13

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 B_{12} 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 well 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 B_9, 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 B-group vitamins. As it is not

[a] Departamento de Tecnologia Bioquímico-Farmacêutica, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, 05508-000, São Paulo, SP, Brasil.
[b] CERELA-CONICET, C.P. T4000ILC, San Miguel de Tucumán, Argentina.
* Corresponding author: leblanc@cerela.org.ar or leblancjeanguy@gmail.com

synthesized by mammals, this vitamin must be obtained through food ingestion (Lin and Young 2000; Sybesma et al. 2003; Padalinno et al. 2007; Kariluoto et al. 2010).

Folate may be synthesized by various plants and some microorganisms. Dairy and non-dairy products are considered important sources of folate (Sybesma et al. 2003; Padalino et al. 2012; Laiño et al. 2013b). The main forms of folate are tetrahydrofolate (THF), 5-formyltetrahydrofolate (5-FmTHF), and 5-methyltetrahydrofolate (5-MeTHF). According to Lin and Young (2000) and Uehara and Rosa (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: DNA replication, repair and 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 (Laiño et al. 2012; Fujimore et al. 2013). 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 (Wyk et al. 2011; Hugenschimidt et al. 2011). Folic acid may, among other things, mask vitamin B_{12} 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. 2013a). 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 and Rosa 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 (Sybesma et al. 2003; Pompei et al. 2007; Kariluoto et al. 2010; D'Aimmo et al. 2012; Laiño et al. 2012; Padalino et al. 2012; Laiño et al. 2013a). 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).

Folate

Chemical structure, bioavailability, and functions

Folate, also known as vitamin B_9, 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 (Laiño et al. 2013a; Fajardo et al. 2015). 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-MTHF), 5-formyltetrahydrofolate (5-formyl-THF), 10-formyltetrahydrofolate (10-formyl-THF), 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 g-carboxyl of glutamate. The main intracellular folates are peteroylpentaglutamates and the major extracellular folates are pteroylmonoglutamates. pteroylpentaglutamates 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 and 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 and Davis 2013). The monoglutamates forms of folate, including FA, are transported across the proximal intestine through a saturable pH-dependent process (Iyer and Tomar 2009). Higher doses of folic acid are absorbed via a non-saturable passive diffusion process (Hendler and Rorvik 2001; Iyer and 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 and 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 and Witthof 2011; Silva and Davis 2013), which is normal since the excess of all water soluble vitamins (including the B group vitamins) is excreted.

Evidences suggest that the polyglutamate form is 60 to 80% bioavailable compared to the monoglutamate form (Gregory 1995; Melse-Boonstra et al. 2004; LeBlanc et al. 2007). 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 (Sauberlich et al. 1987; Gregory 1995; Forssen et al. 2000; McNulty and Pentieva 2004; Iyer and Tomar 2009). 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 and 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 and 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 countries 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 (Truswell and Kounnavong 1997; Shuabi et al. 2009).

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. 2014; 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).

Food rich in folate and folate requirements

As human beings do not synthesize folates, and it is necessary to assimilate this vitamin from exogenous sources. Folate occurs in most foods, with at least 50% being in the polyglutamate form. FA is thermolabile and thus may be destroyed by cooking (Silva and 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 and 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) (Lin and Young 2000; Iyer and Tomar 2009). Scientific evidences suggest that certain strains of LAB may synthesize natural folates (Lin and Young 2000; Aryana, 2003;, Laiño et al. 2014). 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; LeBlanc et al. 2013, 2014). 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 (Wang et al. 2007; Ohrvik and Witthoft 2011). 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 and 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 (Hermann and Obeid 2011; Fajardo et al. 2015). 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 and 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 (McPartlin et al. 1993; FAO/WHO 2002). 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).

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 of America 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 (Rader et al. 2000; Quinlivan and Gregory 2007; Yang et al. 2007; Crider et al. 2011). 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 FA 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 B_{12} 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 (Samaniego-Vaesken et al. 2012; Kariluoto et al. 2014).

