Folate production by lactic acid bacteria and other food-grade microorganisms

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Folate, an important B-group vitamin, participates in many metabolic pathways such as DNA and RNA biosynthesis and amino acid inter-conversions. Mammalian cells cannot synthesize folate; therefore, an exogenous supply of this vitamin is necessary to prevent nutritional deficiency. Many reviews have shown the health benefits associated with increased folate intakes and by consequence many countries now possess mandatory folate enrichment programs. Lately, a number of studies have shown that high intakes of folic acid, the chemically synthesized form of folate, but not natural folates, can cause adverse affects in some individuals such as the masking of the hematological manifestations of vitamin B_{12} deficiency. For these reasons, many researchers have been looking for novel methods to increase concentrations of naturally occurring folate variants in foods. This review will focus on folate production by lactic acid bacteria and levels of folate present in foods fermented by/or containing these valuable microorganisms. The proper selection and use of folate producing microorganisms is an interesting strategy to increase "natural" folate levels in foods.

Keywords folate; vitamin; lactic acid bacteria; fermented foods

1. Introduction

Human life can not exist without folate since this B-group vitamin is involved in essential functions of cell metabolism such as DNA replication, repair and methylation, and synthesis of nucleotides, vitamins and some amino acids. Health of multi-cellular organisms such as humans starts at the individual cell level: if our cells are healthy so are we. Healthy cells, in turn, depend on the continued, faultless replication of our DNA. DNA can be seriously damaged through attacks by free radicals so an adequate antioxidant status is essential to cell health. Folates possess antioxidant properties that protect the genome by inhibiting free radical attack of DNA in addition to their role in DNA repair and replication mechanisms [1]. Folate deficiency has been implicated in a wide variety of disorders from Alzheimer's [2] to coronary heart diseases [3]; osteoporosis [4], increased risk of breast [5] and colorectal cancer [6], poor cognitive performance [7], hearing loss [8], and of course, neural tube defects [9, 10]. Due to the occurrence of problems associated with current folic acid fortification programs, researchers have been looking for novel methods to increase concentrations of naturally occurring folates in foods. This review will focus on folate production by lactic acid bacteria (LAB) and levels of folates present in foods fermented by/or containing these valuable microorganisms. The proper selection and use of folate producing microorganisms is a novel strategy to increase "natural" folate levels in foods.
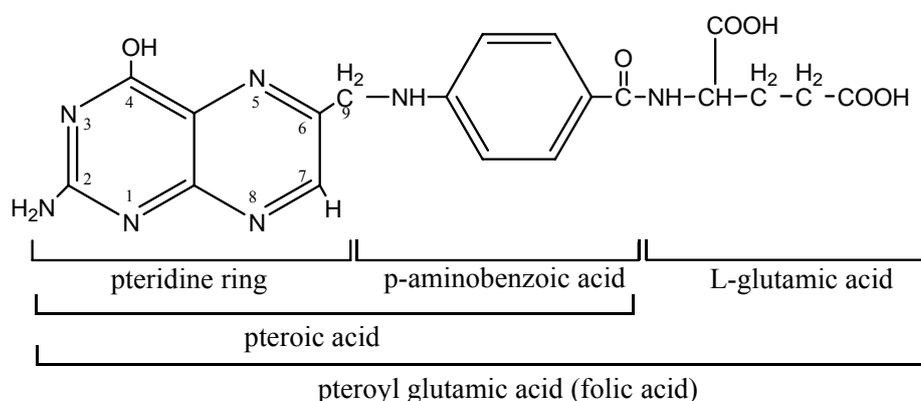
2. Folates

