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Food Technology

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

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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
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1st Examiner

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2nd 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

LIST OF TABLES

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

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.

References

LAIÑO, J.E.; DEL VALLE, M.J.; DE GIORI, G.S.; LEBLANC, J.G.L. Development of a high concentration yogurt naturally bio-enriched using selected lactic acid bacteria. **LWT - Food Science and Technology**, v. 54, p. 1-5, 2013.

ALBUQUERQUE, M.A.C.; BEDANI, R.; VIEIRA, A.D.S.; LEBLANC, J.G.; SAAD, S.M.I. 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, 2016.

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.

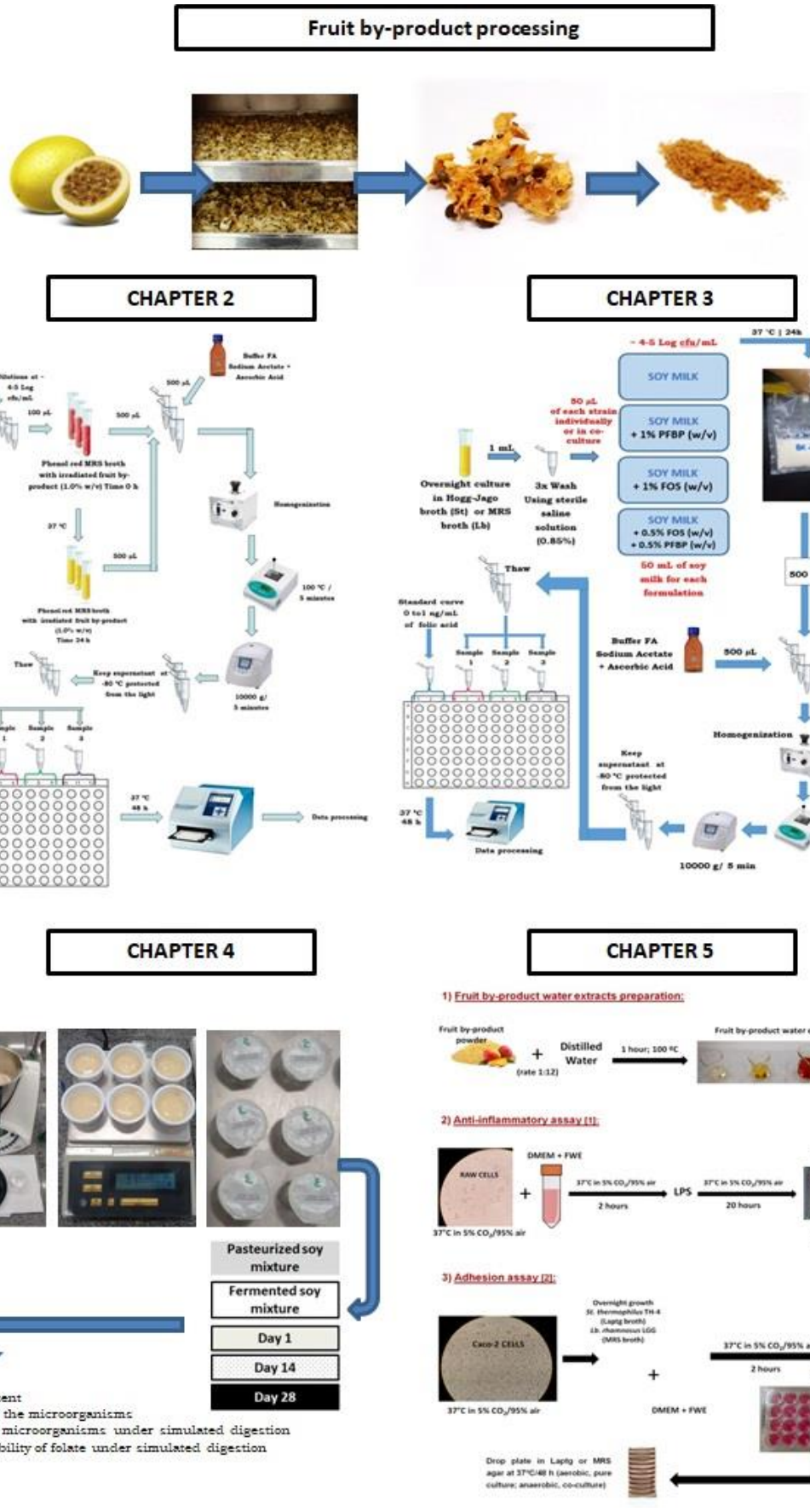
- Evaluate the ability of three strains of *St. thermophilus* and four strains of probiotic Lactobacillus strains (as pure cultures or in co-culture) to produce folates during fermentation of different soy milk formulations containing passion fruit by-product and/or fructo-oligosaccharides.

- 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.

7. References

AACC. AACC Method 86-47 Total Folate in Cereal Products - Microbiological Assay Using Trienzyme Extraction. *Approved Methods of the American Association of Cereal Chemists* (10th ed.). St Paul MN, American Association of Cereal Chemists, 2000.

AISO, K., TAMURA, T. Trienzyme treatment for food folate analysis: optimal pH and incubation time for α-amylase and protease treatments. **Journal of Nutritional Science and Vitaminology**, v. 44, p. 361-370, 1998.

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. Available in: http://portal.anvisa.gov.br/wps/wcm/connect/f851a500474580668c83dc3fbc4c6735/RDC_344_2002.pdf?MOD=AJPERES. Accessed: May 2015.

ANVISA. Agência Nacional de Vigilância Sanitária. Resolução RDC n° 269, de 22 de setembro de 2005. **Regulamento técnico sobre ingestão diária recomendada (IDR) de proteína, vitamina e minerais,** 2005. Available in:

http://portal.anvisa.gov.br/wps/wcm/connect/188497004745781185 7dd53fbc4c6735/RDC_269_2005.pdf?MOD=AJPERES. Accessed: May 2015.

AOAC. *AOAC Official Method 992.05 Total Folate (Pteroylglutamic Acid) in Infant Formula*. AOAC International, 2006a.

ARAYA-FARIAS, M.; GAUDREAU, A.; ROZOY, E.; BAZINET, L. Rapid HPLC-MS method for simultaneous determination of tea catechins and folates. Journal of Agricultural and **Food Chemistry**, v. 62, p. 4241-4250, 2014.

ARYANA, K.J. Folic acid fortified fat-free plain set yogurt. **International Journal of Dairy Technology**, v. 56, p. 219-222, 2003.

CAMILO, E.; ZIMMERMAN, J.; MASON, J.B.; GOLNER, B.; RUSSELL, R.; SELHUB, J.; ROSENBERG, I.H. Folate synthesized by bacteria in the human upper small intestine is assimilated by the host. **Gastroenterology**, 110:991-998, 1996.

CAPOZZI, V.; RUSSO, P.; DUEÑAS, M.T.; LÓPEZ, P.; SPANO, G. Lactic acid bacteria producing B-group vitamins: a great potential for functional cereals products. **Applied Microbiology and Biotechnology**, v. 96, p. 1383-1394, 2012.

CHEW, S.C.; LOH, S.P.; KHOR, G.L. Determination of folate content in commonly consumed Malaysian foods. **International Food Research Journal**, v. 19, p. 189-197, 2012.

CHIANG, F.F.; HUANG, S.C.; WANG, H.M.; CHEN, F.P.; HUANG, Y.C. High serum folate might have a potential dual effect on risk of colorectal cancer. **Clinical Nutrition**, v. 34, p. 986-990, 2015.

CRAVO, M.L.; CAMILO, M.E. Hyperhomocysteinemia in chronic alcoholism: relations to folic acid and vitamins B(6) and B(12) status. **Nutrition**, v. 16, p. 296-302, 2000.

CRIDER, K.S.; BAILEY, L.B.; BERRY, R.J. Folic acid fortification – its history, effect, concerns, and future directions. **Nutrients**, v. 3, p. 370-384, 2011.

CRITTENDEN, R.G.; MARTINEZ, N.R.; PLAYNE, M.J. Synthesis and utilization of folate by yoghurt starter cultures and probiotic bacteria. **International Journal of Food Microbiology**, v. 80, p. 217-222, 2003.

D'AIMMO, M.R.; MATTARELLI, P.; BIAVATI, B.; CARLSSON, N.G.; ANDLID, T. The potential of bifidobacteria as a source of natural folate. **Journal of Applied Microbiology**, v. 112, p. 975-984, 2012.

