed during the storage period.

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 (data not shown). Along the storage period evaluated, a decrease in the *St. thermophilus* population was observed for all FSP, particularly after 21 days of storage. FSP4 presented the highest *St. thermophilus* populations during the entire period of storage ($p < 0.05$).

Regarding the viable counts of *Lb. rhamnosus* LGG during the storage period evaluated, this microorganism was viable during the entire period with counts always above 8 log CFU/mL for all FSP.

Table 3. Viable cell counts of *St. thermophilus* TH-4 and *Lb, rhamnosus* LGG in different fermented soy products during storage at 4 ^{o}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 ($P < 0.05$).

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

According to Figure 1, the pasteurized soy mixture supplemented with FOS presented the highest folate content (227 ± 0 ng/mL) followed by the pasteurized soy mixture supplemented with passion fruit by-product (180 ± 8 ng/mL), pasteurized soy mixture (169 ± 4 ng/mL), and pasteurized soy mixture supplemented with passion fruit by-product and FOS (160 ± 7 ng/mL). At the end of each pasteurized soy mixture fermentation (at pH 5.5), the fermented soy mixture supplemented with both passion fruit by-product and FOS presented the highest folate content (617 ± 58 ng/mL) and was significant different ($p < 0.05$) from the

fermented soy mixture supplemented with FOS (376 ± 10 ng/mL), fermented soy mixture (333 ± 23 ng/mL), and fermented soy mixture supplemented with passion fruit by-product (317 ± 30 ng/mL). The folate content of the last three fermented soy mixtures was not significantly different ($p > 0.05$).

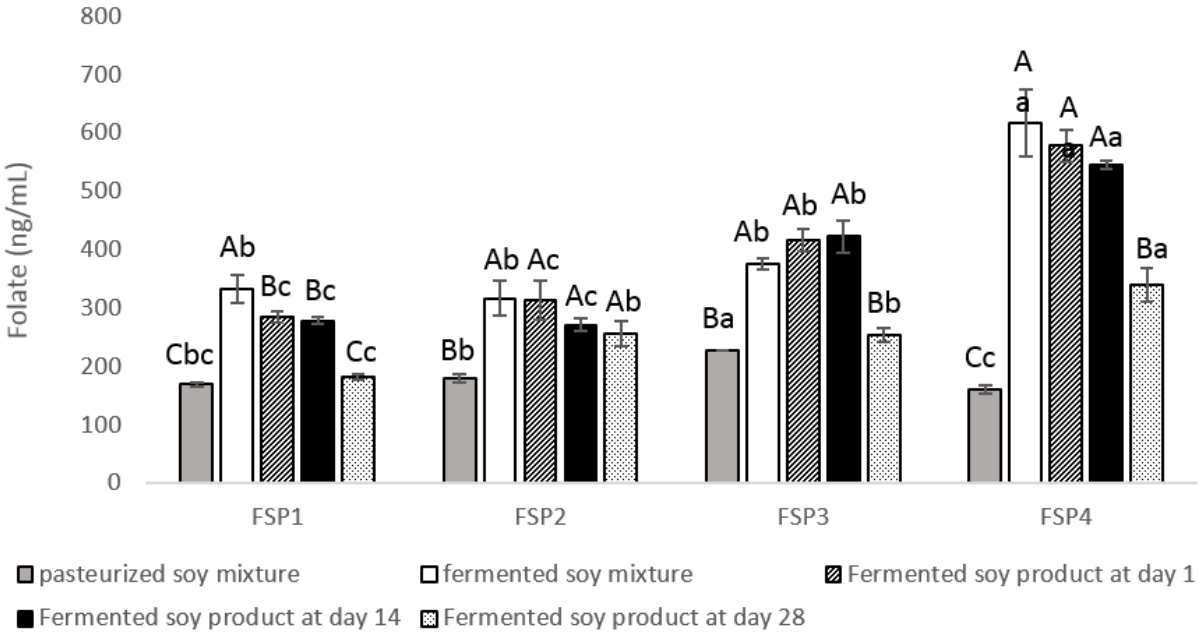


Figure 1. Folate concentrations in fermented soy products during production and shelf-life at 4 ^{o}C. FSP1: Fermented Soy Product. FSP2: Fermented Soy Product supplemented with 1% (w/v) of passion fruit by-product powder. FSP3: Fermented Soy Product supplemented with 1% (w/v) of fructooligosaccharides. 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 ($P < 0.05$).

In general, during the storage period, the folate content of all FSP decreased (Figure 1). After mixing the FSM with concentrated passion fruit juice, the folate content of all FSP was determined in the following day (day 1) and FSP4 presented the highest folate content (578 ± 28 ng/mL) followed by FSP3 (417 ± 19 ng/mL). At day 14, although a significant decrease in the folate content of FSP4 was observed when compared to day 1, 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

the storage period (day 28), the folate content presented by FSP4 (340 ± 28 ng/mL) and FSP2 (256 ± 21 ng/mL) was significantly higher ($p < 0.05$) than the vitamin content 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 significantly different when their folate concentrations were compared to their respective pasteurized soy mixtures. All FSP presented higher folate content at days 1 and 14 of storage when compared to their respective pasteurized soy mixtures ($p < 0.05$).

A comparison of changes in the folate content of soy mixtures after pasteurization and fermentation and of all FSP during the storage periods (days 14 and 28) with the pasteurized soy mixture are shown in Table 4. A slight but significant difference ($p < 0.05$) among all pasteurized soy mixtures was observed. The comparison of changes in the folate content of day 1 and the pasteurized soy mixture was not presented because there was no significant difference from the folate values presented by the comparison of changes of the fermented soy mixture with the pasteurized soy mixture ($p > 0.05$). According to Table 4, the folate content after the fermentation of PSM4 and during its entire storage period was higher and statistically different when compared to the other FSP for the same periods of analysis ($p < 0.05$). Depending on the formulation, the FSP folate content may increase approximately 3.5-fold when compared to the pasteurized unfermented soy mixtures and FSP4 presented the highest bio-enrichment with the highest increase in the folate content ($p < 0.05$).

Table 4. Comparison of changes (from pasteurized soy mixture to fermented soy mixture, 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)	Δ Folate (ng/mL)* $\text{Folate}_{\text{FSM}} - \text{Folate}_{\text{PSM}}$	Δ Folate (ng/mL)** $\text{Folate}_{\text{FSP D14}} - \text{Folate}_{\text{PSM}}$	Δ Folate (ng/mL)*** $\text{Folate}_{\text{FSP D28}} - \text{Folate}_{\text{PSM}}$
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}

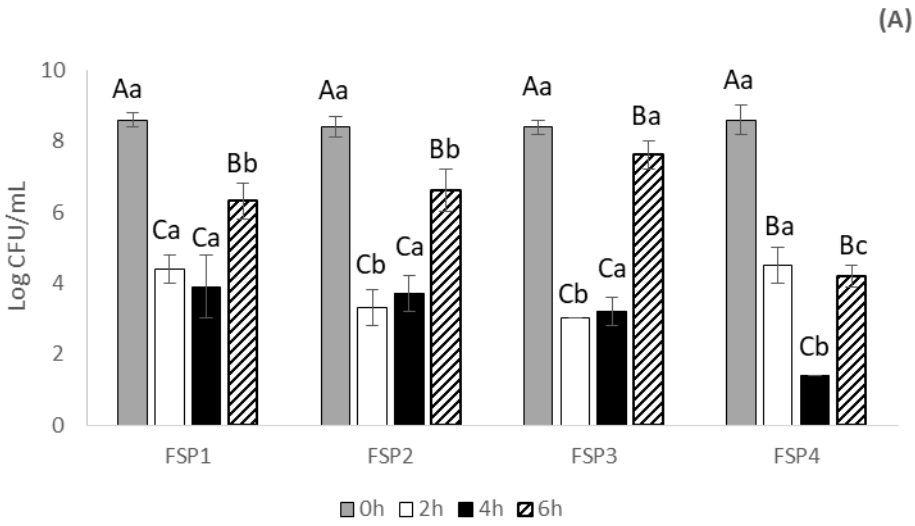
(Caption on next page)

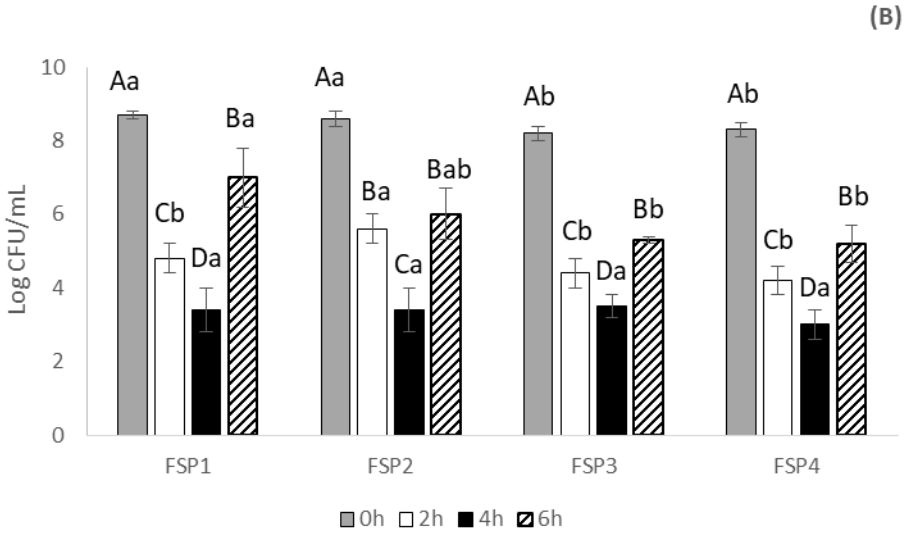
*ΔFolate = Folate $_{FSM}$ (ng/mL) – Folate $_{PSM}$ (ng/mL); **ΔFolate = Folate $_{FSP\ D14}$ (ng/mL) – Folate $_{PSM}$ (ng/mL); ***ΔFolate = Folate $_{FSP\ D28}$ (ng/mL) – Folate $_{PSM}$ (ng/mL); FSM: fermented soy mixture; PSM: pasteurized soy mixture; FSP D14: fermented soy product at day 14; FSP D28: fermented soy product at day 28. 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 ($P < 0.05$).

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 and the results are presented in Figure 2. In general, the highest reductions for all FSP during the period of analysis were observed during the gastric and enteric I phases, followed by an increase in the recovery of *Lb. rhamnosus* LGG in the end of the simulated digestion. At days 1, 14, and 28, the highest recovery of *Lb. rhamnosus* LGG was observed for FSP3, FSP1, and FSP2, respectively.

It was not possible to detect any viable cells of *St. thermophilus* TH-4 during the *in vitro* gastrointestinal assay using the plate count method. The same result was observed when a fermented milk 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 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 0h to 6h of digestion, whereas for FSP4, the reduction in the lactobacilli population (4.5 ± 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 formulations. 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.





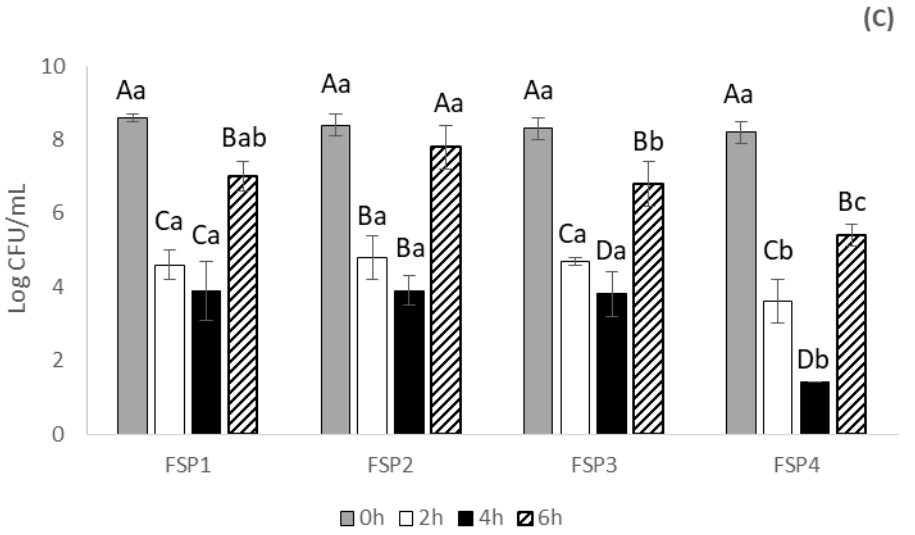


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Figure 2. Survival of *Lactobacillus rhamnosus* LGG (log CFU/mL) in fermented soy products submitted to *in vitro* simulated gastrointestinal conditions during storage at 4 ^{o}C for 1, 14, and 28 days (A, B, and C, respectively). FSP1: Fermented Soy 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. **0h:** lactobacilli populations before the *in vitro* simulated digestion. **2h:** *in vitro* simulated gastric condition. **4h:** *in vitro* simulated enteric I condition. **6h:** *in vitro* simulated enteric II condition. ^{A,B} Different capital letters denote significant differences between different sampling periods (0, 2, 4, and 6h) 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 ($P < 0.05$).