Due to the potential risks of the fortification with FA, there has been growing interest in the fortification of foodstuffs with natural form folates (Scott 1999; LeBlanc et al. 2007; Iyer and Tomar 2009). In such cases, natural folates, such as 5-MTHF, that are usually found in foods and produced by microorganisms do not mask vitamin B_{12} deficiency; therefore they might be considered a promising alternative for fortification with FA (Scott 1999; Kariluoto et al. 2014; Laiño et al. 2014).

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 B_{12} 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.

LAB 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 various types of bread (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 (Lin and Young 2000; Crittenden et al. 2003; Iyer et al. 2010; Laiño et al. 2012). Folates produced by microorganisms are natural (especially 5-MTHF 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 a high value-add 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 13.1). 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 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 becomes electrically neutral. Thus, folate would enhance transport across the membrane, increasing the amount of this vitamin in the extracellular medium. However, for *Lactococcus 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.

TABLE 13.1 Reports on folate production by microorganisms in folate-free medium

Microbial species	Extracellular content	Intracellular content	Total content	Reference
Lactococcus species				
Lactococcus lactis subsp. *cremoris* MG1363	46 μg/L	69 μg/L	115 μg/L	Sybesma et al. (2003)
Lactococcus lactis subsp. *lactis* NZ9000	11 μg/L	245 μg/L	256 μg/L	Sybesma et al. (2003)
Lactobacillus species				
Lactococcus amylovoros CRL887	68.3 ± 3.4 μg/L	12.9 ± 1.3 μg/L	81.2 ± 5.4 μg/L	Laiño et al. (2014)
Lactobacillus plantarum CRL103	16.7 ± 3.4 μg/L	40.5 ± 4.2 μg/L	57.2 ± 5.2 μg/L	Laiño et al. (2014)
Lactobacillus acidophilus CRL1064	21.9 ± 2.3 μg/L	15.3 ± 1.4 μg/L	37.2 ± 3.1 μg/L	Laiño et al. (2014)
Lactobacillus delbrueckii subsp. *bulgaricus* CRL8711	86.2 ± 0.3 μg/L	8.6 ± 0.1 μg/L		Laiño et al. (2012)
Lactobacillus helveticus	1 ng/mL	90 ng/mL	89 μg/L	Sybesma et al. (2003)
Streptococcus species				
Streptococcus thermophilus CRL803	76.6 ± 7.0 μg/L	15.9 ± 0.2 μg/L		Laiño et al. (2012)
Bifidobacterium species				
Bifidobacterium adolescentes ATCC 15703			8865 ± 355 μg/100g DM[1]	D'Aimmo et al. (2012)
Bifidobacterium catenulatum ATCC 27539			9295 ± 750 μg/100g DM[1]	D'Aimmo et al. (2012)
Propionibacterium species				
Propionibacterium freudenreichii	25 ± 3 ng/mL			Hugenschimidt et al (2011)

[1]Dry matter

Lactococcus lactis and *Streptococcus thermophillus* are two industrially important microorganisms that have the ability to produce folate, but other LAB such *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus reuteri*, *Leuconostoc lactis*, *Propionibacterium* spp., and *Bifidobacterium* spp. also have this ability (Lin and Young 2000; Crittenden et al. 2003; Pompei et al. 2007; Santos et al. 2008; Hugenschmidt at al. 2011; D'Aimmo et al. 2012; Laiño et al. 2014). 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).

LAB starter cultures isolated from artisanal Argentinean yogurts were tested by Laiño et al. (2012). In this study certain strains of *Streptococcus thermophillus* and *Lactobacillus 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 *Lactobacillus bulgaricus* consumes this vitamin in a symbiotic relationship. It is now known that some strains of *Lactobacillus 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 13.1). Sybesma et al. (2003) identified a *Lactobacillus plantarum* strain that was able to produce folate. In this context, Nor et al. (2010) verified that the use of *Lactobacillus 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 (*Lactobacillus sakei* CN-3, *Lactobacillus sakei* CN-28 and *Lactobacillus 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.