D'AIMMO, M.R.; MODESTO, M.; MATTARELLI, P.; BIAVATI, B.; ANDLID, T. Biosynthesis and cellular content of folate in bifidobacteria across host species with different diets. **Anaerobe**, v. 30, p. 169-177, 2014.

DANTAS, J.A.; DINIZ, A.S.; ARRUDA, I.C.G. Consumo alimentar e concentração intra-eritrocitárias de folato em mulheres do Recife, Nordeste do Brasil. **Archivos Latino Americano de Nutricion**, v. 60, p. 227-234, 2010.

DELCHIER, N.; RINGLING, C.; MAINGONNAT, J-F.; RYCHLIK, M.; RENARD, C.M.G.C. Mechanisms of folate losses during processing: Diffusion vs. heat degradation. **Food Chemistry**, v. 157, p. 439-447, 2014.

DIVYA, J.B.; NAMPOOTHIRI, K.M. Encapsulated *Lactococcus lactis* with enhanced gastrointestinal survival for the development of folate enriched functional foods. **Bioresource Technology**, v. 188, p. 226-230, 2015.

EITENMILLER, R.R.; LANDEN, W.O. **Folate and folic acid.** *In:* Vitamin Analysis for the Health and Food Sciences. R.R. Eitenmiller and W.O. Landen (eds.). CRC Press, Boca Raton, Chapter 10, pp. 411-466, 2007.

FAJARDO, V.; ALONSO-APERTE, E.; VARELA-MOREIRAS, G. Lack of data on folate in convenience foods: Should ready-to-eat products considered relevant for folate intake? The European challenge. **Journal of food Composition and Analysis**, v. 28, p. 155-163, 2012.

FAJARDO, V.; VARELA-MOREIRAS, G. Efficacy of adding folic acid to foods. **International Journal for Vitamin and Nutrient Research**, v. 82, p. 177-186, 2012.

FAJARDO, V.; ALONSO-APERTE, E.; VARELA-MOREIRAS, G. Folate content in fresh-cut vegetable packed products by 96-well microliter plate microbiological assay. **Food Chemistry**, v. 169, p. 283-288, 2015.

FAO/WHO. **Human vitamin and mineral requirements.** *In:* FAO/WHO (ed.), Human Vitamin and Mineral Requirements (Bangkok, Thailand), 2002.

FAO/WHO. **Human vitamin and mineral requirements**: report of a joint FAO/WHO expert consulation. Rome, Italy: FAO, 341p, 2004.

FAO/WHO. Annex D - **A procedure for estimating feasible fortification levels for a mass fortification programme**. In: L. Allen, B. de Benoist, O. Dary, R.Hurrell (eds.), Guidelines on Food Fortification with Micronutrients. 1st ed. World Health Organization, Portland, p. 294-312, 2006.

FORSSEN, K.M.; JAGERSTAD, M.I.; WIGERTZ, K.; WITTHOFT, C.M. Folates and dairy products: a critical update. **The Journal of the American College of Nutrition**, v. 19, p. 100S-110S, 2000.

FUJIMORI, E.; BALDINO, C.F.; SATO, A.P.S.; BORGES, A.L.V.; GOMES, M.N. Prevalência e distribuição espacial de defeitos do tubo neural no Estado de são Paulo, Brasil, antes e após a fortificação de farinhas com ácido fólico. **Cadernos de Saúde Pública**, v. 29, p. 145-154, 2013.

GANGADHARAN, D.; NAMPOOTHIRI, K.M. Folate production using Lactococcus lactis spp cremoris with implications for fortification of skim milk and fruit juices. **Food Science and Technology**, v. 44, p. 1859-1864, 2011.

GIBSON, G.R.; PROBERT, H.M.; VAN LOO, J.A.E.; RASTALL, R.A.; ROBERFROID, M. B. Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. **Nutrition Research Reviews**, v. 17, p. 259-275, 2004.

GIBSON, G.R.; SCOTT, K.P.; RASTALL, R.A.; TUOHY, K.M.; HOTCHKISS, A.; DUBERT-FERRANDON, A.; et al. Dietary prebiotics: current status and new definition. **Food Science and Technology Bulletin: Functional Foods**, v. 7, p. 1-19, 2010.

GREGORY, J.F. **The bioavailability of folate**. *In:* L.P. Bailey (ed.), Folates in Health and Disease. Marcel Dekker, New York, pp. 195-235, 1995.

HENDLER, S.; RORVIK, D. **Supplement monographs**. *In:* S. Hendler and D. Rorvik (eds.), PDR for Nutritional Supplements. 1st ed. Medical Economics, Montvale, pp. 157-165, 2001.

HERBISON, C.E.; HICKLING, S.; ALLEN, K.L.; O'SULLIVAN, T.A.; ROBINSON, M.; BREMMER, A.P.; HUANG, R.C.; BEILIN, L.J.; MORI, T.A.; ODDY W.H. Low intake of B-vitamins is associated with poor adolescent mental health and behavior. **Preventive Medicine**, v. 55, p. 634-638, 2012.

HERMANN, W.; OBEID, R. The mandatory fortification of staple foods with folic acid. **Deutsches Ärzteblatt International**, v. 108, p. 249-254, 2011.

HOLASOVÁ, M.; FIEDLEROVÁ, V.; ROUBAL, P.; PECHACOVÁ, M. Possibility of increasing natural folate content in fermented milk products by fermentation and fruit componente addition. **Csech Journal of Food Science**, v. 23, p. 196-201, 2005.

HJORTMO, S.; PATRING, J.; JASTREBOVA, J.; ANDLID, T. Biofortification of folates in white wheat bread by selection of strains and process. **International Journal of Food Microbiology**, v. 127, p. 32-36, 2008.

HUGENSCHMIDT, S.; SCHWENNINGER, S.M.; GNEHM, N.; LACROIX, C. Screening of a natural biodiversity of lactic and propionic acid bacteria for folate and vitamin B12 production in supplemented whey permeate. **International Dairy Journal**, v. 20, p. 852-857, 2010.

HUGENSCHMIDT, S.; SCHWENNINGER, S.M.; Lacroix, C. Concurrent high production of natural folate and vitamin B12 using a co-culture process with *Lactobacillus plantarum* SM39 e *Propionibacterium freundenreichii* DF13. **Process Biochemistry**, v. 46, p. 1063-1070, 2011.

INDYK, H.E. An optical biosensor assay for the determination of folate in milk and nutritional dairy products. **International Dairy Journal**, v. 21, p. 783-789, 2011.

IOM. Institute of Medicine. **Dietary Reference Intakes Research Synthesis**: Workshop Summary. National Academy Press, Washington, 2006.

IYER, R.; TOMAR, S.K. Folate: a functional food constituent. **Journal of Food Science**, v. 74, p. R114-R122, 2009.

IYER, R.; TOMAR, S.K. Determination of folate/folic acid level in milk by microbiological assay, immune assay and high performance liquid chromatography. **Journal of Dairy Research**, v. 80, p. 233-239, 2013.

IYER, R.; TOMAR, S.K.; KAPILA, S.; MANI, J.; SINGH, R. Probiotic properties of folate producing Streptococcus thermophilus strains. **Food Research International**, v. 43, p. 103-110, 2010.

IYER, R.; TOMAR, S.K.; SINGH, R.; SHARMA, R. Estimation of folate in milk by microbiological assay using tri-enzyme extraction method. **Milchwissenschaft**, v. 64, p. 125-127, 2009.

IWATANI, Y.; ARCOT, J.; SHRESTHA, A. Determination of folate contents in some Australian vegetables. **Journal of Food Composition and Analysis**, v. 16, p. 37-48, 2003.

KARILUOTO, S.; AITTAMAA, M.; KORHOLA, M.; SALOVAARA, H.; VAHTERISTO, L.; PIIRONEN, V. Effects of yeasts and bactéria on the levels of folates in rye sourdoughs. International Journal of **Food Microbiology**, v. 106, p. 137-143, 2006.

KARILUOTO, S.; EDELMANN, M.; HERRANEN, M.; LAMPI, A.M.; SHMELEV, A.; SALOVAARA, H.; KORHOLA, M.; PIIRONEN, V. Production of folate by bacteria isolated from oat bran. **International Journal of Food Microbiology**, v. 143, p. 41-47, 2010.

KARILUOTO, S.; EDELMANN, M.; NYSTRÖM, L.; SONTAG-STROHM, T.; SALOVAARA, H.; KIVELÄ, R.; HERRANEN, M.; KORHOLA, M.; PIIRONEN, V. *In situ* enrichment of folate by microorganisms in beta-glucan rich oat and barley matrices. **International Journal of Food Microbiology**, v. 176, p. 38-48, 2014.