Table 5. Comparison of changes in the *Lactobacillus rhamnosus* LGG populations of each fermented soy product during 6 hours of the *in vitro* simulated gastrointestinal conditions.

Formulations	$\Delta_{LGG\ (log\ cfu/mL)}$ = T6h $_{LGG\ (log\ cfu/mL)}$ − T0h $_{LGG\ (log\ cfu/mL)}$		
	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}	2.56 ± 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	4.58 ± 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 ($P < 0.05$).

3.5 Determination of folate bioaccessibility 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 Figure 3. In general, there was an increase in the folate content during the simulated gastrointestinal phases considering all FSP formulations during the entire period of analysis. 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 formulations. At day 28, both FSP1 and FSP3 presented a similar concentration of folate at each gastrointestinal phase.

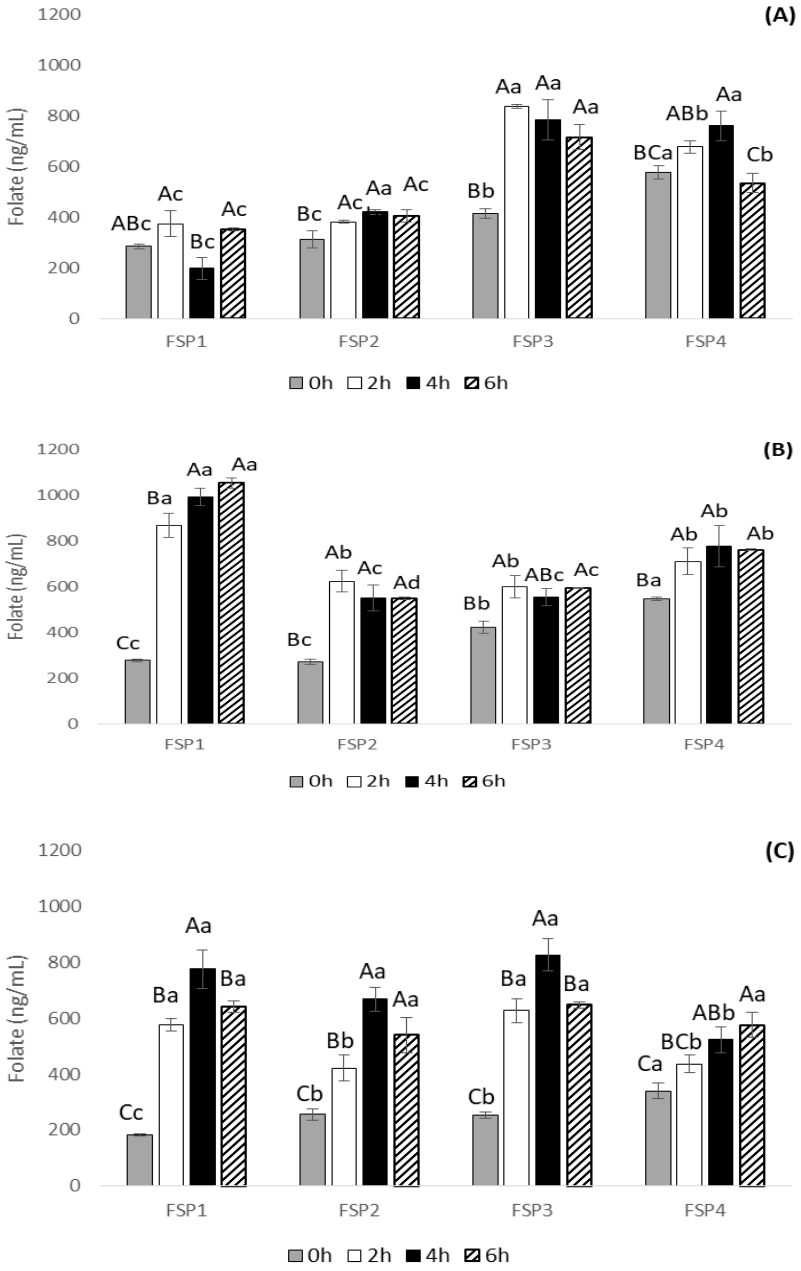

Figure 3. Folate concentration in fermented soy products submitted to *in vitro* simulated gastrointestinal conditions during storage at 4 ^{o}C for 1, 14, and 28 days (A, B, and C, respectively). FSP1: Fermented Soy Product. FSP2: Fermented Soy Product supplemented with 1% (w/v) of passion fruit by-product 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. **0h:** folate content before the *in vitro* simulated digestion. **2h:** folate content at the end of the *in vitro* simulated gastric condition. **4h:** folate content at the end of the *in vitro*

simulated enteric I condition. **6h:** 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 ($P < 0.05$).

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	Δ Folate (ng/mL)* after 2h (Enteric I phase)			
	FSP1	**FSP2**	**FSP3**	**FSP4**
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 4h (Enteric II phase)**			
	FSP1	**FSP2**	**FSP3**	**FSP4**
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}

***Δ Folate (ng/mL) = Folate Enteric I (ng/mL) – Folate T0 (ng/mL) ****Δ Folate (ng/mL) = Folate Enteric II (ng/mL) – Folate T0 (ng/mL); 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} Withing 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 ($P < 0.05$).

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 gastrointestinal conditions increased the bioaccessibility 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; ESPIRITO-SANTO et al., 2012; VIEIRA et al., 2017). Supplementation of soymilk with fruit by-products or commercial prebiotics provides additional carbohydrates content as energy sources, which may result in an increased metabolic activity of starter and probiotic microorganisms. Previously, passion fruit by-product and FOS were used to stimulate the growth of different strains of *St. thermophilus* and *Lactobacillus* spp. and the folate production by these microorganisms during soymilk fermentation (ALBUQUERQUE et al., 2017).

In the present study, four different FSP were prepared using the starter *Streptococcus thermophilus* TH-4 and the probiotic *Lactobacillus rhamnosus* LGG in co-culture to produce a bio-enriched probiotic fermented soy product with natural folates. This microbial combination produced the highest amounts of folate in fermented soymilks especially when supplemented with passion fruit by-product and with the combination of passion fruit by-product and FOS (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 growth and metabolic activity 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 on the *St. thermophilus* growth.

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 FSP (data not shown). The profile and amounts of soymilk carbohydrates may have influenced the growth and the metabolism of lactic acid bacteria. According to CHAMPAGNE et al. (2009), soymilk 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 streptococci 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 in soymilks probably due to a microbial symbiosis (ALBUQUERQUE et al., 2017; CHAMPAGNE et al., 2009). Besides, the folate content produced during the 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 soymilks and that passion fruit by-product and FOS stimulated this production, especially when both microorganisms were grown in co-culture. In our study, the folate contents produced during fermentation of all FSP was widely lower when compared to those produced by the same co-culture in fermented soymilks (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 6-8 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 according to KAPLAN & HUTKINS (2002). 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 24h of soymilks 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 LGG viable and presenting counts above 8 log CFU/mL until the end of the storage period. Another possibility to explain the decrease in the folate content of 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 the storage period, the folate levels of FSP2 and FSP4 at day 28 were higher than the vitamin content 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 amounts of folate. 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 more than 10% of the vitamin compared to the initial concentration of the vitamin for each FSP. At day 14, FSP4 presented an increase of approximately 3.5-fold compared to the initial vitamin content presented by its 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 *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 diarrhea, 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.

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 the 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).

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 the streptococci 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 bioaccessibility 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

bioaccessibility 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 bioaccessibility 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 bioaccessibility of nutrients and are therefore representative for *in vivo* bioavailability in humans. In this study, although FSP4 presented an important increase on the folate content during the gastrointestinal phases contributing to increase the vitamin bioaccessibility (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 (ARKBAGE et al., 2003).

ARKBAGE 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 bioaccessibility of folic acid and (6S)-5- metyltetrahydrofolate 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 soymilks 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 our study, it is important to highlight the increase in the folate bioaccessibility 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 the storage period, probably by the consumption of the vitamin by *Lb. rhamnosus* LGG, during the *in vitro* digestion the folate content increased, being significantly different from the initial folate content (0h) observed at day 1, 14, and 28 of the *in vitro* simulated gastrointestinal assay. Therefore, if the concentration of folate of each FSP before

digestion is considered, an increase of at least 3.5-fold (approximately) was observed, depending on the FSP tested regarding the folate content for day 14 of storage. If the bioaccessibility 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 bioaccessibility of the folate content 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 bioaccessibility 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 bioaccessibility and was important to establish *tempe* as a good source of folates.

5. Conclusions

Our study showed that the use of *St. thermophilus* TH-4 and *Lb. rhamnosus* LGG contributed to bio-enrich different fermented soy products (FSP) with natural folates. The bio-enrichment was increased when passion fruit by-product and FOS were used together to supplement the pasteurized soy mixture achieving, at day 14, about 3.4-fold more folate than its unfermented soy mixture. According to FAO/WHO (2004), the recommended folate intake for an adult is 400 µg/day, which is in agreement with the Brazilian folate intake recommendation (ANVISA, 2017). Therefore, considering the decrease in the folate content during the storage period, it is suggested that all FSP should be consumed up to day 14 of storage. Until this period, FSP may represent a good source of folate contributing with approximately 14% of the recommended daily intake (RDI) if supplemented with both passion fruit by-product and FOS. This percentage may increase if folate bioaccessibility is considered for FSP without supplementation (FSP1) and FSP supplemented with passion fruit by-product and FOS together (approximately 25% and 19% of recommended daily intake, respectively). Therefore, the FSP evaluated in our study may be considered as a good source of folate, especially if supplemented with passion fruit by-product and FOS. Additionally, FSP protected *Lb. rhamnosus* LGG during the *in vitro* simulated gastrointestinal conditions

and this probiotic microorganism was viable during the entire storage period. It is important to point out that folate bioavailability must be further assayed using *in vivo* clinical trials to elucidate if the FSP developed in this study may really contribute for the folate human requirements and if *Lb. rhamnosus* LGG may contribute to increase folate absorption in the small intestine.

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CHAPTER

.5.

Tropical fruit by-products water extracts as sources of soluble fibres associated to phenolic compounds with potential antioxidant, anti-inflammatory, and functional properties

Abstract

Phenolic content, antioxidant and anti-inflammatory activities were evaluated for water extracts of fruit by-products (passion fruit, orange, acerola, and mango). The impact of these extracts on microbial growth, folate production, and adhesion ability of *Streptococcus thermophilus* TH-4 and *Lactobacillus rhamnosus* LGG were investigated. Mango water extract (MWE) presented the highest phenolic content and antioxidant activity. Orange water extract (OWE) presented the greatest phenolic diversity. OWE and MWE showed the highest anti-inflammatory effects on lipopolysaccharide-stimulated macrophages. When TH-4 and LGG were grown together, folate production was only stimulated by MWE. Passion fruit water extract and OWE increased the TH-4 adhesion whereas acerola water extracts and MWE improved LGG adhesion when strains were used individually. These results showed that fruit by-product water extracts (FWE), especially MWE, presented potential biological and functional effects to improve human health. Additionally, FWE could be used to develop new functional antioxidant foods and pharmaceutical ingredients.

Keywords: Probiotic, fruit by-products, dietary fibre, phenolic compounds, anti-inflammatory effect, folate

1. Introduction

Fruit by-products (peel, pulp, and seeds) are produced around the world during fruit processing and represent an important environmental problem (VIEIRA et al, 2017). In contrast, these residues are a natural source not only of nutrients but also of different bioactive molecules. These biocompounds have great potential to be used as functional food ingredients or for application as phytochemical pharmaceutical substances for the prevention or treatment of human diseases (KOWALSKA et al, 2017; O'Shea et al., 2015; Oliveira et al., 2016).

In this context, dietary fibres (DF) are important plant components present in large amounts in fruit by-products. DF are not hydrolysed by the endogenous enzymes in the small intestine of humans (JOINT FAO/WHO, 2010) and their consumption is associated with beneficial health effects, including, regulation of the intestinal transit, prevention or treatment of cancer, diabetes, and cardiovascular diseases (BERES et al., 2016; WANG et al., 2015). In addition, DF from fruits by-products may be good sources of bound phytochemicals including phenolic compounds and other compounds with high biological activity (HAMINIUK et al., 2012). In some cases, the complex DF-phenolic compounds may arise as antioxidant dietary fibre, a category defined as "a dietary fibre concentrate containing significant amounts of natural antioxidants associated with non-digestible compounds" (QUIRÓS-SAUCEDA et al., 2014).

Phenolic compounds are known to be widely present in fruits and their by-products, and they are the most important group of natural antioxidants in the diet. These bioactive molecules act as reducing agents and can improve human health. Besides the structural diversity of phenolic compounds, it is crucial to determine their biological properties such as anti-inflammatory activity, microbial growth stimulation, and their impact on the production of beneficial metabolites by different microorganisms, especially by probiotics (LEBLANC et al., 2017; OH et al., 2012; ROCCHETTI et al., 2018; SANTOS et al., 2017). Probiotics are "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (HILL et al., 2014).