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, *Lactobacillus 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 *Lactobacillus amylovorus*, *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus fermentum*, *Lactobacillus paracasei*, and *Lactobacillus 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 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 (*Bifidobacterium adolescentis* MB 227, *Bifidobacterium adolescentis* MB 239, and *Bifidobacterium 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 et al. 2014). Pasteurization and ultra high temperature (UHT) processes of raw milk also reduce folate levels (Lin and 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 folic acid.

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 *Saccharomyces 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.

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 and 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 *Streptococcus thermophilus* and *Lactobacillus 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 and Nampoothiri (2011) evaluated a fermented skimmed milk using a strain of *Lactococcus 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 and 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 hrs at 37 ºC, the researchers observed that milk sample inoculated with butter starter cultures of *Streptococcus thermophilus* and *Bifidobacterium longum* achieved 3.39 µg/100 g while the milk sample inoculated with butter starter cultures of *Streptococcus thermophilus* and *Propionibacterium* spp. achieved 4.23 µg/100 g 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 *Propioni bacterium* 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 *Lactobacillus amylovorus* strain in co-culture with yogurt starter cultures (*Lactobacillus bulgaricus* CRL871, *Streptococcus thermophilus* CRL803 and CRL415) to produce folate bio-enriched fermented milk was evaluated by Laiño et al. (2014). In the study, a yogurt containing high folate content (263.1 ± 2.4 µg/L) was obtained. Divya and Nampoothirii (2015) identified two strains of *Lactococcus 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 product fermentation. The resulting fermented products showed an enhancement of the folate content; however, the vitamin production by *Lactococcus lactis* CM22 was higher than by *Lactococcus lactis* CM28 demonstrating once again that folate production is a strain-dependent trait.

An interesting study conducted by Iyer and Tomar (2011) assessed the effect of folate-rich fermented milk produced by two strains of *Streptococcus 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 *Streptococcus thermophilus* and *Bifidobacterium animalis* increased more than six fold (72 ng/g) the folate content of milk. The researchers also showed that *Streptococcus 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 *Lactococcus lactis* subsp. *cremoris* to fortify skimed milk and fruit juices (Gangdharan and 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 *Bifidobacterium adolescentis* MB 115 (65 ng/mL) and *Bifidobacterium 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, 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 *Bifidobacterium catenulatum* (23.5 μg/100 mL) and *Lactobacillus plantarum* (11.21 μg/100 mL), after 10

hrs of fermentation, showed the highest folate levels. On the other hand, when milk was supplemented with galactooligosaccharides (GOS), *Streptococcus thermophilus*, *Bifidobacterium adolescentis*, and *Lactobacillus 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.

Folate Analysis in Food

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 *Lactobacillus 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 and Tamura 1998; Iyer et al. 2009; Tomar et al. 2009; Chew et al. 2012). 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 and 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 13.2. 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 and Nampoothiri (2014) used *Lactococcus 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) were 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 amounts of starch and protein and the highest reported folate level was 425 μg/100 g.

TABLE 13.2 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 × 100.

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

Studies of folate measurements in food usually showed folate values from raw vegetables, fruits, milk, and cereal-grain products (Aiso and Tamura 1998; Rader et al. 2000; Yon and Hyun 2003; Chew et al. 2012). 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.

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 and 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 (Phillips et al. 2011; Vishnumohan et al. 2011; Araya-Farias et al. 2014; Tyagi et al. 2015). 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).

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 trienzymatic treatment, would be stimulated since they may provide more accurate values of this vitamin in different food matrices.

Acknowledgments

The authors would like to thank CONICET and ANPCyT (Argentina), and FAPESP (project #2013/50506-8), CNPq, and CAPES for their financial support and fellowships.

References

AACC. (2000). 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.