LAIÑO, J.E.; LEBLANC, J.G.; DE GIORI, G.S. Production of natural folates by lactic acid bacteria starter cultures isolated from artisanal Argentinean yogurts. **Canadian Journal of Microbiology**, v. 58, p. 581-588, 2012.

LAIÑO, J.E.; DEL VALLE, M.J.; DE GIORI, G.S.; LEBLANC, J.G.L. Development of a high concentration yogurt naturally bio-enriched using selected lactic acid bacteria. **Food Science and Technology**, v. 54, p. 1-5, 2013a.

LAIÑO, J.E.; DE GIORI, G.S.; LEBLANC, J.G. **Folate production by lactic acid bacteria**. *In:* R.R. Watson and V.R. Preedy (eds.), Bioactive Food as Dietary Interventions for Liver and Gastrointestinal Disease. Academic Press, San Diego, pp. 251-270, 2013b.

LAIÑO, J.E.; DEL VALLE, M.J.; DE GIORI, G.S.; LEBLANC, J.G.J. Applicability of a *Lactobacillus amylovorus* strain as co-culture for natural folate bio-enrichment of fermented milk. **International Journal of Food Microbiology**, v. 191, p. 10-16, 2014.

LEBLANC, J.G.; MILANI, C.; SAVOY DE GIORI, G.; SESMA, F.; VAN SINDEREN, D.; VENTURA, M. Bacteria as vitamin suppliers to their host: a gut microbiota perspective. **Current Opinion in Biotechnology**, v. 24, p. 160-168, 2013.

LEBLANC, J.G.; SAVOY DE GIORI, G.; SMID, E.J.; HUGENHOLTZ, J.; SESMA, F. **Folate production by lactic acid bacteria and other food-grade microorganisms.** In: A. Méndez-Vilas (ed.), Communicating Current Research and Educational Topics and Trends in Applied Microbiology, vol. 1. Formatex Research Center, Badajoz, pp. 329-339, 2007.

LIN, M.Y.; YOUNG, C.M. Folate levels in cultures of lactic acid bacteria. **International Dairy Journal**, v. 10, p. 409-414, 2000.

LIU, Y.; TOMIUK, S.; ROZOY, E.; SIMARD, S.; BAZINET, L.; GREEN, KITTS, D.D. Thermal oxidation studies on reduced folate, L-5-methyltetrahydrofolic acid (L-5-

MTHF) and strategies for stabilization using food matrices. **Journal of Food Science**, v. 77, p. C236-C243, 2012.

LÓPEZ-NICOLÁS, R.; FRONTELA-SASETA, C.; GONZALÉZ-ABELLÁN, R.; BARADO-PIQUERAS, A.; PEREZ-CONESA, D.; ROS-BERRUEZO, G. Folate fortification of white and whole-grain bread by adding Swiss chard and spinach. Acceptability by consumers. **LWT – Food Science and Technology**, v. 59, p. 263-269, 2014.

MASUDA, M.; IDE, M.; UTSUMI, H.; NIIRO, T.; SHIMAMURA, Y.; MURATA, M. Production potency of folate, vitamin B12 and thiamine by lactic acid bactéria isolated from Japanese pickles. **Bioscience, Biotechnology and Biochemistry**, v. 76, p. 2061-2067, 2012.

MAHARAJ, P.P.P.; PRASAD, S.; DEVI, R.; GOPALAN, R. Folate content and retention in commonly consumed vegetables in the South Pacific. **Food Chemistry**, v. 182, p. 327-332, 2015.

MCNULTY, H.; PENTIEVA, K. Folate bioavailability. **The Proceedings of the Nutrition Society**, v. 63, p. 529-536, 2004.

MCPARTLIN, J.; HALLIGAN, A.; SCOTT, J.M.; DARLING, M.; WEIR, D.G. Accelerated folate breakdown in pregnancy. **Lancet**, v. 341, p. 148-149, 1993.

MELSE-BOONSTRA, A.; WEST, C.E.; KATAN, M.B.; KOK, F.J.; Verhoef, P. Bioavailability of heptaglutamyl relative to monoglutamyl folic acid in healthy adults. **The American Journal of Clinical Nutrition**, v. 79, p. 424-429, 2004.

NYGREN-BABOL, L.; JÄGERSTAD, M. Folate-binding protein in milk: a review of biochemistry, physiology and analytical methods. **Critical Reviews in food Science and Nutrition**, v. 52, p. 410-425, 2012.

NOR, N.M.; MOHAMAD, R.; FOO, H.L.; RAHIM, R.A. Improvement of folate biosynthesis by lactic acid bacteria using response surface methodology. **Food Technology and Biotechnology**, v. 48, p. 243-250, 2010.

OHRVIK, V.E.; WITTHOFT, C.M. HUMAN FOLATE BIOAVAILABILITY. **NUTRIENTS**, V. 3, P. 475-490, 2011.

PADALINO, M.; PEREZ-CONESA, D.; LÓPEZ-NICOLÁS, R.; PRONTELA-SASETA, C. Effect os fructoologosaccharides and galactooligosaccharides on the folate production of some folate-producing bacteria in media cultures or milk. **International Dairy journal**, v. 27, p. 27-33, 2012.

PHILLIPS, K.M.; RUGGIO, D.M.; HAYTOWITZ, D.B. Folate composition of 10 types of mushrooms determined by liquid chromatography-mass spectrometry. **Food Chemistry**, v. 129, p. 630-636, 2011.

POMPEI, A.; CORDISCO, L.; AMARETTI, A.; ZANONI, S.; MATTEUZZI, D.; ROSSI, M. Folate production by bifidobacteria as potential probiotic property. **Applied and Enviromental Microbiology**, v. 73, p. 179-185, 2007.

POUNIS, G.; CASTELNUOVO, A.F.; LORGERIL, M.; KROGH, V.; SIANI, A.; ARNOUT, J.; CAPPUCCIO, F.P.; VAN DONGEN, M.; ZAPPACOSTA, B.; DONATI, M.B.; GAETANO, G.; IACOVIELLO, L. Folate intake and folate serum levels in men and women from two European populations: the IMMIDIET project. **Nutrition**, v. 30, p. 822-830, 2014.

QUINLIVAN, E.P.; GREGORY, J.F. Reassessing folic acid consumption patterns in the United States (1999-2004): potential effect on neural tube defects and overexposure to folate. **The American Journal of Nutrition**, v. 86, p. 1773-1779, 2007.

ROSSI, M.; AMERATTI, A.; RAIMOUNDI, S. Folate productionby probiotic bacteria. **Nutrients**, v. 3, p. 118-114, 2011.

RAHMAN, T.; CHOWDHURY, M.M.H.; ISLAM, M.T.; AKHTARUZZAMAN, M. Microbiological assay of folic acid content in some selected Bangladeshi food stuffs. **International Journal of Biology**, v. 7, p. 35-43, 2015.

RADER, J.I.; WEAVER, C.M.; ANGYAL, G. Use of a microbiological assay with tri-enzyme extraction for measurement of pre-fortification levels of folates in enriched cereal-grain products. **Food Chemistry**, v. 62, p. 451-465, 1998.

RADER, J.; WEAVER, C.M.; ANGYAL, G. Total folate in enriched cereal-grain products in the United States following fortification. **Food Chemistry**, v. 70, p. 275-289, 2000.

SAMANIEGO-VAESKEN, M.L.; ALONSO-APERTE, E.; VARELA-MOREIRAS, G. Vitamin food fortification today. **Food & Nutrition Research**, 56:5459, 2012.

SANTOS, L.M.P.; PEREIRA, M.Z. Efeito da fortificação com ácido fólico na redução dos efeitos do tubo neural. **Cadernos de Saúde Pública**, v. 23, p. 17-24, 2007.

SANTOS, F.; WEGKAMP, A.; DE VOS, W.M.; SMID, E.J.; HUGENHOLTZ, J. High-Level folate production in fermented foods by the B12 producer Lactobacillus reuteri JCM1112. **Applied and Enviromental Microbiology,** v. 74, p. 3291-3294, 2008.

SAUBERLICH, H.F.; KRETSCH, M.J.; SKALA, J.H.; JOHNSON, H.L.; TAYLOR, P.C. Folate requirement and metabolism in nonpregnant women. **The American Journal of Clinical Nutrition**, v. 46, p. 1016-1028, 1987.

SCOTT, J.M. Folate and vitamin B_{12}. **The Proceedings of the Nutrition Society**, v. 58, p. 441-448, 1999.