According to AJILA & RAO (2013), DF associated with polyphenols may enhance the antioxidant effect of these bound phenolic compounds in the human intestine when compared to the non-bound ones. Considering that the DF-phenolic complexes are not hydrolysed by the human digestive enzymes (VELDERRAIN-RODRÍGUEZ et al., 2016), they may exert a positive impact on the gut microbiota modulation and, also, contribute to maintain a balanced oxidant status in the intestinal environment, in that manner, protecting the

intestinal epithelium against inflammatory processes, increasing the adhesion of probiotics microorganisms to intestinal cells, and protecting the intestine against the colonization by pathogens (AJILA & RAO, 2013; DUEÑAS et al., 2015).

According to GIBSON et al (2017), prebiotic is defined as "a substrate that is selectively utilized by host microorganisms conferring a health benefit" extending the prebiotic effect to other substrates such as non-carbohydrate substrates, which could include phenolic compounds. The use of fruit by-products with prebiotic potential to improve not only the growth of beneficial microorganisms but also the production of beneficial metabolites such as the B-group vitamins by probiotic and gut commensal microorganisms has been investigated (ALBUQUERQUE et al., 2016; LEBLANC et al., 2017; VIEIRA et al., 2017).

Therefore, this study aimed was to determine the total dietary fibre and soluble fibre contents, the total phenolic content, the phenolic composition, and the antioxidant activity of four different fruit by-products water extracts (FWE) (from passion fruit, orange, acerola, and mango) and evaluate their anti-inflammatory properties in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. Additionally, the impact of each FWE on the growth and folate production by *Streptococcus thermophilus* TH-4 and *Lactobacillus rhamnosus* LGG and on the *in vitro* adherence abilities of both microorganisms to intestinal human epithelial cells were also investigated. The use of FWE as sources of soluble dietary fibres associated to phenolic compounds with antioxidant and biological activity could be applied as a natural ingredient for the development of functional food, nutraceuticals or natural pharmaceutical drugs.

2. Material and Methods

2.1 Microorganisms and growth conditions

The starter *Streptococcus* (*St.*) *thermophilus* TH-4 and the probiotic *Lactobacillus* (*Lb.*) *rhamnosus* LGG were both supplied by Christian Hansen (Hørsholm, Denmark). The streptococci strain was grown at 37 °C for 24 h in LAPTg broth (LAIÑO et al., 2013) and the lactobacilli in de Man, Rogosa, and Sharp broth (MRS, Oxoid, Basingstoke, UK) at 37 °C for 24 h. Both strains were grown statically under aerobic conditions. Viable streptococci and lactobacilli were counted by plating serial dilutions in LAPTg and MRS agar, respectively. Both strains were incubated (37 °C / 48h) in aerobic atmosphere; however, when in co-culture, *Lb. rhamnosus* LGG was incubated in anaerobic conditions (Anaerogen Anaerobic System, Oxoid) to avoid the concomitant growth of the streptococci strain.

2.2 Fruit by-products processing

Four different fruit by-products [passion fruit (*Passiflora edulis* f. Flavicarpa), orange (*Citrus sinensis*), acerola (*Malpighia emarginata*), and mango (*Mangifera indica*)] were kindly supplied by fruit industries located in the state of São Paulo (Brazil). Passion fruit, orange, and acerola by-products were constituted mainly by peels and seeds, while mango by-product was constituted mainly by peel and a small amount of pulp attached to the peel. These fruit by-products were processed to a fine powder (< 42 μm) and stored according to ALBUQUERQUE et al. (2016) at -20 ºC until analysis.

2.3 Hot-Water extraction

A hot water extraction was performed to obtain soluble fibres from all fruit by-product powders according to BERES et al. (2016). Briefly, each fruit by-product powder was blended with distilled water (1:12 ratio) and boiled (95-100 °C) under agitation for 1 h. Each hot boiled mixture was immediately filtered using a sterile 0.22 μm membrane. The filtered obtained from each hot boiled mixture containing each fruit by-product was denominated fruit by-product water extract (FWE) and stored at 4 ºC until use.

2.4 Physicochemical analysis

Enzymatic-gravimetric methods (991.43 and 991.43) were used to determine the total dietary fibre, soluble fibre, and insoluble fibre contents of each FWE. Values were expressed as g 100 mL^{-1} FWE (AOAC, 2012).

Total phenolic content of each FWE was quantified using the Folin-Ciocalteau assay according to MAGALHÃES et al. (2010). Gallic acid was used as standard and the total phenolic content expressed as GAE mg mL^{-1} FWE.

Antioxidant activity of each FWE was determined based on two methods: the ORAC (hydrophilic oxygen radical absorbance capacity) method according to OU et al. (2001) and DPPH (1,1-diphenyl-2-picrylhydrazyl radical) according to BRAND-WILLIAMS et al. (1995) and the results were expressed as μmol Trolox L^{-1} FWE.

2.5 Evaluation of phenolic composition of FWE by HPLC-DAD

HPLC-DAD of all samples were performed in a Waters® Alliance e2695 HPLC System (Waters®, Milford, USA), equipped with a diode array detector. An aliquot of 5μL of each filtered FWE, prepared according to the item 2.3, was injected without further

preparation. Separations were performed on a Nova-Pak C18 column (250 X 4.6 mm, Waters®, Milford, USA). Mobile phase A consisted of 0.15% phosphoric acid and 99.85% ultrapure water, mobile phase B was 100% acetonitrile. The gradient profile was as follows: 5% B (0min), 5% B (6min), 12%B (12min), 20%B (18min), 30%B (22min), 60% B (25min) and 5%B (26min), with a post-time of 5 min. The flow rate was 1.2 mL min^{-1}. Compounds were identified by comparison to the retention time (Sigma-Aldrich, St. Louis, MO, USA): gallic acid (2.370 min); protocatechuic acid (4.105 min); vanillinic acid (9.760 min); syringic acid (10.767min); epicatechin (11.409 min); rutin (17.535 min); quercitin (22.943 min). Quantification was performed based on each compound concentration curve and expressed as mg 100 g^{-1} dry FWE and purity evaluated by comparing the spectra of the peaks to those of the standards.

2.6 Cell cultures and growth conditions

Caco-2 cell line ATCC HTB-37 and RAW 264.7 macrophages belonging to the collection of the Centro de Referencia para Lactobacilos (CERELA-CONICET, Tucumán, Argentina) were used. Both cells were cultivated in Dulbecco`s modified Eagle`s medium (DMEM) containing high glucose and GlutaMAX™ (Gibco, Gran Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (FBS, Natocor, Córdoba, Argentina), 100 IU/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B (Gibco, Gran Island, NY, USA) and incubated at 37°C in a 5% CO_2 atmosphere. Cells at 70-80% of confluence were washed three times with DMEM without antibiotics, resuspended using the same medium and 1 mL of the cell inoculum (5 x 10^5 cells mL^{-1} for Caco- 2 cells and 4 x 10^4 for RAW cells mL^{-1}) was seeded in each well of a 24-well plate. The microplate was incubated in the same environmental conditions described above until presenting 70-80% of cell confluence to evaluate the effect of FWE on nitric oxide production and bacterial adhesion (NAKAJIMA et al., 2017). The concentration of each FWE solution (ratio 1:10, v/v, in cell culture medium) used to perform the anti-inflammatory and the adhesion assays was not cytotoxic to the cells used in this study (data not shown).

2.7 Evaluation of anti-inflammatory properties of FWE using LPS-stimulated macrophages

The RAW 264.7 macrophage cell line was cultured in DMEM as described in 2.4. The experiment was performed according to CHA et al. (2014) with modifications. RAW cells at

70-80% of confluence in 24 well plate were washed three times with PBS and then co-incubated for two hours with the fruit extracts resuspended in DMEM without antibiotic. After that, the cells were stimulated with LPS from *Salmonella* Typhimurium (Sigma, St. Louis, MO, USA) at a final concentration of 100 ng mL^{-1} in serum free-DMEM for 20 h at 37 °C and 5% CO_2. The NO concentrations were determined by the Griess reaction using a 96 well-plate. Briefly, 50 µL of each culture supernatant was first mixed with an equal volume of Griess reagent A (1% sulfanilamide in 5% phosphoric acid, Britania, Buenos Aires, Argentina) for eight minutes at room temperature in the dark. Next, 50 µL of Griess reagent B (0.1% N-1-naphtylenediaminedihydrochloride, Britania, Buenos Aires, Argentina) were added and incubated for eight minutes at room temperature in the dark. The absorbance was measured at 550 nm in a microplate reader (VERSAmax, Molecular devices, Sunnyvale, CA, USA). The NO concentration was calculated using a sodium nitrite standard curve and the results were expressed as µMol of nitric oxide.

2.8 Influence of FWE on the growth of microorganisms and their folate production during fermentation

The ability of *St. thermophilus* TH-4 and *Lb. rhamnosus* LGG to ferment each FWE was performed according to ALBUQUERQUE et al. (2016) with modifications. Briefly, 5 log CFU mL^{-1} of *St. thermophilus* TH-4 and *Lb. rhamnosus* LGG strains (in pure culture and in co-culture) were inoculated in a modified MRS broth (mMRS) supplemented with 10% (v/v) of each FWE. Viable cell counts were performed before and after (24 h) fermentation as described in 2.1 and the results were expressed as log CFU mL^{-1}. mMRS broth without FWE inoculated with the strains was used as control.

Total folate content was determined using a microbiological method with the indicator strain *Lactobacillus casei* subsp. *rhamnosus* NCIMB 10463 according to ALBUQUERQUE et al. (2016). Samples were taken before (0 h) and after (24 h) the fermentation process. The total folate content was calculated using a folic acid standard curve and expressed as ng mL^{-1}.

2.9 Effect of FWE on the adhesion of microorganisms to Caco-2 cells

The effect of each FWE on *St. thermophilus* TH-4 and *Lb. rhamnosus* LGG adhesion to Caco-2 cells was investigated according to PARKAR et al. (2010) with modifications. The FWE solution was prepared as described in section 2.4. Caco-2 cells were washed using PBS (pH 7.4); then, 500 µL of each FWE solution and 500 µL of each bacterial inoculum (6 log

CFU mL^{-1}), previously washed 3 times with PBS and resuspended in DMEM without antibiotics, were added to each well. Both microorganisms were also tested as co-culture. The 24-wells microplate was incubated at 37 °C in the presence of 5% of CO_2 for 2 h. After that, each well was washed twice using PBS to eliminate unattached bacteria and 1 mL of 1 % Triton X-100 solution (Sigma, ST. Louis, MO, USA) was added to each well to release the microorganisms adhered to Caco-2 cells. Serial dilutions of these samples and also of the initial inoculum were performed and the viable cells counted as described in 2.1. Anaerobic conditions were used for *Lb. rhamnosus* LGG when in co-culture with *St. thermophilus* TH-4. Wells containing FWE solution, each bacterial strain, bacterial co-culture or DMEM were used as controls. The experiment was performed in duplicate and the percentage of adhesion was calculated using the following formula:

% adhesion = (CFU after adhesion x initial CFU^{-1}) x 100

The percentage change in the number of viable adherent bacteria was calculated as the average CFU in treated cells x (average CFU in untreated control)$^{-1}$ x 100.

2.10 Statistical analysis

Total dietary fibre, soluble fibre, insoluble fibre, total phenolic content, and antioxidant activity of each FWE and their effect on the growth of the microorganisms and their folate production were determined in triplicates. The anti-inflammatory activity of each FWE and the adhesion of the microorganisms to Caco-2 cells in the presence of each FWE were performed in duplicates. The results were expressed as mean ± standard deviation. 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. Pearson's correlation was performed to assess the correlation between TPC/ORAC, TPC/DPPH, and ORAC/DPPH. The differences betwen the samples were considered as statistically significant at $p < 0.05$. Principal Components Analysis was performed to evaluate the relationship between the physicochemical characteristics of each fruit by-product water extract and their respective biological effects to determine which fruit by-product water extract presented the best prebiotic potential. For this analysis, the R-Studio version 1.1.453 was employed.

3. Results

3.1 Fibre and total phenolic contents, antioxidant activity, and phenolic composition of each FWE

Fruit by-product water extracts (FWE) were used as sources of dietary fibre associated with phenolic compounds. The total dietary fibres, soluble fibres, insoluble fibres, total phenolic content, phenolic composition, and the antioxidant activity of all FWE were evaluated.

Insoluble fibres were not detected in any of the FWE since all hot boiled mixtures described in section 2.2 were filtered. Regarding Table 1, considering the absence of insoluble fibres, significant differences between the soluble fibres values and between the total dietary fibres contents among the FWE ($p < 0.05$) were observed. Mango water extract (MWE) presented the highest soluble fibres content (0.47 ± 0.01 g 100 mL^{-1}) and, therefore, the highest total dietary fibres content (0.47 ± 0.01 g/100 mL), followed by the orange water extract (OWE) (0.39 ± 0.01 g 100 mL^{-1}), acerola water extract (AWE) (0.26 ± 0.01 g 100 mL^{-1}), and passion fruit water extract (PFWE) (0.12 ± 0.0 g 100 mL^{-1}).