Aiso, K. and Tamura, T. (1998). Trienzyme treatment for food folate analysis: optimal pH and incubation time for α-amylase and protease treatments. Journal of Nutritional Science and Vitaminology 44: 361–370.

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

Araya-Farias, M., Gaudreau, A., Rozoy, E. and Bazinet, L. (2014). Rapid HPLC-MS method for simultaneous determination of tea catechins and folates. Journal of Agricultural and Food Chemistry 62(19): 4241–4250.

Aryana, K.J. (2003). Folic acid fortified fat-free plain set yogurt. International Journal of Dairy Technology 56: 219-222.

ANVISA (2002). 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. Available at: http://portal.anvisa.gov.br/wps/wcm/connect/f851a500474580668c83dc3fbc4c6735/RDC_344_2002.pdf?MOD=AJPERES. Accessed Mai 2015.

Camilo, E., J. Zimmerman, J.B. Mason, B. Golner, R. Russell, J. Selhub and Rosenberg, I.H. (1996). Folate synthesized by bacteria in the human upper small intestine is assimilated by the host. Gastroenterology 110: 991–998.

Capozzi, V., P. Russo, M.T. Dueñas, P. López and G. Spano. (2012). Lactic acid bacteria producing B-group vitamins: a great potential for functional cereals products. Applied Microbiology and Biotechnology 96: 1383–1394.

Chew, S.C., S.P. Loh and G.L. Khor. (2012). Determination of folate content in commonly consumed Malaysian foods. International Food Research Journal 19(1). 189–197.

Chiang, F.F., S.C. Huang, H.M. Wang, F.P. Chen, and Y.C. Huang. (2015). High serum folate might have a potential dual effect on risk of colorectal cancer. Clinical Nutrition 34:986-990.

Cravo, M.L. and M.E. Camilo. (2000). Hyperhomocysteinemia in chronic alcoholism: relations to folic acid and vitamins B(6) and B(12) status. Nutrition 16: 296-302.

Crittenden, R.G., N.R. Martinez and M.J. Playne. (2003). Synthesis and utilization of folate by yoghurt starter cultures and probiotic bacteria. International Journal of Food Microbiology 80: 217–222.

Crider, K.S., L.B. Bailey, and R.J. Berry. (2011). Folic acid fortification – its history, effect, concerns, and future directions. Nutrients 3: 370-384.

D'Aimmo, M.R., P. Mattarelli, B. Biavati, N.G. Carlsson and T. Andlid. (2012). The potential of bifidobacteria as a source of natural folate. Journal of Applied Microbiology 112: 975–984.

D'Aimmo, M.R., M. Modesto, P. Mattarelli, B. Biavati. and T. Andlid. (2014). Biosynthesis and cellular content of folate in bifidobacteria across host species with different diets. Anaerobe 30: 169–177.

Dantas, J.A., A.S. Diniz and I.C.G. Arruda. (2010). Consumo alimentar e concentração intra-eritrocitárias de folato em mulheres do Recife, Nordeste do Brasil. Archivos Latino Americano de Nutricion 60(3): 227–234.

Delchier, N., C. Ringling, J.F. Maingonnat, M. Rychlik and C.M.G.C. Renard. (2014). Mechanisms of folate losses during processing: Diffusion vs. heat degradation. Food Chemistry 157: 439–447.

Divya, J.B. and K.M. Nampoothiri. (2015). Encapsulated *Lactococcus lactis* with enhanced gastrointestinal survival for the development of folate enriched functional foods. Bioresource Technology *in press* 188: 226-30.

Eitenmiller, R.R. and W.O. Landen, (1999). Folate. *In*: Vitamin Analysis for the Health and Food Sciences. R.R. Eitenmiller and W.O. Landen (eds.). CRC Press, Boca Raton, pp. 411-466.

Fajardo, V., E. Alonso-Aperte and G. Varela-Moreiras. (2012). 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 28(2).155–163.