2.1 Chemical structure and bioavailability

In this review, the generic term folate will include the complete group of all folic acid derivatives, including the folylglutamates naturally present in foods and folic acid that is a synthetic folate form

which is commonly used for food fortification and nutritional supplements. Folic acid or pteroyl glutamic acid (PGA) is comprised of *p*-aminobenzoic acid linked at one end to a pteridine ring and at the other end to L-glutamic acid (**Fig. 1a**). The naturally occurring forms of folate 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 (**Fig. 1b**). The naturally occurring 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). Most naturally occurring folates are pteroylpolyglutamates, containing two to seven glutamates joined in amide (peptide) linkages to the γ-carboxyl of glutamate. The principal intracellular folates are pteroylpentaglutamates, while the principal extracellular folates are pteroylmonoglutamates. Pteroylpolyglutamates with up to 11 glutamic acid residues exist naturally.
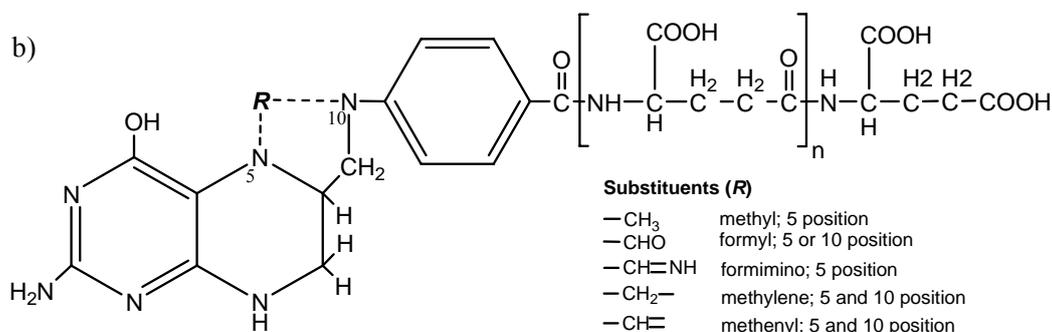
a)

b)

Fig. 1 Structure of a) folic acid (pteroyl-L-glutamic acid), and b) native food folates, e.g. reduced, one-carbon-substituted forms of polyglutamates.

The bioavailability of dietary folate may be hampered by the polyglutamate chain to which most of the natural folate is attached [11]. This polyglutamate chain must be removed, except for the proximal glutamate moiety, by the enzyme γ-glutamyl hydrolase or human conjugase that is present in the brush border of the small intestine. This enzyme is present in sufficient quantity and is not a limiting factor in folate absorption [12]. Folate can be absorbed and transported as a monoglutamate into the portal vein. Bioavailability is defined as the proportion of a nutrient ingested that becomes available to the body for metabolic processes or storage. The available data suggest that the polyglutamate form is 60–80%

bioavailable compared with the monoglutamate form (reviewed in [13]) and was confirmed in a human trial comparing chemically synthesized heptaglutamyl folate to regular, monoglutamyl, folate [14]. The relative bioavailability of dietary folates is estimated to be only 50% compared with synthetic folic acid [15]. In a rat feeding trial, longer polyglutamyl chain folates (containing an average of 8 glutamyl residues) did not show lower, but apparently increased bioavailability compared to shorter folylpolyglutamates [16]. This is clearly different from what has been reported for humans [11, 13, 14]. The rat carboxypeptidase II enzyme that is required for transforming polyglutamyl folates into monoglutamyl folates, which can subsequently be adsorbed by the blood, obviously does not limit absorption of folates with longer glutamyl tail lengths. Alternatively, the affinity of this enzyme for long polyglutamyl folates might even be higher than for short polyglutamyl folates. These results should be confirmed in future animal trials. Furthermore, folate-binding proteins from milk may act to increase efficiency of folate absorption by protecting dietary folates from uptake by bacteria in the gut, thus increasing absorption in the small intestine (reviewed in [17]). Other dietary interactions include effects of foods on intestinal pH with potential modification of conjugase activity, presence of folate antagonists, intestinal changes influenced by dietary factors (alcoholism), chelation, and factors that influence the rate of gastric emptying. In spite of the large amount of information available on folate bioavailability, knowledge of this important part of folate nutrition has been recently described as fragmentary [11, 18, 19].