The Folin-Ciocalteu assay was used to determine the total phenolic content (TPC) of FWE and the results are shown in Table 1. MWE presented the highest TPC (1.31±0.03 mg GAE mL^{-1} FWE) followed by AWE (0.95±0.06 mg GAE mL^{-1} FWE), OWE (0.52±0.01 mg GAE mL^{-1} FWE), and PFWE (0.14±0.00 mg GAE mL^{-1} FWE). The phenolic composition of all FWE is also presented in Table 1 and the chromatograms obtained are shown in the supplementary material I. Considering the phenolic standards, between phenolic acids and flavonoids, used in this study, OWE presented the most diverse phenolic composition (6 from the 7 phenolic standards tested) and the major phenolic compound was gallic acid (45.6 mg GAE mL^{-1} FWE). In contrast, although MWE presented a lower diversity of phenolics (5 from the 7 phenolic standards tested) when compared to OWE and the other FWE, its gallic acid content was expressively higher (238.8 mg GAE mL^{-1} FWE). Among the phenolic standards tested, AWE presented the lowest phenolic composition diversity (2 from the 7 phenolic standards tested) and, vanillic acid and rutin were the only phenolic compounds detected. Considering the amount detected for each phenolic compound evaluated, MWE presented the highest phenolic acids content (240 mg GAE mL^{-1} FWE) and flavonoids content (42 mg GAE mL^{-1} FWE), followed by OWE (phenolic acid content: 79.2 mg GAE mL^{-1} FWE and flavonoids content: 32.4 mg GAE mL^{-1} FWE), PFWE (phenolic acid content: 3.6 mg GAE

mL^{-1} FWE and flavonoids content: 13.2 mg GAE mL^{-1} FWE), and AWE (phenolic acid content: 2.4 mg GAE mL^{-1} FWE and flavonoids content: 9.6 mg GAE mL^{-1} FWE).

Table 1 – Total dietary fibre, soluble fibre, total phenolic content, phenolic profile, and antioxidant activity of fruit by-products water extracts (FWE).

	Fruit by-products water extracts			
	PFWE	**OWE**	**AWE**	**MWE**
Total dietary fibre (g/100 mL)	0.12 (0.0) ^{D}	0.39 (0.01) ^{B}	0.26 (0.01) ^{C}	0.47 (0.01) ^{A}
Soluble fibres (g/100 mL)	0.12 (0.0) ^{D}	0.39 (0.01) ^{B}	0.26 (0.01) ^{C}	0.47 (0.01) ^{A}
Total phenolics (mg GAE mL^{-1} FWE)	0.14 (0.00)^{D}	0.52 (0.01)^{C}	0.95 (0.06)^{B}	1.31 (0.03)^{A}
Phenolic profile (mg 100 g^{-1} dry FWE)				
Phenolic Acids				
Vanillic acid	1.2	9.6	2.4	0
Siringic acid	1.2	19.2	0	0
Gallic acid	1.2	45.6	0	238.8
Protocatechuic acid	0	4.8	0	1.2
Flavonoids				
Rutin	8.4	20.4	9.6	34.8
Quercitin	4.8	12.0	0	4.8
Epicatechin	0	0	0	2.4
Antioxidant activity				
ORAC (µmol Trolox L^{-1} FWE)	46.95 (0.65)^{B}	13.62 (0.48)^{C}	47.03 (1.86)^{B}	61.56 (2.16)^{A}
DPPH (µmol Trolox L^{-1} FWE)	304.7 (36)^{D}	898.9 (94)^{CD}	4431.7 (274)^{B}	18708.7 (1987)^{A}

PFWE: passion fruit water extract, OWE: orange water extract, AWE: acerola water extract, MWE: mango water extract. ^{A,B} Different capital letters in the same row denote significant differences between fruit by-products ($P< 0.05$).

The antioxidant activity of each FWE was evaluated using the ORAC and the DPPH methods and the results are presented in Table 1. MWE presented the highest antioxidant activity when both ORAC and DPPH methods were employed (61.56 ± 2.16 µmol Trolox L^{-1} FWE and 18708.7 ± 1987 µmol Trolox L^{-1} FWE, respectively) followed by AWE (47.03 ± 1.86 µmol Trolox L^{-1} FWE and 4431.7 ± 274 µmol Trolox L^{-1} FWE, respectively). Considering the ORAC method, OWE (13.62 ± 0.48 µmol Trolox L^{-1} FWE) presented the

lowest antioxidant activity, while PFWE (304.7 ± 36 µmol Trolox L^{-1} FWE) presented the lowest antioxidant activity when DPPH method was employed.

3.2 Anti-inflammatory effect of the FWE

The anti-inflammatory effect of each FWE is presented in Figure 1. LPS-stimulated RAW 264.7 macrophages produced high nitric oxide (NO) levels (6.9 ± 0.6 µMol) when compared to the negative control LPS-non stimulated cells (0.7 ± 0.1 µMol). Orange water extract (OWE) showed the highest anti-inflammatory effect by completely decreasing the NO produced by the LPS-stimulated macrophages. There was no significant difference between the anti-inflammatory effect of OWE and MWE on NO reduction ($p > 0.05$). On the other hand, the results suggested that AWE and PFWE had no effect on decreasing NO levels produced by the LPS-stimulated macrophages, since there was no significant difference between the NO levels after the AWE and PFWE treatments and the LPS-stimulated control cells ($p < 0.05$).

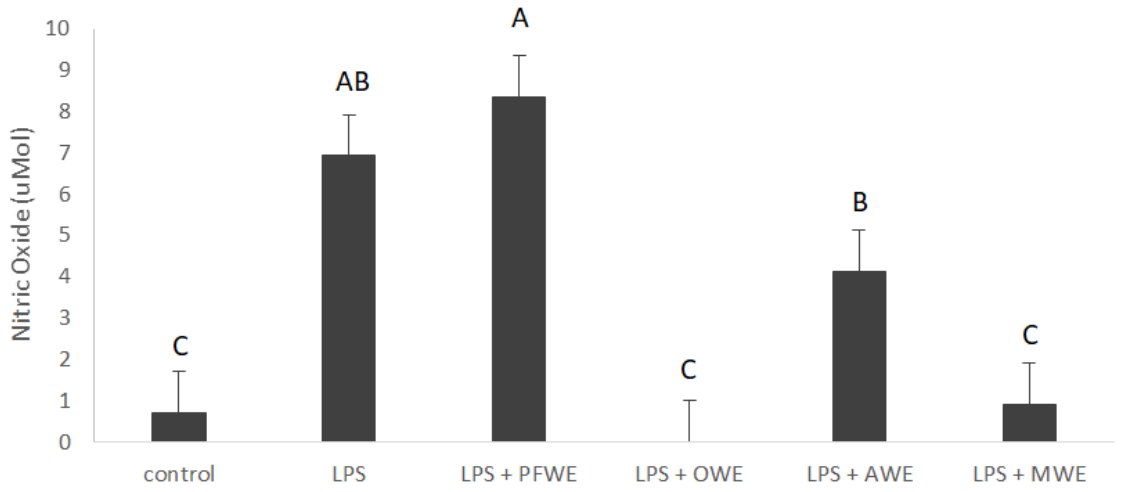

Figure 1. Effect of fruit by-product water extracts on nitric oxide concentration produced by Lipopolysaccharides (LPS)-stimulated RAW 264.7 macrophages. [A,B] Different capital letters denote significant differences between different fruit water extracts on nitric oxide concentration ($P < 0.05$). PFWE: passion fruit water extract, OWE: Orange water extract, AWE: acerola water extract, MWE: mango water extract. Control: RAW 264.7 macrophages without LPS stimulation.

3.3 The impact of FWE on the growth of microorganisms during fermentation

Results regarding the ability of *St. thermophilus* TH-4 and *Lb. rhamnosus* LGG to grow as pure cultures or in co-culture after 24 h of fermentation of each FWE are shown in Table 2. *Lb. rhamnosus* LGG was able to grow in the presence of all fruit extracts when in pure culture and in co-culture with *St. thermophilus* TH-4, reaching counts above 7 log CFU mL^{-1} after 24 h of fermentation. When in pure culture, LGG presented the highest growth in the presence of MWE (7.8 ± 0.5 log CFU mL^{-1}) and OWE (7.6 ± 0.1 log CFU mL^{-1}). Both FWE were significant different when compared to the control ($p < 0.05$). In contrast, *St. thermophilus* TH-4 was not able to grow in the absence of any FWE and, when compared to the growth of LGG, the streptococci strain presented the lowest counts in the presence of all FWE, especially in the presence of AWE when in pure culture (2.2 ± 0.3 log CFU mL^{-1}) and in co-culture (2.7 ± 0.2 log CFU mL^{-1}). The presence of *St. thermophilus* TH-4 had no effect on *Lb. rhamnosus* LGG growth.

Table 2. Viable cell counts of *Streptococcus thermophilus* TH-4 and *Lactobacillus rhamnosus* LGG after 24 h of fermentation of mMRS broth supplemented with fruit by-product water extracts (FWE).

Strains	Fruit by-products water extracts (log CFU mL^{-1})				
	PFWE	**OWE**	**AWE**	**MWE**	**Control**
Pure culture					
St. thermophilus TH-4	6.8 ± 0.1^{Aa}	6.5 ± 0.2^{Aa}	2.2 ± 0.3^{Cb}	5.3 ± 0.3^{Bb}	2.1 ± 0.1^{Ca}
Lb. rhamnosus LGG	$7.4 \pm 0.2^{B\alpha}$	$7.6 \pm 0.1^{AB\alpha}$	$7.4 \pm 0.1^{B\alpha}$	$7.8 \pm 0.5^{A\alpha}$	$7.2 \pm 0.1^{B\alpha}$
Co-culture					
St. thermophilus TH-4	5.3 ± 0.3^{Bb}	6.5 ± 0.3^{Aa}	2.7 ± 0.2^{Ca}	6.6 ± 0.4^{Aa}	1.1 ± 0.1^{Db}
Lb. rhamnosus LGG	$7.5 \pm 0.2^{A\beta}$	$7.5 \pm 0.2^{A\alpha}$	$7.4 \pm 0.1^{A\alpha}$	$7.7 \pm 0.1^{A\alpha}$	$7.2 \pm 0.2^{B\alpha}$

^{A,B} Different capital letters in the same row denote significant differences between fruit by-products ($P< 0.05$). ^{a,b} Different small letters in the same column denote significant differences between *St. thermophilus* TH-4 growth in pure culture and in co-culture ($P< 0.05$). $^{\alpha,\beta}$ Different Greek letters in the same column denote significant differences between *Lb. rhamnosus* LGG growth in pure culture and co-culture ($P< 0.05$). PFWE: passion fruit water extract, OWE: Orange water extract, AWE: acerola water

extract, MWE: mango water extract. Control: Modified MRS broth without any fruit by-product water extract.

3.4 Folate production by the microorganisms during FWE fermentation

The results regarding the production of folate by *St. thermophilus* TH-4 and *Lb. rhamnosus* LGG strains after 24 h of FWE fermentation are presented in the Figure 2. The folate content of each FWE and of the mMRS broth were determined (PFWE, 14 ± 4 ng mL^{-1}; OWE, 18 ± 0 ng mL^{-1}; AWE, 22 ± 2 ng mL^{-1}; MWE, 14 ± 1 ng mL^{-1}, and mMRS broth, 7 ± 1 ng mL^{-1}). Among all FWE, AWE presented the highest amount of folate and the mMRS broth without FWE supplementation (control) presented the lowest vitamin content. *St. thermophilus* TH-4 produced the highest amount of folate in the presence of MWE (41 ± 7 ng mL^{-1}), followed by OWE (23 ± 3 ng mL^{-1}), and PFWE (20 ± 3 ng mL^{-1}), while *Lb. rhamnosus* LGG was not able to produce folate in the presence of any FWE. When both strains and lactobacilli were tested in co-culture, the highest production of folate was observed in the presence of MWE (50 ± 7 ng mL^{-1}) followed by PFWE (21 ± 7 ng mL^{-1}), and OWE (18 ± 2 ng mL^{-1}). Although the co-culture was able to produce folate in the presence of PFWE and OWE, no significant difference was observed when both FWE were compared to the co-culture folate production observed for the non-supplemented mMRS broth. In the absence of FWE, the co-culture was able to produce the vitamin (30 ± 6 ng mL^{-1}); however, the folate production by the co-culture in the presence of MWE was significantly higher, when compared to the control ($p < 0.05$).