Fajardo, V., E. Alonso-Aperte, and G. Varela-Moreiras. (2015). Folate content in fresh-cut vegetable packed products by 96-well microliter plate microbiological assay. Food Chemistry 169: 283-288.

FAO/WHO. Food and Agricultural Organization of the United Nations and World Health Organization. Human vitamin and mineral requirements: report of a joint FAO/WHO expert consulation. Rome, Italy: FAO, 290 p. 2002.

FAO/WHO (2006). 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, pp. 294-312.

Fujimori, E., C.F. Baldino, A.P.S. Sato, A.L.V. Borges and M.N. Gomes. (2013). 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 29(1): 145–154.

Gangadharan, D. and K.M. Nampoothiri. (2011). Folate production using *Lactococcus lactis* spp. cremoris with implications for fortification of skim milk and fruit juices. Food Science and Technology 44: 1859–1864.

Gibson, G.R., H.M. Probert, J.A.E. Van Loo, R.A. Rastall and M.B. Roberfroid. (2004). Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. Nutrition Research Reviews, 17(2): 259–275.

Gibson, G.R., K. P. Scott, R. A. Rastall, K. M. Tuohy, A. Hotchkiss, A. Dubert-Ferrandon, et al. (2010). Dietary prebiotics: current status and new definition. Food Science and Technology Bulletin: Functional Foods, *7*(1): 1–19.

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

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

Herbison, C.E., S. Hickling, K.L. Allen, T.A. O'Sullivan, M. Robinson, A.P. Bremmer, R.C. Huang, L.J. Beilin, T.A. Mori, and W.H. Oddy. (2012). Low intake of B-vitamins is associated with poor adolescent mental health and behavior. Preventive Medicine 55: 634-638.

Hermann, W. and R. Obeid. (2011). The mandatory fortification of staple foods with folic acid. Deutsches Ärzteblatt International 108: 249-254.

Holasová, M., V. Fiedlerová, P. Roubal and M. Pechacová. (2005). Possibility of increasing natural folate content in fermented milk products by fermentation and fruit component addition. Csech Journal of Food Science 23(5): 196–201.

Hjortmo, S., J. Patring, J. Jastrebova and T. Andlid. (2008). Biofortification of folates in white wheat bread by selection of strains and process. International Journal of Food Microbiology 127: 32–36.

Hugenschmidt, S., S.M. Schwenninger and C. Lacroix. (2011). Concurrent high production of natural folate and vitamin B_{12} using a co-culture process with *Lactobacillus plantarum* SM39 and *Propionibacterium freundenreichii* DF13. Process Biochemistry 46: 1063–1070.

Indyk, H.E. (2011). An optical biosensor assay for the determination of folate in milk and nutritional dairy products. International Dairy Journal 21(10): 783–789.

Iyer, R., S.K. Tomar, (2009). Folate: A functional food constituent. Journal of Food Science 74(9): R114-122.

Iyer, R., S.K. Tomar. (2011). Dietary effect of folate-rich fermented milk produced by Streptococcus thermophilus strains on hemoglobin level. Nutrition 27: 994-997.

Iyer, R. and S.K. Tomar. (2013). Determination of folate/folic acid level in milk by microbiological assay, immune assay and high performance liquid chromatography. Journal of Dairy Research 80(2): 233–239.

Iyer, R., S.K. Tomar, S. Kapila, J. Mani and R. Singh. (2010). Probiotic properties of folate producing Streptococcus thermophilus strains. Food Research International 43: 103–110.

Iyer, R., S.K. Tomar, R. Singh and R. Sharma. (2009). Estimation of folate in milk by microbiological assay using tri-enzyme extraction method. Milchwissenschaft 64(2): 125–127.

Iwatani, Y., J. Arcot and A. Shrestha. (2003). Determination of folate contents in some Australian vegetables. Journal of Food Composition and Analysis 16(1): 37–48.

Kariluoto, S., M. Aittamaa, M. Korhola, H. Salovaara, L. Vahteristo and V. Piironen. (2006). Effects of yeasts and bacteria on the levels of folates in rye sourdoughs. International Journal of Food Microbiology 106: 137–143.