2.2 Folate requirements. Fortification programs

Humans cannot synthesize folate; in consequence, it is necessary to assimilate this vitamin exogenously. Folate is present in most foods such as legumes (beans, nuts, peas, etc.), leafy greens (such as spinach), citrus, some fruits, vegetables (broccoli, cauliflower), liver, and (fermented) dairy products [17]. Although beans and green vegetables like spinach are good sources of folates, relatively few people eat lots of these foods. The recommended daily intake (RDI) of folate in an adult is 200-400 µg [20, 21]. For pregnant women, 400-600 µg is recommended. Although folate is omni-present in a normal human diet, folate deficiencies still occur frequently, even in well-developed countries [22, 23]. Recent reports have indicated that folate intake levels are inadequate among various population groups including women of childbearing age [24]. Studies such as these have obligated the governing bodies of many countries to advocate mandatory supplementation with folic acid. In Canada and in the USA, fortification of flours is mandatory since 1998 in order to reduce the incidences of neural tube malformations in newborns. Many other countries have afterwards developed similar programs; Argentina, for example, has been fortifying its commercial flours with some vitamins, including folic acid, since 2002. Even with mandatory folic acid fortification, recent reports have shown that one-third of pregnant and lactating women may not be meeting their folate requirements from diet alone [25]. Many countries have not adopted a national folic acid fortification program because of the potential adverse effects of folic acid, in particular, that high intakes can delay the diagnosis of vitamin B_{12} deficiency [26]. The concern is that folic acid is added at levels where individuals with low folate intakes can meet folate RDI in order to prevent neural-tube defects (NTD) and/or lowering plasma homocysteine; however, others, with normal or higher folate intakes could inadvertently become exposed to very high levels of folic acid. Excessive folic acid intake has the potential to mask the early haematological manifestations of vitamin B_{12} deficiency such as pernicious anaemia (reviewed in [27]). Other safety considerations of excess folic acid consumption highlighted by the FDA [28] include potential unknown risks for pregnant women, and persons on anti-epileptic and anti-folate medication. The FDA also noted the uncertainties regarding the effects of chronic elevated exposure in children, whose requirements for folate are lower than those of adults. Further concerns include the potential to promote cancer [29, 30] and the recent hypothesis that exposure of the foetus to excess folic acid may favor the selection of the methylentetrahydrofolate polymorphism, associated with a range of debilitating illnesses [31]. Natural folates (such as tetrahydrofolates produced by microorganims) do not cause "masking" of pernicious anemia that occurs at high concentrations of folic acid and should thus be considered as a viable alternative to folic acid fortification programs [32].

2.3 Measurement of folate in foods

During the last four decades, numerous reports have been published regarding food folate content and the various methods of folate determination (reviewed in [33]) including: microbiological, radiobinding or radiometric assay, and fluorometric, electrochemical or spectrophotometric methods, some of which are carried out in combination with gel or high-pressure-liquid chromatography (HPLC). Microbiological assay has been considered to be one of the best and most versatile methods for determining food folates. *Lactobacillus* (*L.*) *rhamnosus* ATCC 7469 (formerly known as *L. casei*) is the most commonly used and most accepted organism for folate analysis of natural products. It responds to natural folate forms present in foods, and does not respond to pteroic acid, a common folate degradation product. *L. rhamnosus* has greater capacity for response to the γ-glutamyl folate polymers compared to the other assay organisms; however, its response is limited to no greater than three glutamates with much lower response to higher polymeric folates. Lack of response to the higher γ-glutamyl folate polymers requires treatment with pteroyl-*y*-glutamyl carboxypeptidase (folate conjugase, EC 3.4.19.9) in order to hydrolyze folate polyglutamates to folates with shorter glutamyl residues such as mono- or diglutamates, which can be utilized by the assay microorganism. Most current studies now determine food folate concentrations in response to growth of *L. rhamnosus* using 96-well microtiter plates [34]. Nowadays, HPLC methods for folate analysis have been refined to the point that most analytical laboratories equipped with modern LC systems and detectors, specifically a fluorescent or photodiode array detector, can successfully assay naturally occurring folates. The primary advantage of LC analysis is the ability to quantify the different folate forms, a specificity not obtainable by other methods. Several excellent reviews of HPLC methods are available [35]. A serious limitation of HPLC methods would be the need to use all the known forms of folates in order to be able to quantify total folates in a given sample, some of which are hard to find and costly making this technique out-of reach for smaller analytical laboratories in the food industry.