SHUAIBI, A.M.; SEVENHUYSEN, G.P.; HOUSE, J.D. The importance of using folate intake expressed as dietary folate equivalents in predicting folate status. **Journal of Food Composition and Analysis**, v. 22, p. 38-43, 2009.

SILVA, N., DAVIS, B. Iron, B_{12}, and folate. **Medicine**, v. 41, p. 204-207, 2013.

SMITH, A.D.; KIM, Y.I.; REFSUM, H. Is folic acid good for everyone?. **The American Journal of Clinical Nutrition**, v. 87, p. S17-S33, 2008.

SYBESMA, W.; STARRENBURG, M.; TIJSSELING, L.; HOEFNAGEL, M.H.N.; HUGENHOLTS, J. Effects of cultivation conditions on folate production by lactic acid bacteria. **Applied and Enviromental Microbiology**, v. 69, p. 4542-4548, 2003.

SREENIVAS, K.M.; LELE, S.S. Prebiotic activity of gourd family vegetable fibres using *in vitro* fermentation. **Food Bioscience**, v. 1, p. 26-30, 2013.

SOONGSONGKIAT, M.; PUWASTIEN, P.; JITTINANDANA, S.; DEE-UAM, A.; SUNGPUAG, P. Testing of folate conjugase from chicken pancreas vs. commercial enzyme and studying the effect of cooking on folate retention in Thai foods. **Journal of food Composition and Analysis**, v. 23, p. 681-688, 2010.

STAMM, R.A.; HOUGHTON, L.A. Nutrient intake values for folate during pregnancy and lactation vary widely around the world. **Nutrients**, v. 5, p. 3920-3947, 2013.

STRANDLER, H.S.; PATRING, J.; JÄGERSTAD, M.; JASTREBOVA, J. Challenges in the determination of unsubstituted food folates: Impact of stabilities and conversions on analytical results. **Journal of Agricultural and Food Chemistry**, v. 63, p. 2367-2377, 2015.

TAMURA, T.; MIZUNO, Y.; JOHNSTON, K.E.; JACOB, R.A. Food folate assay with protease, α-amylase and folate conjugase treatments. **Journal of Agricultural and Food Chemistry**, v. 45, p. 135-139, 1997.

TOMAR, S.K.; SRIVATSA, N.; IYER, R.; SINGH, R. Estimation of folate production by Streptococcus thermophilus using modified microbiological assay. **Milchwissenschaft**, v. 64, p. 260-263, 2009.

TRUSWELL, A.; KOUNNAVONG, S. Quantitative responses of serum folate to increasing intakes of folic acid in healthy women. **European Journal of Clinical Nutrition**, v. 51, p. 839-845, 1997.

TYAGI, K.; UPADHYAYA, P.; SARMA, S.; TAMBOLI, V.; SREELAKSHMI, Y.; SHARMA, R. High performance liquid chromatography coupled to mass spectrometry for profiling and quantitative analysis of folate monoglutamates in tomato. **Food Chemistry**, v. 179, p.76-84, 2015.

UEHARA, S.K.; ROSA, G. Associação da deficiência de ácido fólico com alterações patológicas e estratégias para sua prevenção: uma visão crítica. **Revista de Nutrição**, v. 23, p. 881-894, 2010.

VISHNUMOHAN, S.; ARCOT, J.; PICKFORD, R. Naturally-occorring folates in foods: Method development ans analysis using liquid chromatography-tandem mass spectrometry (LC-MS/MS). **Food Chemistry**, v. 125, p. 736-742, 2011.

YON, M.; HYUN, T.H. Folate content of foods commonly consumed in Korea measured after trienzyme extraction. **Nutrition Research**, v. 23, p. 735-746, 2003.

WALKEY, C.J.; KITTS, D.D.; LIU, Y.; VAN VUUREN, H.J.J. Bioengineering yeast to enhance folate levels in wine. **Process Biochemistry**, v. 50, p. 205-210, 2015.

WANG, X.; QIN, X.; DEMIRTAS, H.; LI, J.; MAO, G.; HUO, Y.; SUN, N.; LIU, L.; XU, X. Efficacy of folic acid supplementation in stroke prevention: a meta-analysis. **Lancet**, v. 369, p. 1876-1882, 2007.

WYK, J.V.; WITTHUHN, R.C.; BRITZ, T.J. Optimisation of vitamin B12 and folate production by *Propionibacterium freundenreichii* strains in kefir. **International Dairy Journal**, v. 21, p. 69-74, 2011.

YANG, Q.H.; CARTER, H.K.; MULINARE, J.; BERRY, R.J.; FRIEDMAN, J.M.; ERICKSON, J.D. Race-ethnicity differences in folic acid intake in women of childbearing age in the United States after folic acid fortification: findings from the National Health and Nutrition Examination Survey, 2001-2002. **The American Journal of Clinical Nutrition**, v. 85, p. 1409-1416, 2007.

ZHAO, R.; DIOP-BOVE, N.; VISENTIN, M.; GOLDMAN, I.D. Mechanisms of membrane transport of folates into cells and across epithelia. **Annual Review of Nutrition**, v. 31, p. 177-201, 2011.

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).

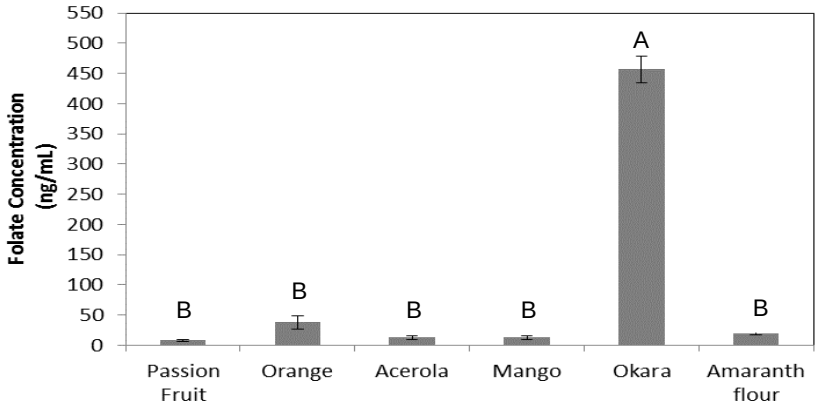


Figure 1. Folate concentration in vegetable by-products and amaranth flour. [A,B] Different capital letters denote significant differences between the tested by-products and/or amaranth flour.

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.