Kariluoto, S., M. Edelmann, L. Nyström, T. Sontag-Strohm, H. Salovaara, R. Kivelä, M. Herranen, M. Korhola, and V. Piironen. (2014). In situ enrichment of folate by microorganisms in beta-glucan rich oat and barley matrices. International Journal of Food Microbiology 176: 38-48.

Kariluoto, S., M. Edelmann, M. Herranen, A.M. Lampi, A. Shmelev, H. Salovaara, M. Korhola and V. Piironen. (2010). Production of folate by bacteria isolated from oat bran. International Journal of Food Microbiology 143: 41–47.

Laiño, J.E., J.G. LeBlanc and G.S. De Giori. (2012). Production of natural folates by lactic acid bacteria starter cultures isolated from artisanal Argentinean yogurts. Canadian Journal of Microbiology 58: 581–588.

Laiño, J.E., M.J. Del Valle, G.S. De Giori and J.G.L. LeBlanc. (2013a). Development of a high concentration yogurt naturally bio-enriched using selected lactic acid bacteria. Food Science and Technology 54: 1–5.

Laiño, J.D., G.S. De Giori and J.G. LeBlanc. (2013b). Folate production by lactic acid bacteria. *In*: Watson RR and VR (eds.), Bioactive Food as Dietary Interventions for Liver and Gastrointestinal Disease. Academic Press, San Diego, pp. 251–270.

Laiño, J.E., M.J. del Valle, G.S. de Giori and J.G.J. LeBlanc. (2014). Applicability of a *Lactobacillus amylovorus* strain as co-culture for natural folate bio-enrichment of fermented milk. International Journal of Food Microbiology 191: 10–16.

LeBlanc, J.G., C. Milani, G.S. De Giori, F. Sesma, D. Van Sinderen and M. Ventura. (2013). Bacteria as vitamin suppliers to their host: a gut microbiota perspective. Current Opinion in Biotechnology 24: 160–168.

LeBlanc, J.G., G.S. de Giori, E.J. Smid, J. Hugenholtz and F. Sesma. (2007). Folate production by lactic acid bacteria and other food-grade microorganisms. Communicating Current Research and Educational Topics and Trends in Applied Microbiology.

Lin, M.Y. and C.M. Young. (2000). Folate levels in cultures of lactic acid bacteria. International Dairy Journal 10: 409–413.

Liu, Y., Tomiuk, S., Rozoy, E., Simard, S., Bazinet, L., Green, Kitts, D.D. (2012). Thermal oxidation studies on reduced folate, L-5-methyltetrahydrofolic acid (L-5-MTHF) and strategies for stabilization using food matrices. Journal of Food Science 77: C236-C243.

López-Nicolás, R., C. Frontela-Saseta, R. Gonzaléz-Abellán, A. Barado-Piqueras, D. Perez-Conesa, and G. Ros-Berruezo. (2014). Folate fortification of white and wholegrain bread by adding Swiss chard and spinach. Acceptability by consumers. LWT – Food Science and Technology 59: 263-269.

Masuda, M., M. Ide, H. Utsumi, T. Niiro, Y. Shimamura and M. Murata. (2012). Production potency of folate, vitamin B_{12} and thiamine by lactic acid bacteria isolated from Japanese pickles. Bioscience, Biotechnology and Biochemistry 76: 2061–2067.

Maharaj, P.P.P., S. Prasad, R. Devi and R. Gopalan. (2015). Folate content and retention in commonly consumed vegetables in the South Pacific. Food Chemistry 182. 327–332.

McNulty, H. and K. Pentieva, (2004). Folate bioavailability. The Proceedings of the Nutrition Society 63: 529-536.

McPartlin, J., A. Halligan, , J.M. Scott, M. Darling, and D.G. Weir. (1993). Accelerated folate breakdown in pregnancy. Lancet 341: 148-149.