3. Folate concentration in fermented foods

3.1 Dairy products

Milk is not a rich source of dietary folate (**Table 1**). However, many dairy products are processed using microbial fermentations in which folate can be synthesized, significantly increasing folate concentrations in the final product (**Table 1**). In most regions of the world, yogurt constitutes the main portion of the *per capita* consumption of fermented milk products [36]; nevertheless, cultured milk, cultured buttermilk, and cultured cream are also commonly consumed in European countries (reviewed in [37]). In addition to several attractive sensory aspects attributed to these fermented milk products, the public is becoming increasingly aware of their nutritional and physiological properties. In addition, consumers also understand that their typical Western diet falls short of the recommended daily allowance of some nutrients. In some population groups, there is a veritable risk of vitamin deficiency, especially among the elderly since their food intake is lower, and in young children consuming a restricted variety of foods (reviewed in [38]). Additionally, increased dietary intakes of folate are suggested especially for women of childbearing age [28]. For these reasons it has been suggested that the fortification of fermented milks with B-complex vitamins seems to be a good option to prevent vitamin deficiencies [38]. Among dairy products, fermented milks are considered as a potential matrix for folate fortification because folate-binding proteins of milk improve folate stability and the bioavailability of both 5-methyltetrahydrofolate and folic acid may be enhanced [39, 40, 41]. However, due to the potential risks of fortification with folic acid, the elaboration of fermented milks containing elevated levels of natural form folates would be a better suited alternative.

Table 1   Folate concentrations in dairy products and its contribution to the reference daily intake (RDI)

Product	Folate (µg/l) [42]	Folate per serving (µg/240ml)	% RDI (3 servings)
Milk	40 ± 10	10 ± 2	6-8
Buttermilk	90 ± 20	22 ± 5	13-20
Yogurt	80 ± 20	19 ± 5	11-18
Kefir	50 ± 10	12 ± 2	8-11
Ropy-milk	110 ± 20	26 ± 5	16-23
Sour buttermilk	75 ± 15	18 ± 4	11-17
Acidophilus milk	50 ± 10	12 ± 2	8-11
Bifidus milk	75 ± 15	18 ± 4	11-17

MyPyramid (www.mypyramid.gov), the modified Food Guide Pyramid released in 2005 by the United States Department of Agriculture (USDA) recommends the consumption of at least 3 servings of milk products as part of a healthy daily diet. Taking into account this recommendation, and considering that a normal serving consists of 240 ml, currently available fermented milk products could contribute significantly to the reference daily intake of folates (up to 23% of RDI based on **Table 1** values). This intake could be even higher if properly selected starter cultures capable of producing elevated levels of natural folates were used in the elaboration of these products.

3.2   Non-dairy foods

Besides fermented dairy products, microorganisms are capable of increasing folate content in a wide variety of foods. For example, fermentation of rye dough is often accompanied with an increase in folate content [43]. In these studies, the increase of folate concentrations during fermentation was mainly due to folate synthesis by yeasts; LAB did not produce folates but rather consumed them. Variations in starter cultures lead to great differences in folate content of sourdoughs but their effects have been scantly investigated. Proper strain selection, i.e. exchanging folate consuming LAB with folate producing ones could significantly increase folate content in these breads.