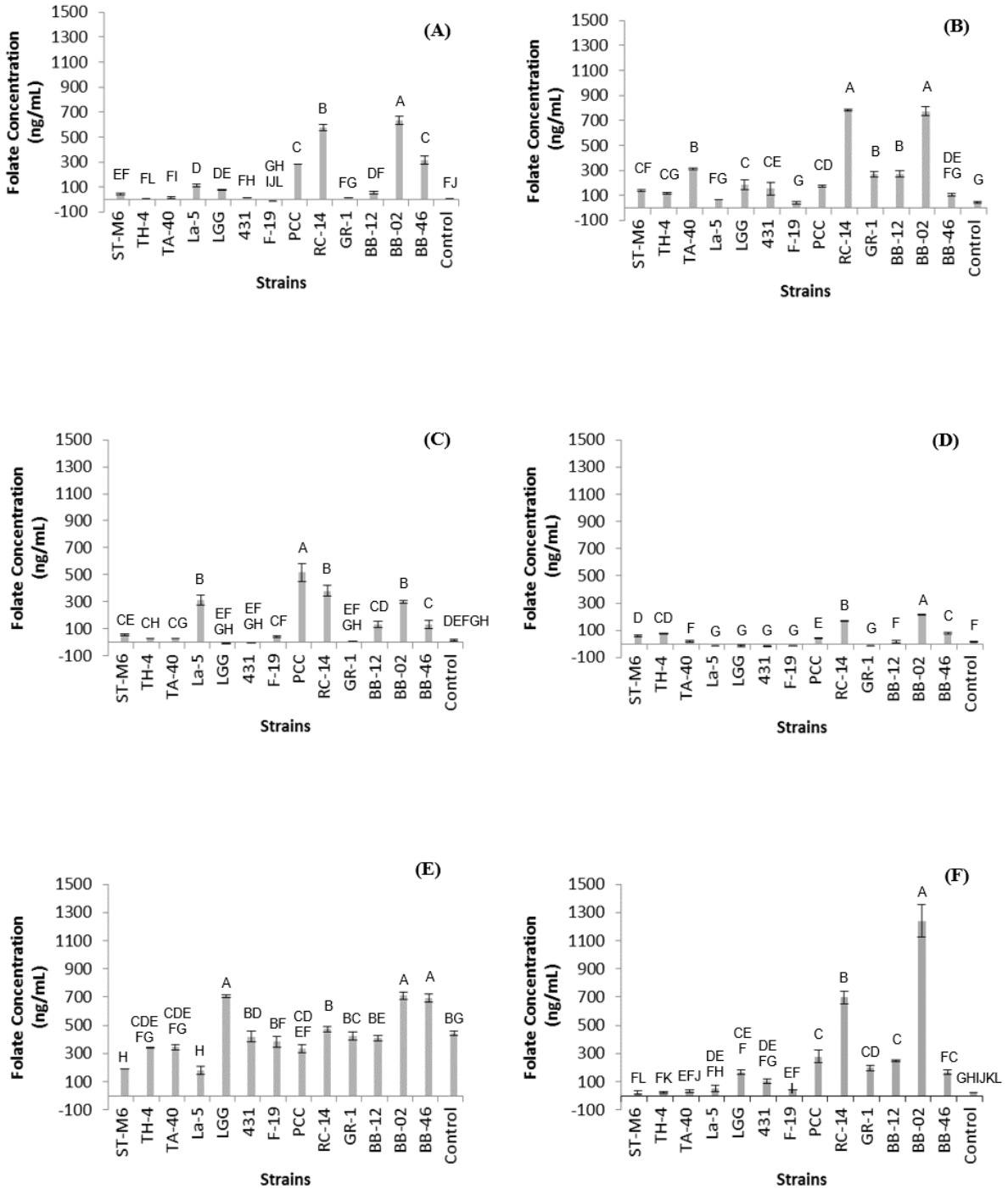


Figure 2. Folate production by starter and probiotic strains* after 24 h of fermentation of a Modified MRS Phenol Red Broth supplemented with fruit by-products, soybean okara by-product and amaranth flour. (A) Modified MRS Phenol Red Broth supplemented with 1% of passion fruit by-product, (B) Modified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

5. References

AGUEDO, M.; KOHNEN, S.; RABETAFIKA, N.; BOSSCHE, S.V.; STERCKX, J.; BLECKER, C.; BEAUVE, C.; PAQUOT, M. Composition of by-products from cooked fruit processing and potential use in food products. **Journal of Food Composition and Analysis**, v. 27, p. 61-69, 2012.

ALVAREZ-JUBETE, L.; WIJNGAARD, H.; ARENDT., E.K.; GALLAGHER, E. Polyphenol composition and *in vitro* antioxidant activity of amaranth, quinoa buckwheat and wheat as affected by sprouting and baking. **Food Chemistry**, v. 119, p. 770-778, 2010.

AYALA-ZAVALA, J.F.; ROSAS-DOMINGUEZ, C.; VEGA-VEGA, V.; GONZALEZ-AGUILAR, G.A. Antioxidant enrichment and antimicrobial protection of fresh-cut fruits using their own by-products: Looking for integral exploitation. **Journal of Food Science**, v. 25, p. 175–181, 2010.

BAILEY, S.W.; AYLING, J.E. The extremely slow and variable activity of dihydrofolate reductase in human liver and its implications for high folic acid intake. **PNAS - Proceedings of the National Academy of Sciences of the United States of America**, v. 106, p. 15424-15429, 2009.

BEDANI, R.; ROSSI, E.A.; SAAD, S.M.I. Impact of inulin and okara on *Lactobacillus acidophilus* La-5 and *Bifidobacterium animalis* Bb-12 viability in a fermented soy product and probiotic survival under *in vitro* simulated gastrointestinal conditions. **Food Microbiology**, v. 34, p. 382-389, 2013.

BLOMQVIST, T.; STEINMOEN, H.; HÅVARSTEIN, L.S. Natural genetic transformation: a novel tool for efficient genetic engineering of the dairy bacterium *Streptococcus thermophilus*. **Applied and Environmental Microbiology**, v. 72, p. 6751–6756, 2006.

BURITI, F.C.C.; SANTOS, K.M.O.; SOMBRA, V.G.; MACIEL, J.S.; SÁ, D.M.A.T.; SALLES, H.O.; PAULA, R.C.M.; FEITOSA, J.P.A.; MOREIRA, A.C.O.M.; MOREIRA, R.A.; EGITO, A.S. Characterisation of partially hydrolysed galactomannan from *Caesalpinia pulcherrima* seeds as a potential dietary fibre. **Food Hydrocolloids**, v. 35, p. 512-521, 2014..

CASELATO-SOUSA, V.M.; AMAYA-FARFÁN, J. State of knowledge on amaranth grain: a comprehensive review. **Journal of Food Science**, v. 77, p. R93-R104, 2012.

CRITTENDEN, R.G.; MARTINEZ, N.R.; PLAYNE, M.J. Synthesis and utilization of folate by yoghurt starter cultures and probiotic bacteria. **International Journal of Food Microbiology**, v. 80, p. 217-222, 2003.

D'AIMMO, M.R.; MATTARELLI, P.; BIAVATI, B.; CARLSSON, N.G.; ANDLID, T. The potential of bifidobacteria as a source of natural folate. **Journal of Applied Microbiology**, v. 112, p. 975-984, 2012.

DE MAN, J.D.; ROGOSA, M.; SHARPE, M.E. A medium for the cultivation of lactobacilli. **Journal of Applied Bacteriology**, v. 23, p. 130–135, 1960..

ESPÍRITO SANTO, A.P.; PEREGO, P.; CONVERTI, A.; OLIVEIRA, M.N. Influence of milk type and addition of passion fruit peel powder on fermentation kinetics, texture profile and bacterial viability in probiotic yogurts. **LWT – Food Science and Technology**, v. 47, p. 393-399, 2012a.

ESPÍRITO SANTO, A.P.; CARTOLANO, N.S.; SILVA, T.F.; SOARES, F.A.S.M.; GIOIELLI, L.A.; PEREGO, P.; CONVERTI, A.; OLIVEIRA, M.N. Fibres from fruit by-products enhance probiotic viability and fatty acid profile and increase CLA content in yogurts. **International Journal of Food Microbiology,** v. 154, p. 135-144, 2012b.

ESPÍRITO-SANTO, A.P.; CARLIN, F.; RENARD, C.M.G.C. Apple, grape or orange juice: Which one offers the best substrate for lactobacilli growth? – A screening study on bacteria viability, superoxide dismutase activity, folates production and hedonic characteristics. **Food Research International**, v. 78, p. 352-360, 2015.

FAJARDO, V.; ALONSO-APERTE, E.; VARELA-MOREIRAS, G. Lack of data on folate in convenience foods: Should ready-to-eat products considered relevant for folate intake? The European challenge. **Journal of Food Composition and Analysis**, v. 28, p. 155-163, 2012.

GANGADHARAN, D.; NAMPOOTHIRI, K.M. Folate production using *Lactococcus lactis* spp. *cremoris* with implications for fortification of skim milk and fruit juices. **LWT – Science and Technology**, v. 44, p. 1859-1864, 2011.

HILL, C.; GUARNER, F.; REID, G.; GIBSON, G.R.; MERENSTEIN, D.J.; POT, B.; MORELLI, L.; CANANI, R.B.; FLINT, H.J.; SALMINEN, S.; CALDER, P.C.; SANDERS, M.E. Expert consensus document: The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the

term probiotic. **Nature Reviews Gastroenterology & Hepatology**, v.11, p. 506–514, 2014.

HOLASOVÁ, M.; FIEDLEROVÁ, V.; ROUBAL, P.; PECHACOVÁ, M. Possibility of increasing natural folate contente in fermented milk products by fermentation and fruit component addition. **Czech Journal of Food Sciences**, v. 23, p. 196-201, 2005.

IYER, R.; TOMAR, S.K.; SINGH, R.; SHARMA, R. Estimation of folate in milk by microbiological assay using tri-enzyme extraction method. **Milchwissenschaft**, v. 64, p. 125-127, 2009.

KARILUOTO, S.; AITTAMAA, M.; KORHOLA, M.; SALOVAARA, H.; VAHTERISTO, L.; PIIRONEN, V. Effects of yeasts and bacteria on the levels of folates in rye sourdoughs. International Journal of Food Microbiology, v. 106, p. 137-143, 2006.

JIMÉNEZ-ESCRIG, A.; TENORIO, M.D.; ESPINOSA-MARTOS, I.; RUPEREZ, P. Health-promoting effects of a dietary fibre from the soybean by-products okara in rats. **Journal of Agricultural and Food Chemistry**, v.56, p. 7495-7501, 2008.