Melse-Boonstra, A., C.E. West, M.B. Katan, F.J. Kok, and P. Verhoef, (2004). Bioavailability of heptaglutamyl relative to monoglutamyl folic acid in healthy adults. The American Journal of Clinical Nutrition 79: 424-429.

Nygren-babol, L. and Jägerstad. (2012). Folate-binding protein in milk: a review of biochemistry, physiology and analytical methods. Critical Reviews in food Science and Nutrition 52(5): 410–425.

Nor, N.M., R. Mohamad, H.L. Foo and R.A. Rahim. (2010). Improvement of folate biosynthesis by lactic acid bacteria using response surface methodology. Food Technology and Biotechnology 48: 243–250.

Ohrvik, V.E. and C.M. Witthoft, (2011). Human folate bioavailability. Nutrients 3: 475-490.

Padalino, M., D. Perez-Conesa, R. López-Nicolás and C. Prontela-Saseta. (2012). Effect os fructoologosaccharides and galactooligosaccharides on the folate production of some folate-producing bactéria in media cultures or milk. International Dairy Journal 27: 27–33.

Phillips, K.M., D.M. Ruggio and D.B. Haytowitz. (2011). Folate composition of 10 types of mushrooms determined by liquid chromatography-mass spectrometry. Food Chemistry 129(2): 630–636.

Pompei, A., L. Cordisco, A. Amaretti, S. Zanoni, D. Matteuzzi and M. Rossi, (2007). Folate production by bifidobacteria as potential probiotic property. Applied and Enviromental Microbiology 73(1): 179–185.

Pounis, G., A.F. Castelnuovo, M. Lorgeril, V. Krogh, A. Siani, J. Arnout, F.P. Cappuccio, M. van Dongen, B. Zappacosta, M.B. Donati, G. Gaetano, and L. Iacoviello, (2014). Folate intake and folate serum levels in men and women from two European populations: the IMMIDIET project. Nutrition 30: 822-830.

Quinlivan, E.P. and Gregory, J.F., III. (2007). 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 86: 1773-1779.

Rossi, M., A. Ameratti and S. Raimoundi. (2011). Folate productionby probiotic bacteria. Nutrients 3: 118–114.

Rahman, T., M.M.H. Chowdhury, M.T. Islam and M. Akhtaruzzaman. (2015). Microbiological assay of folic acid content in some selected Bangladeshi food stuffs. International Journal of Biology 7(2): 35–43.

Rader, J.I., C.M. Weaver and G. Angyal. (1998). Use of a microbiological assay with trienzyme extraction for measurement of pre-fortification levels of folates in enriched cereal-grain products. Food Chemistry 62(4): 451–465.

Rader, J., C.M. Weaver and G. Angyal. (2000). Total folate in enriched cereal-grain products in the United States following fortification. Food chemistry 70(3):275–289.

Samaniego-Vaesken, M.L., E. Alonso-Aperte, and G. Varela-Moreiras. (2012). Vitamin food fortification today. Food & Nutrition Research 56:5459. DOI: 10.3402/fnr.v56i0.5459.

Santos, L.M.P. and M.Z. Pereira. (2007). Efeito da fortificação com ácido fólico na redução dos efeitos do tubo neural. Cadernos de Saúde Pública 23(1): 17–24.

Santos, F., A. Wegkamp, W.M. de Vos, E.J. Smid, and J. Hugenholtz. (2008). High-level folate production in fermented foods by the B_{12} producer *Lactobacillus reuteri* JCM1112. Applied and Enviromental Microbiology 74: 3291–3294.

Sauberlich, H.F., M.J. Kretsch, J.H. Skala, H.L. Johnson, and P.C. Taylor. (1987). Folate requirement and metabolism in nonpregnant women. The American Journal of Clinical Nutrition 46: 1016-1028.

Scott, J.M. (1999). Folate and vitamin B_{12}. The Proceedings of the Nutrition Society 58: 441-448.