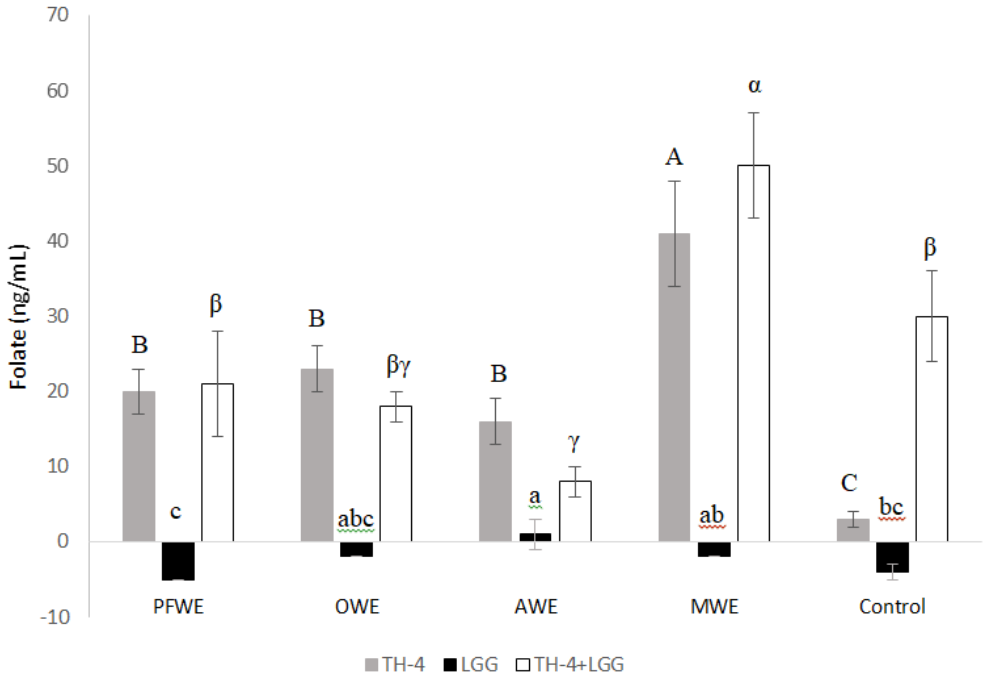

Figure 2. Folate content after fruit water extracts fermentation. Grey bars present the production of folate by *Streptococcus thermophilus* TH-4. Black bars present the folate production by *Lactobacillus rhamnosus* LGG. White bars present the production of folate by the co-culture (TH-4 + LGG). Textured bars present the folate content of each fruit by-product water extract. Control: Modified MRS broth without fruit by-product water extract ^{A,B} Different capital letters denote significant differences between folate production by *St. thermophilus* TH-4 among different fruit water extracts fermentation ($P < 0.05$). ^{a,b} Different small letters denote significant differences between folate production by *Lb. rhamnosus* LGG among different fruit water extracts fermentation ($P < 0.05$). $^{\alpha,\beta}$ Different Greek letters denote significant differences between folate production by the co-culture among different fruit water extracts fermentation ($P < 0.05$).

3.5 Adhesion of microorganisms in the presence of FWE

The results regarding the impact of each FWE on *St. thermophilus* TH-4 and *Lb. rhamnosus* LGG adhesion to Caco-2 cells are shown in Figure 3. When in pure culture, the adhesion of *St. thermophilus* TH-4 was strongly enhanced by the presence of PFWE (64.3 ± 2.7%) and OWE (64.3 ± 1.4%). In contrast, MWE decreased the adhesion of TH-4 (2.1 ± 2.7%), even when compared to the control (9.3 ± 1.4%) ($p < 0.05$). The adhesion of *Lb. rhamnosus* LGG was positively influenced by the presence of MWE (13.5 ± 2.7%) and AWE (12.7 ± 2.7%) when compared to the control without FWE (3.5 ± 1.4%). In co-culture, AWE (41.4 ± 12.9%) and MWE (28.6 ± 2.9%) increased the adhesion of *St. thermophilus* TH-4 when compared to the adhesion of this strain in pure culture for the same both FWE (32.9 ± 3.5% and 2.1 ± 2.7%, respectively). In contrast, the adhesion of *Lb. rhamnosus* LGG in co-

culture with the streptococci strain was enhanced only by the presence of MWE (27 ± 5.4%) when compared to the adhesion of the lactobacilli strain individually (13.5 ± 2.7%) and to the control (4.3 ± 1.5%). According to Figure 3, *St. thermophilus* TH-4 did not interfered with *Lb. rhamnosus* LGG adhesion. In contrast, LGG negatively affected the TH-4 adhesion in the presence of PFWE and OWE.

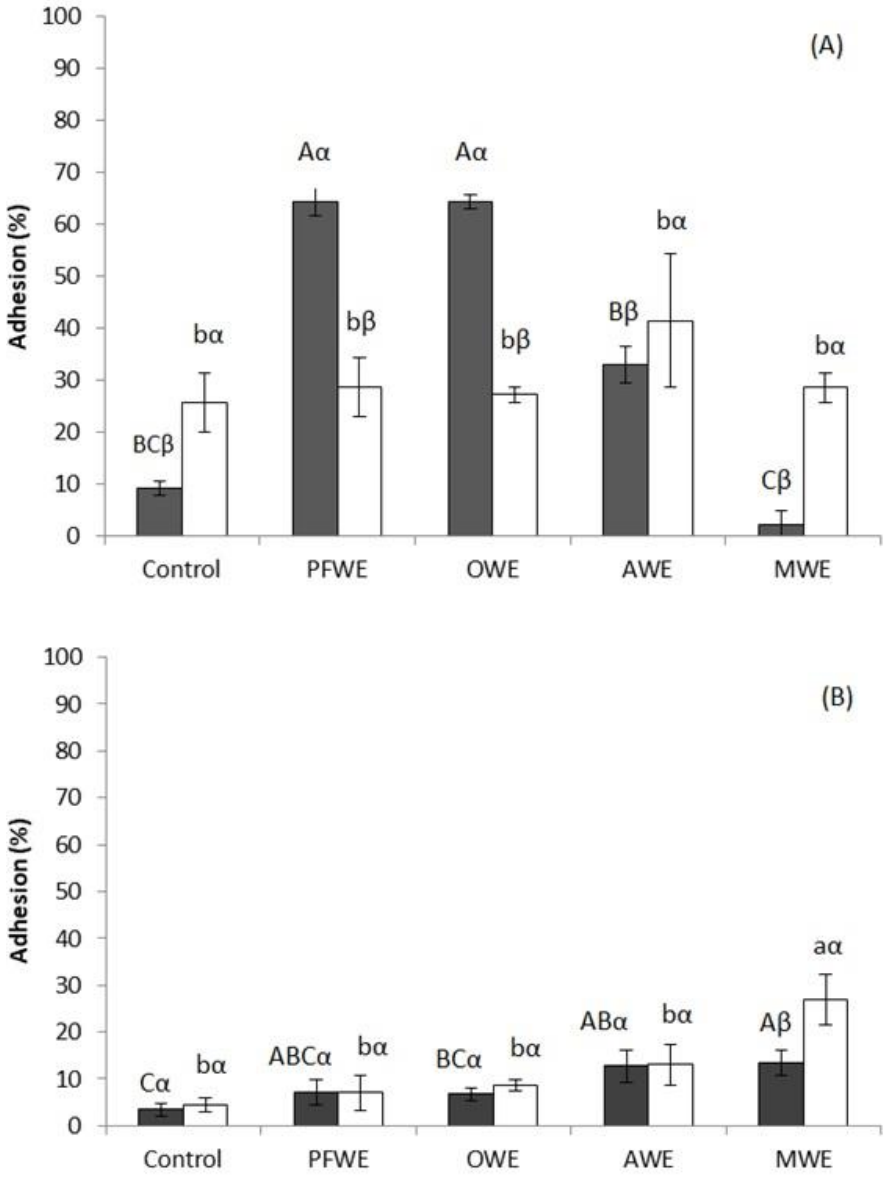

Figure 3. Effect of fruit by-product water extracts (FWE) on the adhesion ability of *Streptococcus thermophilus* TH-4 and *Lactobacillus rhamnosus* LGG, as pure cultures and in co-culture in a model of human colon cell line (Caco-2). (A) Adhesion of *St. thermophilus* TH-4 as pure culture (grey bars) and in co-culture (white bars) with *Lb. rhamnosus* LGG. (B) Adhesion of *Lb. rhamnosus* LGG as pure culture (grey bars) and in co-culture (white bars) with *St. thermophilus* TH-4. [A,B] Different capital

letters denote significant differences between *St. thermophilus* TH-4 or *Lb. rhamnosus* LGG adhesion to Caco-2 cells as pure culture ($P < 0.05$). [a,b] Different small letters denote significant differences between *St. thermophilus* TH-4 or *Lb. rhamnosus* LGG adhesion to Caco-2 cells as co-culture ($P < 0.05$). [α,β] Different greek letters denote significant differences between *St. thermophilus* TH-4 or *Lb. rhamnosus* LGG adhesion to Caco-2 cells as pure cultures and co-culture ($P < 0.05$). PFWE: passion fruit water extract, OWE: orange water extract, AWE: acerola water extract, MWE: mango water extract. Control: without FWE supplementation.

3.6 Effect of physicochemical parameters of FWE on biological effects

To determine which FWE presented the best biological effect considering the physicochemical parameters evaluated, a principal components analysis (PCA) was performed and the results are presented in Figure 4. The first principal component (PC1) and the second component (PC2), explained, respectively, 54.4% and 28.5% of the total variance between the physicochemical parameters and the biological effects. Considering these results, soluble fibres content, flavonoids and phenolic acids composition, TPC, and the antioxidant activity (ORAC and DPPH) contributed to determine the highest anti-inflammatory effect of MWE. Additionally, the physicochemical characteristics of MWE were able not only to stimulate folate production, especially by *St. thermophilus* TH-4 and by the co-culture, but also to increase the growth of *St. thermophilus* TH-4 when in co-culture with LGG and the adhesion of *Lb. rhamnosus* LGG to Caco-2 cells.

4. Discussion

Recently, the use of fruit by-products as sources of bioactive compounds has increased aiming a sustainable use of the large amounts of these fruit residues generated and discarded by the fruit industry (VIEIRA et al., 2017). Several studies have been characterizing and quantifying these fruit by-products bioactive molecules to develop new food products and/or new pharmaceutical preparations (BERES et al., 2016; DA SILVA et al., 2013). Among these fruit bioactive compounds, dietary fibres are one of the greatest interest to the food industry due to their physicochemical characteristics, such as solubility, viscosity, water holding capacity, and fermentability (MACAGNAN et al., 2015). Besides, dietary fibres may be associated with other compounds such as proteins, lipids, and antioxidant substances creating an important vegetable dietary complex that may exert some biological and functional effects (QUIRÓS-SAUCEDA et al., 2014). Phenolic compounds are one of the most abundant antioxidants present in fruits and studies have been performed to elucidate the biological

importance of the association between these compounds and dietary fibres (ZHU et al., 2015; QUIRÓS-SAUCEDA et al., 2014; OLEJNIK et al., 2015).