LAIÑO, J.E.; LEBLANC, J.G.; DE GIORI, G.S. Production of natural folates by lactic acid bacteria starter cultures isolated from artisanal Argentinean yogurts. **Canadian Journal of Microbiology**, v. 58, p. 581-588, 2012.

LAIÑO, J.E.; DEL VALLE, M.J.; DE GIORI, G.S.; LEBLANC, J.G.L. Development of a high concentration yogurt naturally bio-enriched using selected lactic acid bacteria. **LWT - Food Science and Technology**, v. 54, p. 1-5, 2013a.

LAIÑO, J.D. ; DE GIORI, G.S. ; LEBLANC, J.G. **Folate production by lactic acid bacteria**. In: Watson, R.R., Preedy, V.R. (Eds.), Bioactive Food as Dietary Interventions for Liver and Gastrointestinal Disease. Academic Press, San Diego, pp. 251-270, 2013b.

LAIÑO, J.E.; DEL VALLE, M.J.; DE GIORI, G.S.; LEBLANC, J.G.J. Applicability of a *Lactobacillus amylovorus* strain as co-culture for natural folate bio-enrichment of fermented milk. **International Journal of Food Microbiology**, V. 191, p. 10-16, 2014.

LEBLANC, J.G.; MILANI, C.; DE GIORI, G.S.; SESMA, F.; VAN SINDEREN, D.; VENTURA, M. Bacteria as vitamin suppliers to their host: a gut microbiota perspective. **Current Opinion in Biotechnology**, v. 24, p. 160-168, 2013.

LIN, M.Y.; YOUNG, C.M. Folate levels in cultures of lactic acid bacteria. **International Dairy Journal**, v. 10, p. 409-413, 2000.

LÓPEZ-VARGAS, J.H.; FERNÁNDEZ-LÓPEZ, J.; PÉREZ-ÁLVAREZ, J.A.; VIUDA-MARTOS, M. Chemical, physico-chemical, technological, antibacterial and antioxidant

properties of dietary fibre powder obtained from yellow passion fruit (*Passiflora edulis* var. *flavicarpa*) co-products. **Food Research International**, v. 51, p. 756-763, 2013.

MAGNÚSDÓTTIR, S.; RAVCHEEV, D.; DE CRÉCY-LAGARD, V.; THIELE, I. Systematic genome assessment of B-vitamin biosynthesis suggests co-operation among gut microbes. **Frontiers in Genetics**, v. 6, p. 1-18, 2015.

MATEOS-APARICIO, I.; REDONDO-CUENCA, A.; VILLANUEVA-SUÁREZ, M.J.; ZAPATA-REVILLA, M.A.; TENORIO-SANZ, M.D. Pea pod, broad bean and okara, potential sources of functional compounds. **LWT - Food Science and Technology**, v. 43, p. 1467-1470, 2010.

O'SHEA, N.; ARENDT, E.K.; GALLAGHER, E. Dietary fibre and phytochemical characteristics of fruit and vegetable by-products and their recent applications as novel ingredients in food products. **Innovative Food Science and Emerging Technologies**, v.16, p. 1-10, 2012.

O'SHEA, N.; KTENIOUDAKI, A.; SMYTH, T.P.; MCLOUGHLIN, P.; DORAN, L.; AUTY, M.A.E.; ARENDT, E.; GALLAGHER, E. Physicochemical assessment of two fruit by-products as functional ingredients: Apple and orange pomace. **Journal of Food Engineering**, v. 153, p. 89-95, 2015.

PACHECO DA SILVA, F.F.; BISCOLA, V.; LEBLANC, J.G.; GOMBOSSY DE MELO FRANCO, B.D. Effect of indigenous lactic acid bacteria isolated from goat milk and cheeses on folate and riboflavin content of fermented goat milk. **LWT - Food Science and Technology**, v. 71, p. 155-161, 2016.

PAINE-WILSON, B.; CHEN, T.S. Thermal destruction of folacin: effect of pH and buffer ions. **Journal of Food Science**, v. 44, p. 717-722, 1979.

POMPEI, A.; CORDISCO, L.; AMARETTI, A.; ZANONI, S.; MATTEUZZI, D.; ROSSI, M. Folate production by bifidobacteria as potential probiotic property. **Applied and Environmental Microbiology**, v. 73, p. 179-185, 2007.

PADALINO, M.; PEREZ-CONESA, D.; LÓPEZ-NICOLÁS, R.; PRONTELA-SASETA, C. Effect of fructoologosaccharides and galactooligosaccharides on the folate production of some folate-producing bacteria in media cultures or milk. **International Dairy Journal**, v. 27, p. 27-33, 2012.

REZENDE, A.C.B.; IGARASHI, M.C.; DESTRO, M.T.; FRANCO, B.D.G.; LANDGRAF, M. Effect of gamma radiation of *Salmonella* strains, *Listeria monocytogenes*, and Shiga

Toxin-Producing *Escherichia coli* and sensory evaluation of minimally processed spinach (*Tetragonia expansa*). **Journal of Food Protection**, v. 10, p. 1656-1833, 2014.

ROSSI, M.; AMERATTI, A.; RAIMOUNDI, S. Folate production by probiotic bacteria. **Nutrients**, v. 3, p. 118-114, 2011.

RYU, S.I.; KIM, B.G.; PARK, M.S.; LEE, Y.B.; LEE, S.B. Evaluation of enhanced hygroscopicity, bifidogenicity, and anticariogenicity of enzymatically synthesized β-galactosyl-trehalose oligosaccharides. **Journal of Agricultural and Food Chemistry**, v. 55, p. 4184-4188, 2007.

STANOJEVIC, S.P.; BARACT, M.B.; PESIC, M.B.; JANKOVIC, V.S.; VUCELIC-RADOVIC, B.V. Bioactive proteins and energy value of okara as a by-product in hydrothermal processing of soymilk. **Journal of Agricultural and Food Chemistry**, v. 61, p. 9210-9219, 2013.

SYBESMA, W.; STARRENBURG, M.; TIJSSELING, L.; HOEFNAGEL, M.H.N.; HUGENHOLTS, J. Effects of cultivation conditions on folate production by lactic acid bacteria. **Applied and Environmental Microbiology**, v. 69, p. 4542-4548, 2003.

TIENGO, A.; FARIA, M.; NETTO, F.M. Characterization and ACE-inhibitory activity of amaranth proteins. **Journal of Food Science**, v. 74, p. H121-H126, 2009.

TOMAR, S.K.; SRIVATSA, N.; IYER, R.; SINGH, R. Estimation of folate production by *Streptococcus thermophilus* using modified microbiological assay. **Milchwissenschsft**, v. 64, p. 260-263, 2009.

VILLANUEVA, M.J.; YOKOYAMA, W.H.; HONG, Y.J.; BARTTLEY, G.E.; RUPÉREZ, P. Effect of high-fat diets supplemented with okara soybean by-product on lipid profiles of plasma, liver and faeces in Syrian hamsters. **Food Chemistry**, v. 124, p. 72-79, 2011.

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).