Shuaibi, A.M., G.P. Sevenhuysen, and J.D. House. (2009). The importance of using folate intake expressed as dietary folate equivalents in predicting folate status. Journal of Food Composition and Analysis 22: 38-43.

Silva, N. and B. Davis. (2013). Iron, B_{12}, and folate. Medicine 41: 204-207.

Smith, A.D., Y.I. Kim, and H. Refsum. (2008). Is folic acid good for everyone? The American Journal of Clinical Nutrition 87: S17-S33.

Soongsongkiat, M., P. Puwastien, S. Jittinandana, A. Dee-Uam and P. Sungpuag. (2010). 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 23: 681–688.

Stamm, R.A. and L.A. Houghton. (2013). Nutrient intake values for folate during pregnancy and lactation vary widely around the world. Nutrients 5: 3920-3947.

Strandler, H.S., J. Patring, M. Jägerstad and J. Jastrebova. (2015). Challenges in the determination of unsubstituted food folates: Impact of stabilities and conversions on analytical results. Journal of Agricultural and Food chemistry 63(9): 2367–2377.

Sybesma, W., M. Starrenburg, L. Tijsseling, M.H.N. Hoefnagel and J. Hugenholts. (2003). Effects of cultivation conditions on folate production by lactic acid bacteria. Applied and Enviromental Microbiology 69(8): 4542–4548.

Tyagi, K., P. Upadhyaya, S. Sarma, V. Tamboli, Y. Sreelakshmi and R. Sharma. (2015). High performance liquid chromatography coupled to mass spectrometry for profiling and quantitative analysis of folate monoglutamates in tomato. Food Chemistry 179: 76–84.

Tomar, S.K., N. Srivatsa, R. Iyer and R. Singh. (2009). Estimation of folate production by *Streptococcus thermophilus* using modified microbiological assay. Milchwissenschaft 64(3): 260–263.

Tamura, T., Y. Mizuno, K.E. Johnston and R.A. Jacob. (1997). Food folate assay with protease, α-amylase and folate conjugase treatments. Journal of Agricultural and Food chemistry 45(1):135–139.

Truswell, A. and S. Kounnavong. (1997). Quantitative responses of serum folate to increasing intakes of folic acid in healthy women. European Journal of Clinical Nutrition 51: 839-845.

Uehara, S.K. and G. Rosa. (2010). Associação da deficiência de ácido fólico com alterações patológicas e estratégias para sua prevenção: uma visão crítica. Revista de Nutrição 23(5): 881–894.

Vishnumohan, S., J. Arcot and R. Pickford. (2011). Naturally-occorring folates in foods: Method development ans analysis using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Food Chemistry 125: 736–742.

Yon, M., and T.H. Hyun. (2003). Folate content of foods commonly consumed in Korea measured after trienzyme extraction. Nutrition Research 23(6): 735–746.

Walkey, C.J., D.D. Kitts, Y. Liu, and H.J.J. van Vuuren. (2015). Bioengineering yeast to enhance folate levels in wine. Process Biochemistry 50: 205-210.

Wang, X., X. Qin, H. Demirtas, J. Li, G. Mao, Y. Huo, N. Sun, L. Liu, and X. Xu. (2007). Efficacy of folic acid supplementation in stroke prevention: a meta-analysis. Lancet 369: 1876-1882.

Wyk, J.V., R.C. Witthuhn and T.J. Britz. (2011). Optimisation of vitamin B_{12} and folate production by *Propionibacterium freundenreichii* strains in kefir. International Dairy Journal 21: 69–74.

Yang, Q.H., H.K. Carter, J. Mulinare, R.J. Berry, J.M. Friedman, and J.D. Erickson. (2007). 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 85: 1409-1416.

Zhao, R., Diop-Bove, N., Visentin, M., Goldman, I.D. (2011). Mechanisms of membrane transport of folates into cells and across epithelia. Annual Review of Nutrition 31: 177-201.