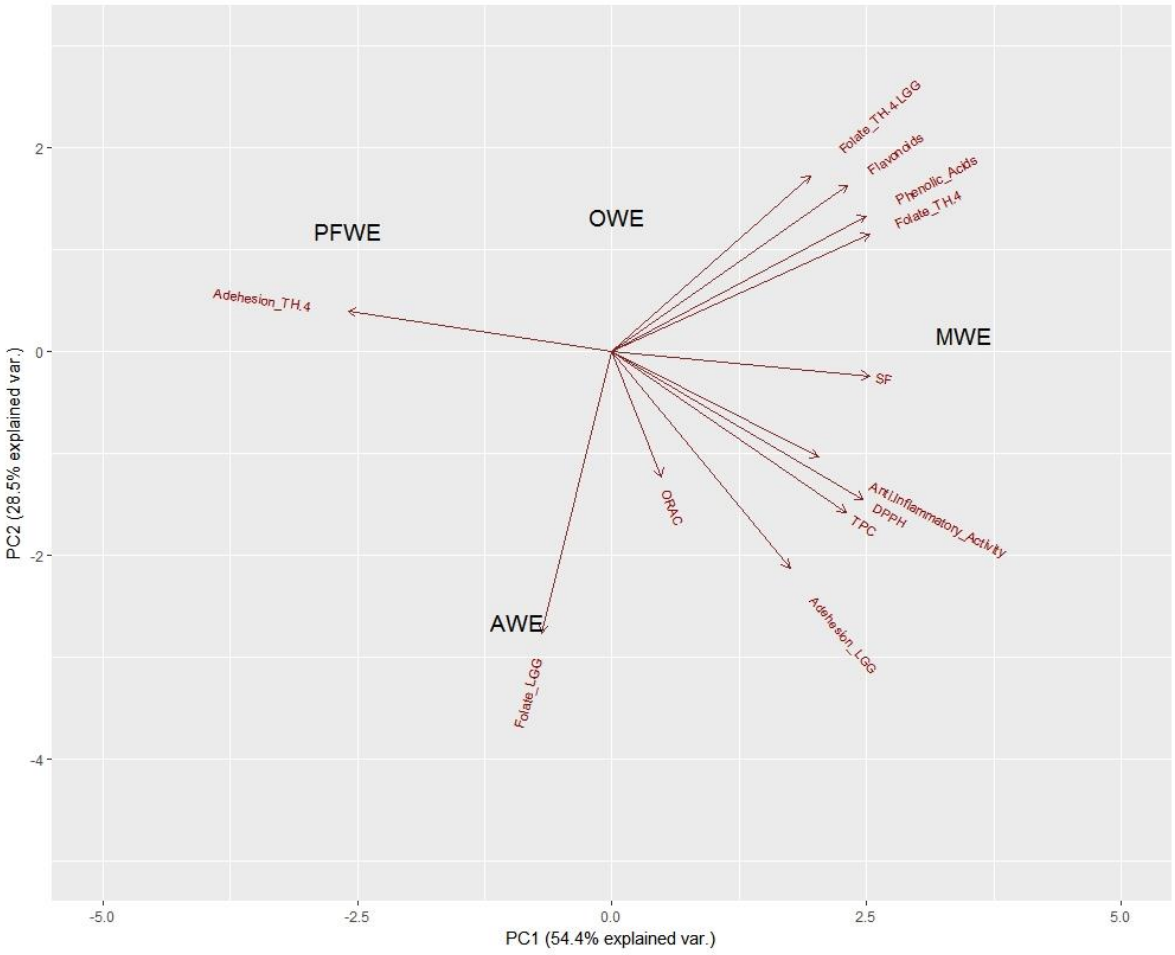

Figure 4. Principal Component Analysis (PCA) graph of the influence of physicochemical parameters of fruit by-product water extracts (FWE) on the biological and functional effects evaluated. *Streptococcus thermophilus* TH-4; *Lactobacillus rhamnosus* LGG. PFWE: passion fruit by-product water extract; OWE: orange by-product water extract; AWE: acerola by-product water extract; MWE: mango water extract. SF: soluble dietary fibres content; TPC: total phenolic compounds; Folate_TH-4: folate produced by TH-4; Folate_LGG: folate produced by LGG; Folate_TH-4 LGG: folate produced by the co-culture TH-4+LGG; Adhesion_TH-4: adhesion of TH-4 to Caco-2 cells; Adhesion_LGG: adhesion of LGG to Caco-2 cells; Adhesion_TH-4cc: adhesion of TH-4 in co-culture with LGG to Caco-2 cells; Adhesion_LGGcc: adhesion of LGG in co-culture with TH-4 to Caco-2 cells; Growth_TH-4: growth of TH-4 during fermentation of FWE; Growth_LGG: growth of LGG during fermentation of FWE; Growth_TH-4cc: growth of TH-4 in co-

culture with LGG during fermentation of FWE; Growth_LGGcc: growth of LGG in co-culture with TH-4 during fermentation of FWE.

Previously, the total dietary fibre and the soluble fibre fraction of passion fruit, orange, acerola, and mango by-products were evaluated (VIEIRA et al., 2017). The authors observed that the total dietary fibre content of all fruit residues ranged from 48.46 g/100g of dry matter (acerola by-product) to 41.53 g/100 g of dry matter (mango by-product). Mango by-product presented the highest soluble fibres content, followed by orange, acerola, and passion fruit by-products. As expected, after the hot water extraction, the soluble fibres content of all FWE was proportionally in agreement with the correspondent amounts of soluble fibres obtained for their respective fruit by-products, with MWE presenting the higher amount of soluble fibres content (VIEIRA et al., 2017).

MARTÍNEZ et al. (2012) evaluated the total dietary fibre and soluble fibre content of mango and passion fruit by-products. Both fruit by-products presented higher total dietary fibre content (> 70 g/ 100 g of dry matter) and soluble fibres (> 28g/100 g of dry matter) than the fruit by-products evaluated in our study. Although other studies also presented higher total dietary fibre and soluble fibres contents for different fruit by-products (MACAGNAN et al., 2015), considering the pre-treatment employed during the processing of the fruit by-products before the hot water extraction, the washing and bleaching treatments were probably responsible for decreasing the amount of the total dietary fibres and soluble fibres contents of the fruit by-products evaluated (CHANTARO et al., 2008). Thus, the dietary fibres content of the FWE may be increased if an improvement of the fruit by-products processing is considered.

Regarding the association of dietary fibres with antioxidant bioactive molecules, the total phenolic content of each FWE was evaluated and considerable amounts of total phenolic were observed especially for MWE. According to DA SILVA et al. (2014), MACAGNAN et al. (2015), and MARTINEZ et al. (2012), large amounts of phenolic compounds were detected in orange, acerola, mango, and passion fruit by-products. Although studies described the mixture of alcohol-water one of the best solvents when compared to only water to extract phenolic compounds (MARTINEZ et al., 2012), we observed that the hot water extraction significantly increased the total phenolic content of both OWE and AWE ($p < 0.05$), and did not present any significant differences from MWE and PFWE total phenolic content when compared to the extraction with methanol 70% ($p > 0.05$) (data not shown). These data are in

agreement with KABIR et al. (2015), who compared the extraction of total phenolics from fruit and vegetable wastes using hot water and ethanol as solvents and observed that the hot water extractions yielded higher amounts of total phenolics than those with ethanol. These results suggest that the kind of extracted compounds may be different depending on the extraction methods and that some phenolic compounds are more soluble in the presence of water (WU et al., 2004).

Our data suggests that the FWE may be a good source of soluble fibres associated to phenolic compounds. AJILA & RAO (2013) investigated the bound phenolics to mango peel dietary fibre and observed significant amounts of bound phenolics. To evaluate the potential of all FWE as dietary fibre-phenolic complex with antioxidant activity, ORAC and DPPH techniques were used to determine the antioxidant capacity of each fruit by-product water extract. A strong correlation (r^2=0.87) was observed for TPC and DPPH. The antioxidant activity observed for all FWE using the DPPH method was directly proportional to the TPC amounts of the respective FWE. In contrast, TPC and ORAC (r^2=0.52) and ORAC and DPPH (r^2=0.69) presented a lower correlation. Considering the correlation observed between the TPC and the ORAC data, the low correlation value may indicate that some non-phenolic compounds (such as, antioxidant water-soluble vitamins) probably contributed to the total antioxidant activity, especially for OWE. This data is in agreement with BABBAR et al. (2011), who observed a low correlation between TPC and different methods to determine the antioxidant activity of some fruit by-product extracts. Another possibility is the kind of technique employed to determine the antioxidant activity of the FWE. According to ZULUETA et al. (2009) the nature and the time spent to perform the method to determine the antioxidant activity may contribute to underestimate the final values obtained.

Studies regarding the use of vegetable residues rich in bioactive compounds to evaluate their biological and functional effects have been investigated (VIEIRA et al., ALBUQUERQUE et al., 2016; NAKAJIMA et al., 2017; LI et al., 2017). The use of dietary fibres and antioxidants is recognized in the prevention of several chronic diseases (SAURA-CALIXTO, 2011). Inflammatory processes may start in the gut due to an unbalanced intestinal microbiota that may contribute to action of pathogens and improvement of inflammation since macrophages are able to recognize endotoxin produced by pathogenic microorganisms, the lipopolysaccharides (LPS), by their Toll-like receptors (AMBRIZ-PÉREZ et al., 2016).

The potential of phenolic compounds as natural alternatives to the use of non-steroid anti-inflammatory drugs for the treatment of inflammation has been investigated. In this context, OH et al. (2012) observed that Lycium Fruit water extract inhibited the production of many pro-inflammatory mediators, including NO, produced by LPS-stimulated RAW 264.7 macrophages. Moreover, OLEJNIK et al. (2015) evaluated the anti-inflammatory effect of *Sambucus nigra* fruit extract, submitted to a gastrointestinal digestion, and observed that the production of NO was controlled and the expression of major genes of the inflammatory pathway were down-regulated.

Phenolic compounds may act by inhibiting the production or action of pro-inflammatory mediators resulting in an anti-inflammatory response. Different mechanisms of action might be related to the anti-inflammatory properties of the phenolic compounds. Among them, up/downregulation of transcriptional factors (e.g, NF-kβ), inhibition of pro-inflammatory mediators (e.g, interleukin IL-6), inhibition of activated immune cells (e.g, macrophages), and inhibition of the inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) are discussed (AMBRIZ-PÉREZ et al., 2016).

Additionally, the immunomodulatory effect of dietary fibres has also to be considered (WISMAR et al., 2010). Macrophages present carbohydrate-binding pattern recognition receptors (PRRs) which can associate with different polysaccharide structures. The macrophage activity depends not only on the structure, but also of the molecular weight and solubility of the carbohydrate. POPOV et al. (2013) evaluated the anti-inflammatory effect of citrus pectins and observed that pectin with low degrees of methylesterification was found to inhibit local and systemic inflammation whereas pectin with high degrees of methylesterification was found to prevent intestinal inflammation. VOGT et al. (2016) showed that the activation of immune cells by lemon pectin is Toll-like receptor dependent and that the epithelial barrier protective effect may be improved. The amount of pectin of all fruit by-products was determined and mango by-product presented the highest pectin content (supplementary material II). This fact probably contributed to the highest soluble fibres content of MWE and might be related to the positive biological and functional effects exerted by this FWE in our study.

The concept of antioxidant dietary fibres (QUIRÓS-SAUCEDA et al., 2014) may indicates that the FWE evaluated in the present study might have health benefits conferred by the interaction of the dietary fibre and the antioxidant capacity from phenolic compounds. Our results suggest that the fruit by-products used to prepare their respective water extracts rich in

soluble fibres associated to phenolic compounds present great potential to act or to be source of antioxidant dietary fibres. In this context, FWE evaluated in the present study have the potential to act as prebiotic (GIBSON et al., 2017) with antioxidant activity and may be fermented by probiotics or other beneficial commensal gut microorganisms. The FWE may improve the growth of these microorganisms and, also, their metabolic activity by the production of beneficial metabolites (VIEIRA et al., 2017; ALBUQUERQUE et al., 2017; LEBLANC et al., 2017). Although dietary fibres may entrap associated phenolic compounds and reduce their antioxidant and/or prebiotic potential, the bio-accessibility and bioavailability of these molecules could be improved by the beneficial microorganisms from the intestinal microbiota. Thus, these microorganisms could contribute to the release of the phenolic compounds by fermenting the dietary fibres portion (QUIRÓS-SAUCEDA et al., 2014). Therefore, the released phenolic compounds could improve the antioxidant characteristics of the gut environment and, additionally, these bioactive molecules could be metabolized by the intestinal microbiota resulting in the production of several beneficial catabolites, such as folates, that are efficiently absorbed by the intestinal cells or used as nutritional source by the intestinal microbiota (GONZÁLEZ-SARRÍAS, ESPÍN & TOMÁS-BARBERÁN, 2017). The non-absorbed phenolics may contribute to an intestinal antioxidant environment, by scavenging the free radicals and counteracting the effects of dietary pro-oxidants, preventing the growth and action of pathogenic bacteria, due to the phenolic antimicrobial effect (CETIN-KARACA & NEWMAN, 2015), and contributing to improve the adhesion of beneficial microorganisms to intestinal epithelium (PARKAR et al., 2008).

GLISZCZYŃSKA-ŚWIGŁO (2006) showed that folates present antioxidant activity. VIEIRA et al. (2017) and ALBUQUERQUE et al. (2016) evaluated, respectively, the impact of different fruit by-products on the growth and folate production by starter and probiotic strains. ALBUQUERQUE et al. (2016) observed that all strains were able to produce the vitamin in the presence of orange by-product and amaranth flour and that the folate production cannot be associated to the growth ability of the strains once the vitamin production was strain-dependent and might be influenced by the nutritional and environmental conditions. Although the most *St. thermophilus* and lactobacilli strains were reported as being able to ferment the fruit by-products, VIEIRA et al. (2017) also observed that *St. thermophilus* TH-4 was not able to grow in the presence of the acerola by-product after 24 h of fermentation which corroborates our findings. *St. thermophilus* TH-4 was not able to grow individually in the presence of AWE, probably due to the absence of sucrose in

this FWE once acerola by-product did not present this carbohydrate in its composition (supplementary material II). This fact was confirmed once TH-4 was not able to ferment the modified MRS broth without any FWE supplementation (control). Therefore, considering the fermentation of AWE and its absence of sucrose, it seems that *St. thermophilus* TH-4 was not able to grow nor produce folate when in pure culture or in co-culture with *Lb. rhamnosus* LGG.