It has also been reported that it is possible to select lactic acid-producing starter cultures that produce significant amounts of 5-MTHF (almost doubling its concentration) during fermentation of vegetables [44]. The conclusion of this study was that further work needs to be performed to optimize the whole process of enhancing folate concentrations in fermented vegetables. To achieve this, it is important to carefully check the folate concentration in the raw vegetables (starting material). In addition, the loss of folate during processing must be limited as much as possible and the conditions favoring microbiological biosynthesis of folate needs to be better understood and controlled.

4. Folate producing microorganisms

4.1   Native strains

Numerous researchers have reported that lactic acid bacteria, such as the industrial starter bacteria *Lactococcus* (*Lc.*) *lactis* and *Streptococcus* (*S.*) *thermophilus* have the ability to synthesize folate [38, 45, 46, 47, 48, 49]. For this reason, some fermented dairy products, including yogurt, are reported to contain even higher concentrations of folate than non-fermented milk (**Table 1**). The ability of these commercial yogurt starter culture mixtures to produce or utilize folate production levels has been shown to vary considerably (**Table 2**, [36, 37, 42, 48, 50, 51, 52]. On the other hand, it has been published that lactobacilli are known for their inability to synthesize this essential vitamin [47, 53]. However, it was demonstrated that *L. plantarum* has the ability to produce folate, although at low levels, when grown in chemically defined folate-free medium [46].

Table 2 Folate produced by microorganisms grown in chemically defined folate-free medium

Microbial species	Extracellular (µg/l)	Intracellular (µg/l)	Total (µg/l)	Reference
Lactococcus lactis subspecies				
Lc. lactis subsp. *cremoris*	8 - 46	59 - 99	92 - 116	[46]
Lc. lactis subsp. *lactis*	5 - 26	47 - 269	57 - 291	[46]
Lc. lactis subsp. *lactis* biovar diacetylactis	14 - 21	65 - 84	79 - 100	[46]
Lactobacillus species				
L. plantarum	27	18	45	[46]
L. helveticus	-1 - 3	-1 - 90	2 - 89	[46]
L. acidophilus	0	1	1	[46]
L. casei	-45	32	-13	[46]
L. casei subsp. *rhamnosus*	-98	34	-63	[46]
L. delbrueckii subsp. *bulgaricus*	12	41	54	[46]
Propionibacterium species				
P. thoenii	28	8	36	[49]
P. acidipropionici	58	–22	36	[49]
P. jensenii	51	–11	40	[49]
P. freudenreichii ssp. *shermanii*	0 - 93	-20 - 41	17 - 78	[49]
P. sp.	13 - 31	-22 - 16	9 - 29	[49]
Bifidus species				
B. adolescentis	1 - 65	10 - 40	70 - 110	[54]
B. animalis	26	-	-	[54]
B. bifidum	1	-	-	[54]
B. breve	1 - 3	-	-	[54]
B. catenulatum	3	-	-	[54]
B. dentium	29	-	-	[54]
B. infantis	27	-	-	[54]
B. longum	2	-	-	[54]
B. pseudocatenulatum	12 - 82	5 - 35	75 - 90	[54]
Streptoccoccus species				
S. thermophilus	23 - 40	4 - 179	29 - 202	[46]
Other species				
Leuconostoc lactis	37	7	45	[46]
Leuconostoc paramesenteroides	33	10	44	[46]

The amount of folic acid found in cow's milk ranges from 20 to 60 µg/l, whereas its concentration in yogurts may be increased depending on the starter cultures used and on the storage conditions to values above 200 µg/l [55]. This level depends on the strain of *S. thermophilus* and *L. bulgaricus* used, because the latter organism has been shown to use and to degrade folate during its growth. It is therefore of utmost importance to select the optimal combination of *S. thermophilus* and *L. bulgaricus* strains leading to an organoleptically acceptable yoghurt with a concomitantly increased folic acid concentration. The pools of available strains will undoubtedly harbor the desired strains. It is now known that not only the yogurt starter cultures and *Lc. lactis* have the ability to produce folate but also this important property exists in other LAB. *L. acidophilus* is reported as being able to increase folate concentrations in fermented milks [45]. It has been published that *L. plantarum,* is capable of producing folate in chemically-defined medium (**Table 2**) and thus should be evaluated to see if this LAB can increase folate levels in milk. Additionally, recent reports have shown that some probiotic microorganisms have

the ability to synthesize folate (**Tables 2 and 3**) such as bifidobacteria [45, 47, 54, 56] and propionibacteria [49, 56].