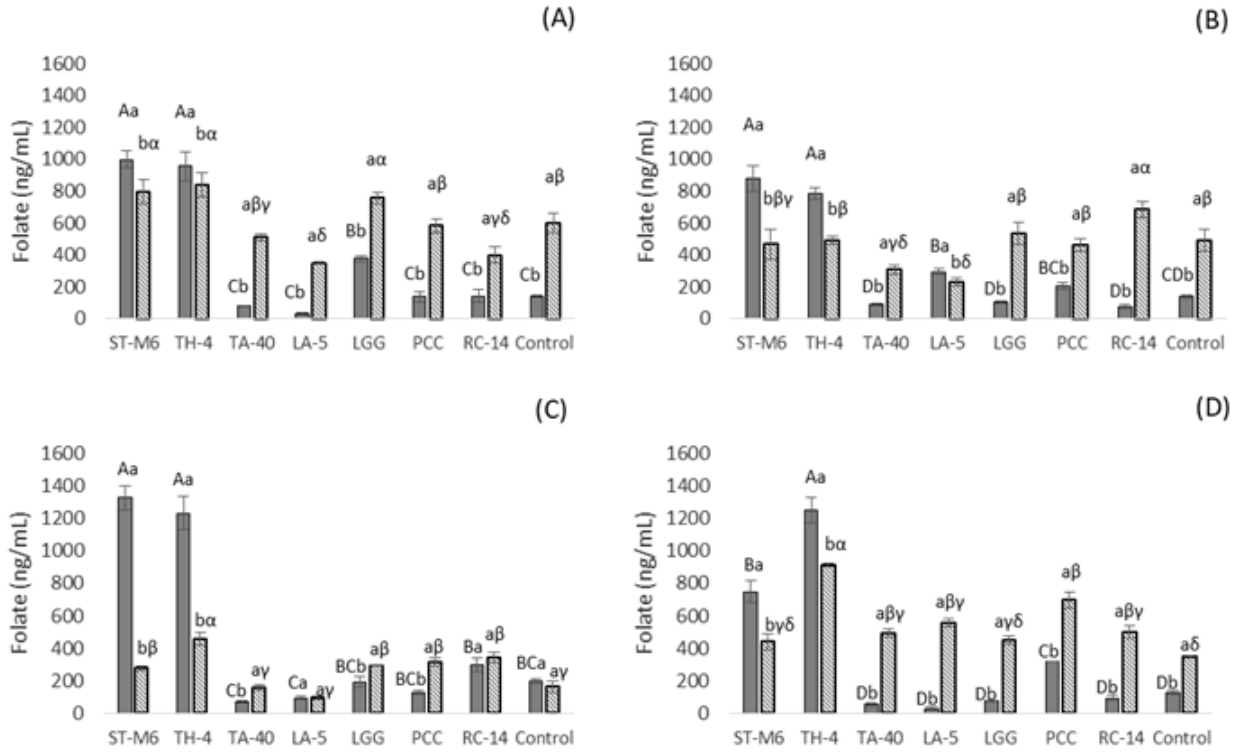


Figure 1. Total folate content of different soymilk formulations after 24 h of fermentation by starter and probiotic strains as pure cultures. Traditional microbiological assay (grey bars); Tri-enzymatic treatment (textured bars). ^{A,B} Different capital letters denote significant differences between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

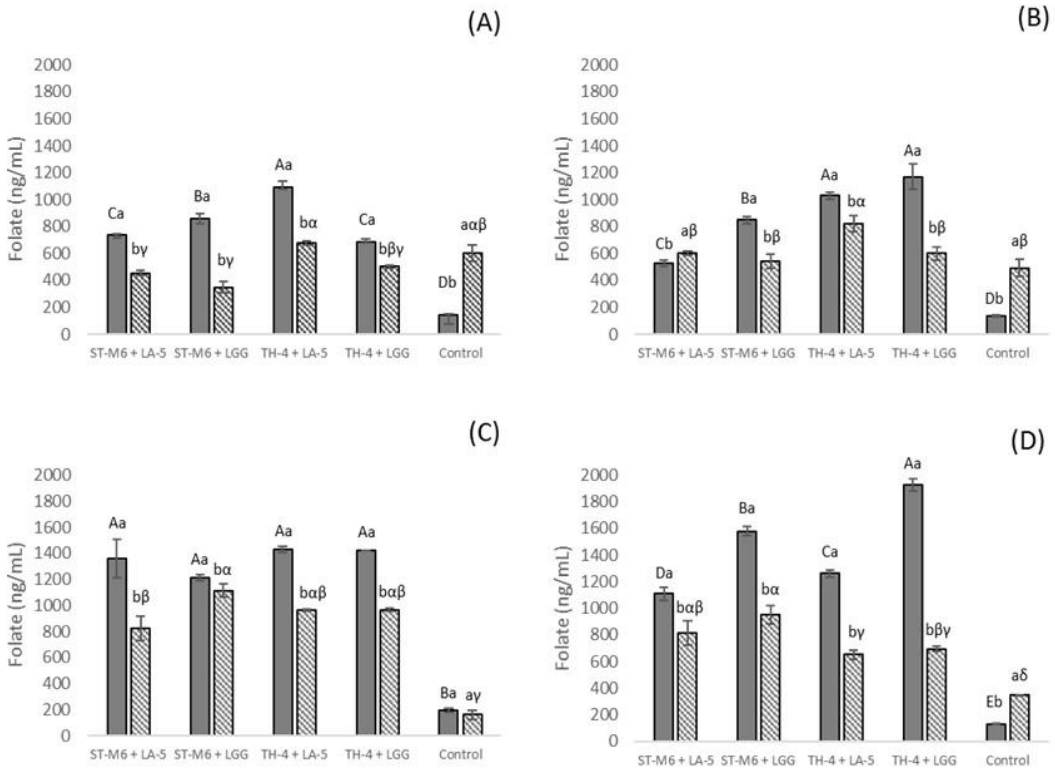


Figure 2. Total folate content of different soymilk formulations after 24 h of fermentation by *Lactobacillus* spp. strains with *Streptococcus thermophilus* strains. Traditional microbiological assay (grey bars); Tri-enzymatic treatment (textured bars). ^{A,B} Different capital letters denote significant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

6. References

AISO, K.; TAMURA, T. Trienzyme treatment for food folate analysis: optimal pH and incubation time for alfa-amylase and protease treatment. **Journal of Nutritional Science and Vitaminology**, v. 44, p. 361-370, 1998.

ALBUQUERQUE, M.A.C.; BEDANI, R.; VIEIRA, A.D.S.; LEBLANC, J.G.; SAAD, S.M.I. 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, 2016.

ASSOCIATION OF ANALYTICAL COMMUNITIES (AOAC). **Official methods of analysis**. Rev. 2. AOAC International, Gaithersburg, MD, USA. 18th ed. 2007.

ARCOT, J.; WONG, S.; SHERESTHA, A.K. Comparison of folate losses in soybean during the preparation of tempeh and soymilk. **Journal of the Science of Food and Agriculture**, v. 82, p. 1365-1368, 2002.

BEDANI, R.; ROSSI, E.A.; SAAD, S.M.I. Impact of inulin and okara on *Lactobacillus acidophilus* La-5 and *Bifidobacterium animalis* Bb-12 viability in a fermented soy product and probiotic survival under *in vitro* simulated gastrointestinal conditions. **Food Microbiology**, v. 34, p. 382–389, 2013.

BEDANI, R.; VIEIRA, A.D.S.; ROSSI, E.A.; SAAD, S.M.I. Tropical fruit pulps decrease probiotic survival to in vitro gastrointestinal stress in symbiotic soy yoghurt with okara during storage. **LWT - Food Science and Technology**, v. 55, p. 436-443, 2014.

BLOMQVIST, T.; STEINMOEN, H.; HÅVARSTEIN, L.S. Natural genetic transformation: a novel tool for efficient genetic engineering of the dairy bacterium *Streptococcus thermophilus.* **Applied and Environmental Microbiology**, v. 72, p. 6751–6756, 2006.

CAPOZZI, V.; RUSSO, P.; DUEÑAS, M.T.; LÓPEZ, P.; SPANO, G. Lactic acid bacteria producing B-group vitamins: a great potential for functional cereals products. **Applied Microbiology and Biotechnology**, v. 96, p. 1383–94, 2012.

CHAMPAGNE, C.P.; GREEN-JOHNSON, J.; RAYMOND, Y.; BARRETTE, J.; BUCKLEY, N. Selection of probiotic bacteria for the fermentation of a soy beverage in combination with Streptococcus thermophiles. **Food Research International**, v. 42, p. 612-621, 2009.

CORRÊA, R.C.G.; PERALTA, R.M.; HAMINIUK, C.W.I.; MACIEL, G.M.; BRANCHT, A.; FERREIRA, I.C.F.R. The past decade findings related with nutritional composition,

bioactive molecules and biotechnological applications of *Passiflora* spp. (passion fruit). **Trends in Food Science & Technology**, v. 58, p. 79-95, 2016.

DONKOR, O.N.; HENRIKSSON, A.; VASILJEVIC, T.; SHAH, N.P. α-Galactosidase and proteolytic activities of selected probiotic and dairy cultures in fermented soymilk. **Food Chemistry**, 104, 10–20, 2007.

ESPÍRITO SANTO, A.P.; CARTOLANO, N.S.; SILVA, T.F.; SOARES, F.A.S.M.; GIOIELLI, L.A.; PEREGO, P.; CONVERTI, A.; OLIVEIRA, M.N. Fibres from fruit by-products enhance probiotic viability and fatty acid profile and increase CLA content in yogurts. **International Journal of Food Microbiology**, v. 154, p. 135–144, 2012.

ESPÍRITO-SANTO, A.P.; CARLIN, F.; RENARD, C.M.G.C. Apple, grape or Orange juice: which one offers the best substrate for lactobacilli growth? – a screening study on bacteria viability, superoxide dismutase activity, folates production and hedonic characteristics. **Food Research International**, v. 78, p. 352-360, 2015.