The ability of *St. thermophilus* and probiotic lactobacilli strains to grow and produce folate in soymilk supplemented with passion fruit by-product and fructooligosaccharides (FOS) was also evaluated (ALBUQUERQUE et al., 2017). According to the authors, *St. thermophilus* ST-M6 and TH-4 produced the highest amounts of folate and the co-culture of *St. thermophilus* TH-4 and *Lb. rhamnosus* LGG produced the highest folate content when stimulated by passion fruit by-product and the concomitant presence of passion fruit by-product and FOS. Additionally, the synergism of the both microorganisms in co-culture might also contribute to improve the folate production. Our results suggest that MWE stimulated the folate production by *St. thermophilus* TH-4, in accordance with ALBUQUERQUE et al. (2016), and also by the co-culture (TH-4+LGG), probably due to the phenolic and soluble fibres content and the synergic stimulation of LGG to TH-4 as indicated by the principal components analysis. In contrast, none of the FWE stimulated the production of folate by *Lb. rhamnosus* LGG which is in agreement with LAIÑO et al. (2013), who highlighted lactobacilli strains as usually folate consumers.

The use of vitamin producing microorganisms, such as probiotics, could be an innovative strategy to improve not only the folate content of new fermented functional food products, but also the folate content in the gut (LEBLANC et al., 2017). Thus, the amount of folate produced by probiotics and other beneficial intestinal bacteria could provide this vitamin as nutrient to keep the metabolic activities of the intestinal microorganisms, to be absorbed by the intestinal cells and, additionally, to improve the antioxidant characteristic of the intestinal lumen.

In this regard, the adhesion ability to intestinal cells is another important characteristic of probiotics strains to promote their health benefits, including the production of beneficial metabolites and the immunoregulatory activity (AMBRIZ-PÉREZ et al. 2016; PARKAR et al., 2014). Once vitamin producing beneficial microorganisms, such as probiotics, may adhere and colonize the intestinal epithelium, they also may exert anti-inflammatory effects. These microorganisms may be potentially used to decrease inflammation in people with intestinal

inflammatory disease. Besides, these vitamin producing microorganisms could improve nutritional deficiency of patients with intestinal inflammatory diseases by producing different kind of vitamins, such as vitamins from group B, once these patients present low nutrient absorption due to changes in their intestinal epithelium (DE MORENO DE LEBLANC et al., 2018). Regarding the microorganism adhesion abilities, PARKAR et al. (2008) investigated the effect of different polyphenols on the growth of intestinal microorganisms and their adhesion to intestinal cells. The authors observed that most polyphenols tested affected the viability of the microorganisms and that the phenolic compounds phloridzin and rutin enhanced the adhesion of probiotic lactobacilli and decreased the adherence of harmful microorganisms. PARKAR et al. (2014) investigated the effect of the blackcurrant juices on the modulation of pathogenic and probiotic proliferation and their adhesion to intestinal cells. They observed that blackcurrant juices are rich in various polyphenols with functional antioxidant benefits, which contributed to inhibit the adhesion of *Salmonella* to intestinal cells. According to the authors, the adhesion of the probiotic was not impaired. PARKAR et al. (2010) evaluated the benefits of kiwi fruit pectins on the bacterial adhesion to intestinal epithelial using Caco-2 cells. The authors observed that monoK pectin was the most effective kiwi fruit pectin when compared to the prebiotic inulin, enhancing the adhesion of *Lb. rhamnosus* and decreasing the adhesion of *Salmonella* Typhimurium to Caco-2 cells. Additionally, the authors observed that depending on the kind and amount of carbohydrates, these compounds may have pro- or anti-adhesive effects.

5. Conclusions

Our results suggest that water extracts from fruit by-products as sources of dietary fibres associated with phenolic compounds with antioxidant activity may present biological potential on inflammatory processes by decreasing the production of pro-inflammatory mediators such as nitric oxide by LPS-stimulated macrophages. Besides, most of FWE contributed to the growth of *St. thermophilus* TH-4 and the probiotic *Lb. rhamnosus* LGG, the folate production by these microorganisms, and their adhesion to Caco-2 cells when used individually or in co-culture. Mango water extract (MWE) presented the best influence on the biological and functional parameters evaluated, especially in the reduction of NO levels and regarding the production of folate by the beneficial microorganisms tested. Taken together, the results highlight the use of MWE as the most promising natural water extract from tropical fruit by-products considering its dietary fibres content, total phenolic content,

phenolic composition, and antioxidant activity in order to improve anti-inflammatory effects, the growth of TH-4 and LGG and their folate production and adhesion to Caco-2 cells. The use of fruit by-products as source of natural compounds with antioxidant, biological, and functional activity is increasing in the last few years. Thus, further studies will be required to better characterize the different FWE components and elucidate how these compounds, especially the dietary fibres and phenolic compounds, affect beneficially the human health including their effect on inflammatory processes and the modulation of the intestinal microbiota activity to improve intestinal homeostasis.

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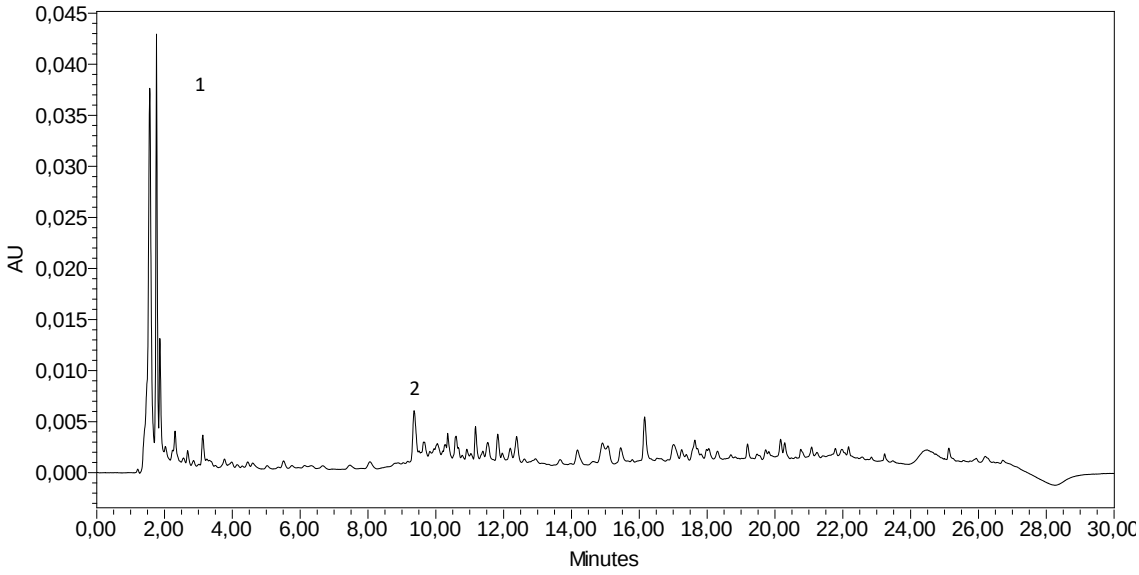
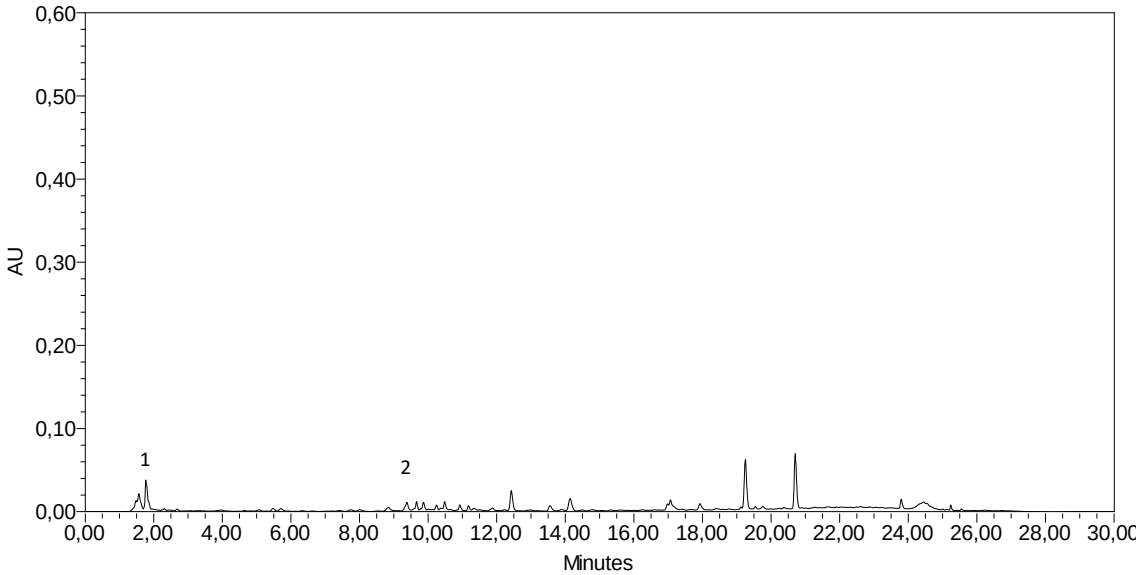
Supplementary material I: Chromatograms from fruit by-product water extracts

HPLC-DAD chromatograms of all fruit by-products water extracts (FWEs)

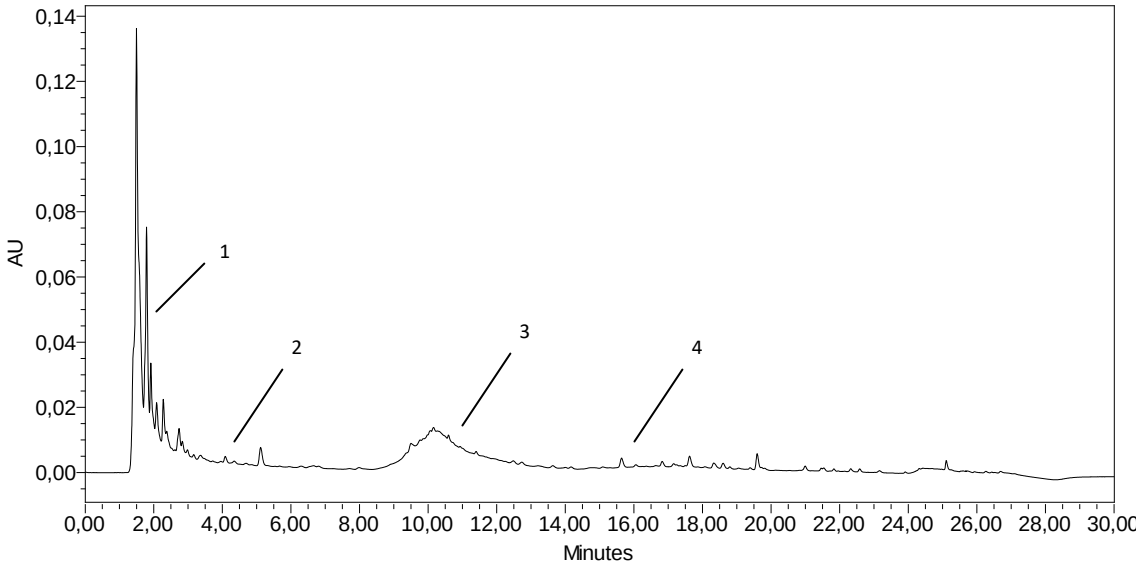
a) Passion fruit water extract (PFWE). Numbers refer to the phenolic compounds identified as: 1- Gallic acid, 2- Siringic acid



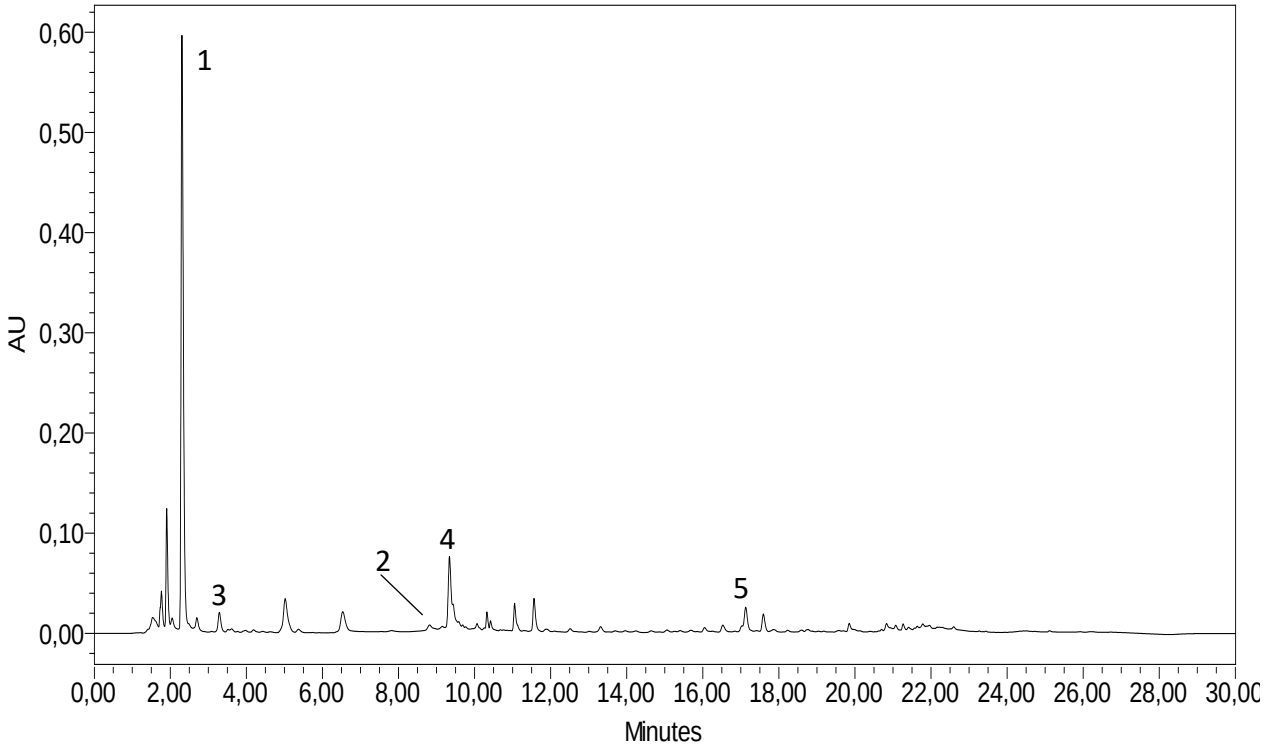
b) Orange water extract (OWE). Numbers refer to the phenolic compounds identified as: 1- Gallic acid, 2- Vanilic acid



c) Acerola water extract (AWE). Numbers refer to the phenolic compounds identified as: 1 – Gallic acid, 2 – Protocatecuic acid, 3 – Epicatechin, 4 – Rutin



d) Mango water extract (MWE). Numbers refer to the phenolic compounds identified as: 1- Gallic acid, 2- Syringe acid, 3- Protocatechuic acid, 4- Catechin, 5- Rutin.