Table 3   Folate concentrations in milk fermented with a unique folate producing microorganism.

Microbial species	Method	Folate (ng/g)	Reference
Lactobacillus species			
L. helveticus	MA[1]	10 – 15	[47]
L. acidophilus	MA	2 – 12	[47]
	MA	48 - 69	[45]
L. casei	MA	3 - 10	[47]
L. casei subsp. *rhamnosus*	MA	0 - 10	[47]
L. delbrueckii subsp. *bulgaricus*	MA	0 - 60	[55]
	MA	0 - 20	[47]
	MA	60 - 70	[45]
L. reuteri	MA	0 - 10	[47]
L. fermentum	MA	2 - 10	[47]
L. johnsonii	MA	4 - 10	[47]
Bifidus species			
B. animalis	MA	15 - 25	[47]
B. bifidum	HPLC[2]	5 - 6	[56]
B. breve	MA	40 - 55	[47]
B. infantis	MA	25 - 45	[47]
B. longum	HPLCF	9 - 12	[56]
	MA	69 - 103	[45]
B. lactis	MA	20 - 30	[47]
B. sp.	MA	15 - 30	[47]
Streptococcus species			
St. thermophilus	MA	15 -140	[55]
	MA	35 - 55	[47]
	MA	42 – 62	[45]
	HPLC	14 - 45	[56]
Enterococcus species			
E. faecium	MA	18 - 28	[47]

[1] microbiological assay; total folates
[2] HPLC, only 5-MTHF

Bifidobacteria have been shown to exert a number of beneficial effects on the heath of consumer such as stimulating the immune system, relieving lactose intolerance, increasing resistance to microbial infections, prevention of inflammatory bowel diseases and cancer to name a few [57]. Propionibacteria in turn are able to produce vitamin B_{12} and inhibit the undesirable microflora in fermented foods via the release of organic acids and bacteriocins, they were shown to beneficially modulate the colon flora both in animals [58] and humans [59], mainly by enhancing the indigenous bifidobacteria population. A recent study provided new perspectives on the specific uses of probiotics, such as to prevent the localized folate deficiency that is associated with premalignant changes in the colonic epithelia [54]. The oral administration of folate-producing probiotic strains may confer a more efficient protection against inflammation and cancer, both by exerting the beneficial effects and by delivering folate to colonic-rectal cells [54]. In humans, folate produced by the microbiota in the small intestine is assimilated by the host [60]. Although it is believed to supply only a minor source of total absorbed folate in humans [61], the contribution of the microbiota to the folate requirements of the high cell turnover intestinal epithelium is unknown. A mechanism for luminal folate absorption by cells in the human colon has been reported [62], which suggests that folate produced *in situ* by the colonic microbiota may be utilized by cells in the colonic epithelium. It has recently been shown that bacterially synthesized folate are absorbed across the

large intestine and incorporated into the liver and kidneys of piglets [63]. These authors predicted that approximately 18% of the dietary folate requirement for the piglet could be met by folate absorption across the large intestine. Increased intestinal *Bifidobacterium* populations, induced by consumption of human breast milk, have been correlated with an enhanced folate status in rats [64]. It is therefore possible that probiotic bacteria active in the intestinal tract may be able to contribute to the folate requirement of colonic epithelial cells. However, further research is required to determine if these bacteria produce folate in the intestinal environment; the form in which this folate occurs; the availability of this folate for transport and utilization by colonocytes from the lumen; and the contribution of the intestinal microbiota to the total folate requirement of colonic epithelial cells.