FAO/WHO. (2002). **Human vitamin and mineral requirements**. In FAO/WHO (Ed.), *Human vitamin and mineral requirements*. Bangkok, Thailand.

FARNWORTH, E.R.; MAINVILLE, I.; DESJARDINS, M.P.; GARDNER, N.; FLISS, I.; CHAMPAGNE, C. Growth of probiotic bacteria and bifidobacteria in a soy yogurt formulation. **International Journal of Food Microbiology**, v. 116, p. 174–181, 2007.

FUNG, W.Y.; LIONG, M.T. Evaluation of proteolytic and ACE-inhibitory activity of Lactobacillus acidophilus in soy whey growth medium via response surface methodology. **LWT – Fod Science and Technology**, v. 43, p. 563-567, 2010.

GANGADHARAN, D.; NAMPOOTHIRI, K.M. Folate production using *Lactococcus lactis*spp. *cremoris* with implications for fortification of skim milk and fruit juices. **LWT – Fod Science and Technology**, v. 44, p. 1859–1864, 2011.

GIBSON, G.R.; HUTKINS, R.; SANDERS, M.E.; PRESCOTT, S.L.; REIMER, R.A.; SALMINEN, S.J.; SCOTT, K.; STANTON, C.; SWANSON, K.S.; CANI, P.D.; VERBEKE,K.; REID,G. The international Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. **Nature Reviews Gastroenterology & Hepatology**, v. 14, p. 491-502, 2017.

HILL, C.; GUARNER, F.; REID, G.; GIBSON, G.R.; MERENSTEIN, D.J.; POT, B.; MORELLI, L.; CANANI, R.B.; FLINT, H.J.; SALMINEN, S.; CALDER, P.C.; SANDERS, M.E. Expert consensus document: The International Scientific Association

for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. **Nature Reviews Gastroenterology & Hepatology**, 11, 506–514, 2014.

HOLASOVÁ, M.; FIEDLEROVÁ, V.; ROUBAL, P.; PECHACOVÁ, P. Possibility of increasing natural folate contente in fermented milk products by fermentation and fruit component addition. **Czech Journal of Food Sciences**, v. 23, p. 196–201, 2005.

HYUN, T.H.; TAMURA, T. Trienzyme extraction in combination with microbiologic assay in food folate analysis: an update review. **Experimental Biology and Medicine**, v. 230, p. 444-454, 2005.

IYER, R.; TOMAR, S.K.; KAPILA, S.; MANI, J.; SINGH, R. Probiotic properties of folate producing *Streptococcus thermophilus* strains. **Food Research International**, v. 43, p. 103-110, 2010.

LAIÑO, J.E. ; LEBLANC, J.G. ; DE GIORI, G.S. Production of natural folates by lactic acid bacteria starter cultures isolated from artisanal Argentinean yogurts. **Canadian Journal of Microbiology**, v. 58, p. 581-588, 2012.

LAIÑO, J.E.; JUAREZ DEL VALLE, M.; SAVOY DE GIORI, G.; LEBLANC, J.G. Development of a high concentration yogurt naturally bio-enriched using selected lactic acid bacteria. **LWT – Fod Science and Technology**, v. 54, p. 1–5, 2013.

LAIÑO, J.E.; JUAREZ DEL VALLE, M.; SAVOY DE GIORI, G.; LEBLANC, J.G. Applicability of a *Lactobacillus amylovorus* strain as co-culture for natural folate bio-enrichment of fermented milk. **International Journal of Food Microbiology**, v. 191, p. 10–16, 2014.

LEBLANC, J.G.; LEDUE-CLIER, F.; BENSAADA, M.; SAVOY DE GIORI, G.; GUEREKOBAYA, T.; SESMA, F.; JUILLARD, V.; RABOT, S.; PIARD, J.C. Ability of *Lactobacillus fermentum* to overcome host alpha-galactosidase deficiency, as evidenced by reduction of hydrogen excretion in rats consuming soya alpha-galacto-oligosaccharides. **BMC Microbiology**, v. 8, p. 1-9, 2008.

LEBLANC, J.G.; CHAIN, F.; MARTÍN, R.; BERMÚDEZ-HUMARÁN, L.G.; COURA, S.; LANGELLA, P. Beneficial effects on host energy metabolism of short-chain fatty acids and vitamins produced by commensal and probiotic bacteria. **Microbial Cell Factories**, v. 16, p. 79, 01-10, 2017.

LEE, B-H.; LO, Y-H.; PAN, T-M. Anti-obesity activity of *Lactobacillus* fermented soy milk products. **Journal of Functional Foods**, v. 5, p. 905-913, 2013.

MO, H.; KARILUOTO, S.; PIIRONEN, V.; ZHU, Y.; SANDERS, M.G.; VINCKEN, J.P.; WOLKERS-ROOIJACKERS, J.; NOUT, M.J.R. Effect of soybean processing on content and bioacessibility of folate, vitamin B12 and isoflavones in tofu and tempe. **Food Chemistry**, v. 141, p. 2418-2425, 2013.

O'SHEA, N.; KTENIOUDAKI, A.; SMYTH, T.P.; MCLOUGHLIN, P.; DORAN, L.; AUTY, M.A.E.; ARENDT, E.; GALLAGHEr E. Physicochemical assessment of two fruit by-products as functional ingredients: apple and orange pomace. **Journal of Food Engineering**, v. 153, p. 89–95, 2015.

PADALINO, M.; PEREZ-CONESA, D.; LÓPEZ-NICOLÁS, R.; PRONTELA-SASETA, C. Effect of fructoologosaccharides and galactooligosaccharides on the folate production of some folate-producing bacteria in media cultures or milk. **International Dairy Journal**, v. 27, p. 27–33, 2012.

PACHECO DA SILVA, F.F.; BISCOLA, V.; LEBLANC, J.G.; FRANCO, B.D.G.M. Effect of indigenous lactic acid bacteria isolated from goat milk and cheese on folate and riboflavin content of fermented goat milk. **LWT – Fod Science and Technology**, v. 71, p. 155-161, 2016.

PATRING, J.D.M.; JOHANSSON, M.S.; YAZYNINA, E.; JASTREBOVA, J.A. Evaluation of impact of different antioxidants on stability of dietary folates during food sample preparation and storage of extracts prior to analysis. **Analytica Chimica Act**, v. 553, p. 36-42, 2005.

POMPEI, A.; CORDISCO, L.; AMARETTI, A.; ZANONI, S.; MATTEUZZI, D.; ROSSI, M. Folate production by bifidobacteria as potential probiotic property. **Applied and Environmental Microbiology**, v. 73, p. 179–185, 2007.

VIEIRA, A.D.S.; BEDANI, R.; ALBUQUERQUE, M.A.C.; BÍSCOLA, V.; SAAD, S.M.I. The impact of fruit and soybean by-products and amaranth flour on the growth of probiotic and starter microorganisms. **Food Research International**, v., p. 356-363, 2017.

SAMINATHAN, M.; SIEO, C.C.; KALAVATHY, R.; ABDULLAH, N.; HO, Y.W. Effect of prebiotic oligosaccharides on growth of Lactobacillus strains used as a probiotic for chickens. **African Journal of Microbiology Research**, v. 5, p. 57-64, 2011.

SYBESMA, W.; STARRENBURG, M.; TIJSSELING, L.; HOEFNAGEL, M.H.N.; HUGENHOLTS, J. Effects of cultivation conditions on folate production by lactic acid bacteria. **Applied and Environmental Microbiology**, v. 69, p. 4542-4548, 2003.

VALDÉS-VARELA, L.; RUAS-MADIEDO, P.; GUEIMONDE, M. *In vitro* fermentation of diferente fructo-oligosaccharides by *Bifidobacterium* strains for the selection of synbiotic combinations. **International Journal of Food Microbiology**, v. 242, p. 19-23, 2017.

WATSON, D.; MOTHERWAY, M. O. C.; SCHOTERMAN, M. H. C.; NEERVEN, R. J. J. V.; NAUTA, A.; SINDEREN, D. V. Selective carbohydrate utilization by lactobacilli and bifidobacteria. **Journal of Applied Microbiology**, v. 114, p. 1132-1146, 2012.

YON, M.; HYUN, T.H. Folate content of foods commonly consumed in Korea measured after trienzyme extraction. **Nutrition Research**, v. 23, p. 735-746, 2003.

YEO, S-K.; LIONG, M-T. Effect of prebiotics on viability and growth characteristics of probiotic in soymilk. **Journal of the Science of Food and Agriculture**, 90, 267-275, 2010.

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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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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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

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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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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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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
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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
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