Supplementary material II: Soluble carbohydrates and Pectin contents

1. Material and Methods

1.1 Fruit by-products soluble sugars content

Fructose, glucose and sucrose were extracted, filtered and determined by high performance liquid chromatography (HPLC), as described by Feinberg & Burgner (1992). The analysis were performed using a Young Lin (YL 9100) HPLC system equipped with Refractive Index detector, and a normal phase column (Luna NH2. 250 × 4.6 mm, 5 μm, Phenomenex Inc., Torrance, USA) and thermostated at 40 ˚C. The HPLC mobile phase consisted of acetonitrile and water (80:20 v/v) a 1.0 mL/min flow rate. The results were expressed in g/100 g of dry matter of fruit by-product and the analysis were performed in triplicate.

1.2 Determination of pectin content

Pectin was determined according to Pearson (1976) and expressed in gram of calcium pectate per 100 g of dry matter of fruit by-product.

2. Results

2.1 Fruit by-products soluble sugars and pectin content

Soluble sugars (fructose, glucose, and sucrose) and pectin content of all fruit by-products are shown in Table 1. Mango by-product presented the highest amount of fructose (5.65 ± 0.13 g/100 g), sucrose (2.37 ± 0.07 g/100 g) and, pectin (17.59 ± 0.40 g/100 g) while acerola by-product presented the lowest amount of pectin (2.91 ± 0.10 g/100g) and sucrose which was not detected by the method used. Orange by-product presented the highest amount of glucose (4.86 ± 0.04 g/100 g) followed by mango by-product (3.09 ± 0.05 g/100g). There was no significant difference ($P > 0.05$) between the pectin content of orange (12.49 ± 0.18 g/100 g) and passion fruit by-products (12.64 ± 0.29 g/100 g).

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Table 1 – Soluble carbohydrates (glucose, fructose, and sucrose) and pectin content of fruit by-products.

Fruit by-product flour	Fructose (g/100g)	Glucose (g/100g)	Sucrose (g/100g)	Pectin (g calcium pectate/100g)
Passion fruit	1.87 ± 0.04^{D}	1.68 ± 0.05^{D}	2.01 ± 0.03^{B}	12.64 ± 0.29^{B}
Orange	3.34 ± 0.02^{C}	4.86 ± 0.04^{A}	2.30 ± 0.04^{A}	12.49 ± 0.18^{B}
Acerola	3.60 ± 0.12^{B}	2.94 ± 0.06^{C}	ND	2.91 ± 0.10^{C}
Mango	5.65 ± 0.13^{A}	3.09 ± 0.05^{B}	2.37 ± 0.07^{A}	17.59 ± 0.40^{C}

^{A,B} Different capital letters in the same column denote significant differences between fruit by-products ($P < 0.05$). ND – not detectable (< 0.20 g/100 g).

GENERAL CONCLUSIONS

In this study, the potential prebiotic effect of different vegetable by-products was evaluated. Moreover, their capacity to enhance folate production by this vitamin B producing lactic acid bacteria in a soy base and in fermented soy products was also evaluated, especially when these food products were supplemented with passion fruit by-product and/or FOS. These vegetable ingredients, especially the fruit by-products, improved not only the growth of starter and probiotic strains, but also enhanced the metabolic activity of these microorganisms, which were able to produce natural folates during fermentation. The microbial folate production was strain-dependent, and the environmental and nutritional conditions were also relevant to stimulate the vitamin production by the microorganisms tested. The combination of proper strains and vegetable substrates allowed to bio-enrich both soy milk and fermented soy products, which could be considered innovative and functional alternatives to deliver high folate content products to consumers. Additionally, the fermented soy products protected *Lactobacillus rhamnosus* LGG and increased the folate bioaccessibility under simulated gastrointestinal conditions. Finally, fruit by-products, especially mango by-product, confirmed to be sources of bioactive compounds that possess, beyond the nutritional values, biological and functional properties.

ATTACHMENTS

ATTACHMENT 1

Food Biology Series

Fermented Foods of Latin America

From Traditional Knowledge to Innovative Applications

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Increasing Folate Content Through the Use of Lactic Acid Bacteria in Novel Fermented Foods

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

Abstract

Folate is an essential B-group vitamin that plays a key role in numerous metabolic reactions such as energy usage and the biosynthesis of DNA, RNA, and some amino acids. Since humans cannot synthesize folate, an exogenous supply of this vitamin is necessary to prevent nutritional deficiency. For this reason, many countries possess mandatory folic acid enrichment programs in foods of mass consumption; however, there is evidence that high intakes of folic acid, the synthetic form of folate, but not natural folates, can cause adverse effects in some individuals such as the masking of the hematological manifestations of vitamin B12 deficiency. Currently, many researcher groups are evaluating novel alternatives to increase concentrations of natural folates in foods. Lactic acid bacteria (LAB), widely used as starter cultures for the fermentation of a large variety of foods, can improve the safety, shelf life, nutritional value, flavor, and overall quality of the fermented products. Although most LAB are auxotrophic for several vitamins, it is now known that certain strains have the capability to synthesize some B-group vitamins. In this Chapter, the use of specific strains of folate producing LAB for the design of novel fermented food products will be discussed as will their use as an important strategy to help in the prevention of folate deficiency and as a safer alternative to mandatory folic acid fortification programs.

Introduction

Folic acid or vitamin B9, is an essential component of the human diet and is involved in many metabolic pathways (Rossi et al. 2011; LeBlanc et al. 2013). This micronutrient is a water-soluble vitamin and is part of the group of B vitamins. As it may not be synthesized by mammals, this vitamin is mainly obtained through food ingestion

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ATTACHMENT 2

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

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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.

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

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ATTACHMENT 3

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

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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

ATTACHMENT 4

Scientific article to be submitted to International Journal of Food Microbiology

Fermented soy products bio-enriched with folates and containing probiotic *Lactobacillus rhamnosus* LGG may improve the bioaccessibility of folate under *in vitro* simulated gastrointestinal digestion

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ATTACHMENT 5

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Tropical fruit by-products water extracts as source of soluble fibres associated to phenolic compounds with potential antioxidant, anti-inflammatory, and functional properties

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ATTACHMENT 6

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The impact of fruit and soybean by-products and amaranth on the growth of probiotic and starter microorganisms

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ABSTRACT

The ability of different fruit by-products, okara, and amaranth flour, to support the growth of probiotic and non-probiotic strains was evaluated. The tests were conducted with three commercial starter cultures (*Streptococcus thermophilus*), ten probiotic strains (seven *Lactobacillus* spp. and three *Bifidobacterium* spp. strains), and two harmful bacteria representative of the intestinal microbiota (*Escherichia coli* and *Clostridium perfringens*). *In vitro* fermentability assays were performed using a modified MRS broth supplemented with different fruits (acerola, orange, passion fruit, and mango), and soy (okara) by-products or amaranth flour. Orange and passion-fruit by-products were the substrates that most promoted the growth of bacterial populations, including pathogenic strains. On the other hand, the acerola by-product was the substrate that showed the highest selectivity for beneficial bacteria, since the *E. coli* and *Cl. perfringens* populations were lower in the presence of this fruit by-product. Although the passion fruit by-product, okara, and amaranth stimulated the probiotic strains, the growth of the pathogenic strains studied was higher compared to other substrates. Different growth profiles were verified for each substrate when the different strains were compared. Although pure culture models do not reflect bacterial interaction in the host, this study reinforces the fact that the ability to metabolize different substrates is strain-dependent, and acerola, mango, and orange by-products are the substrates with the greatest potential to be used as prebiotic ingredients.

1. Introduction

For more than two decades, Brazilian agriculture has registered strong growth. Brazil has become a major exporter of agricultural products, with a surplus of USD 78.6 billion in 2013. The country is one of the largest fruit exporters and the largest exporter of processed citrus, particularly concentrated frozen orange juice. In addition to citrus fruits, the main fruits produced include bananas, apples, grapes, melons, and tropical fruits, particularly papayas, mangoes, avocados, and pineapples. These last three are the most important in terms of volume. In the grain sector, soybeans are expected to continue to be one of the most important agricultural products. Currently, Brazil is the second largest producer, only behind the USA, but this scenario is expected to change by 2024, with Brazil overtaking the USA (OECD/FAO, 2015).

By-products generated in the fruit and vegetable processing industries are also an important environmental problem, resulting in significant economic losses for the sector. These facts have increased the interest of the food industries in discovering and applying strategies to improve the sustainability of food processing, such as the use of these by-products for livestock feeding and fuel production (Villanueva-Suárez, Pérez-Cózar, & Redonco-Cuenca, 2013). Even though they are frequently treated as industrial waste, they might be good sources of nutrients and bioactive compounds and improve the nutritional and functional properties of food products. A good example of this is okara, a by-product of soymilk and tofu (bean curd) processing, which presents high amounts of dietary fibres, proteins, lipids, and minerals, along with unspecified monosaccharides and oligosaccharides (Jiménez-Escrig, Tenorio, Espinosa-Martos, & Rupérez, 2008; Mateos-Aparicio, Mateos-Peinado, Jiménez-Escrig, & Rupérez, 2010). In general, okara may be considered a good and cheap source of dietary fibres, since they are its major component (Lu, Liu, & Li, 2013), and could be used to increase the content of high-value compounds in different products (Bedani, Campos, Castro, Rossi, & Saad, 2014; Villanueva-

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FICHA DO ALUNO

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Programa:	Tecnologia Bioquímico-Farmacêutica
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FBT5705-2/2	Tecnologia de Produtos Lácteos Funcionais	21/07/2014	31/08/2014	90	6	95	A	N	Concluída
FBA5741-3/2	Química e Bioquímica de Alimentos I	12/09/2014	16/10/2014	60	0	-	-	N	Matrícula cancelada
FBT5781-4/2	Culturas Probióticas: Aplicações Tecnológicas	21/10/2014	13/11/2014	60	4	100	A	N	Concluída
ICB5738-1/1	Gestão de Projetos em Inovação (Instituto de Ciências Biomédicas - Universidade de São Paulo)	24/11/2014	30/11/2014	30	0	-	-	N	Pré-matrícula indeferida
FBT5773-7/5	Tópicos Especiais em Tecnologia Bioquímico-Farmacêutica	02/03/2015	10/05/2015	30	0	-	-	N	Pré-matrícula indeferida
EDM5791-7/1	Metodologia do Ensino Superior (Faculdade de Educação - Universidade de São Paulo)	10/03/2015	20/04/2015	60	0	-	-	N	Pré-matrícula indeferida
FBA5728-3/11	Aprimoramento Didático	14/04/2015	11/05/2015	60	4	85	A	N	Concluída
FBT5700-3/2	Preparo de Artigos Científicos na Área de Tecnologia Bioquímico-Farmacêutica	08/05/2015	09/07/2015	90	0	-	-	N	Matrícula cancelada
FBF5779-2/3	Preparo de Artigos Científicos na Área de Farmácia	04/09/2015	05/11/2015	90	6	90	A	N	Concluída
FBT5773-7/7	Tópicos Especiais em Tecnologia Bioquímico-Farmacêutica	07/03/2016	15/05/2016	30	2	90	A	N	Concluída
FBA5896-7/2	Tópicos em Ciência dos Alimentos e Nutrição II	17/11/2017	25/01/2018	30	2	100	A	N	Concluída

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