4.2 Metabolic engineering of production strains

According to the Integrated Microbial Genomes (IMG) System, more than 250 microbial genomes (including more than 20 LAB genomes) have been sequenced to date, with several other projects ongoing and more in the process of being launched [65]. With information from these sequencing projects, such as those published by the Joint Genome Institute Microbial Sequencing program (http://genome.jgi-psf.org/mic_home.html), it is now possible to analyze specific bacterial genomes *in silico* in order to determine if they contain the complete metabolic pathways necessary for *de novo* vitamin biosynthesis. A number of useful tools can be found on the internet that are of great help in analyzing microbial genomes. One of the more useful sites is that of the Genome Analysis and System Modeling Group of the Life Sciences Division of Oak Ridge National Laboratory (http://genome.ornl.gov/microbial/). This site contains tools that allow organizing genes in function of metabolic pathways, functional categories, taxonomic distribution, etc. In the metabolism of cofactors and vitamins sub-category, it is possible to see which enzymes are present in the genome compared to the reference metabolic pathways, thus permitting the user to determine if a specific microorganism has the ability to biosynthesize folate without performing laboratory assays. Similar tools are available at the KEGG (Kyoto Encyclopedia of Genes and Genomes) databases (http://www.genome.jp/kegg/)

The genes for folate biosynthesis have been identified in some LAB such as *Lc. lactis* [66] and *L. plantarum* [67], and recently it has been shown that *L. bulgaricus* possesses all the folate biosynthesis genes [68]. The biosynthetic pathway in LAB includes seven consecutive steps, in which the precursor guanosine triphosphate is converted into tetrahydrofolate [69]. However, some LAB, cannot synthesize folate, because at least some of the genes involved in folate biosynthesis are lacking in the genome, this is the case for *L. gasseri* [70], *L. salivarius* [71], *L. acidophilus* and *L. johnsonii* [68].

Recently, it has been shown that metabolic engineering can be used to increase folate levels in *Lc. lactis* [66, 72], *L. gasseri* [70], and *L. reuteri* (Santos et al., pers. comm.). *Lc. lactis* strains have been modified to produce intracellularly folates with a short glutamyl tail length (average polyglutamyl tail length of 3) or with a long polyglutamyl tail length (average polyglutamyl tail length of 8), which generates an increased retention of folate in the cells [73]. These strains were evaluated in animal models in order to determine if the folate glutamyl tail length affects bioavailability. In contrast to monoglutamyl folate, polyglutamyl folates cannot be transported across the cell membrane. Hence, the release of intracellular polyglutamyl folate depends on the disruption of the cells during passage through the gastro-intestinal tract. The clear responses of lactococcal cells added to a folate free diet of deficient rodents on the folate levels in organs and blood indicate that these cells lyse after consumption and that the bacterial folate becomes available for absorption in the gastro-intestinal tract of the rat [16]. This study provided the first animal trial with food containing living bacteria that were engineered in order to increase the intracellular accumulation of folate or to change the average polyglutamyl tail length compared to a wild-type lactococcal strain. This study revealed that *Lc. lactis* could be used to deliver and release folates in the gastrointestinal tract, a result also obtained previously with riboflavin in *Lc. lactis* [74] and *Propionibacterium freudenreichii* [75].

5. Conclusions

In this review it has been shown that increased folate levels in yogurts and fermented milks are possible through judicious selection of the microbial species and cultivation conditions. The food industry should now take the next step to use this information for selecting folate-producing strains as part of their starter cultures in order to produce fermented products with elevated levels of this essential vitamin. Such products would provide economic benefits to food manufacturers since increased "natural" folate concentrations would be an important value-added effect without increasing production costs. Consumers would obviously benefit from such products since they could increase their folate intakes while consuming products that form part of their normal lifestyle. The proper selection of probiotic folate producing strains provides a strategy for the development of novel functional foods with increased nutritional value.

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