Production of natural folates by lactic acid bacteria starter cultures isolated from artisanal Argentinean yogurts

Jonathan Emiliano Laiño, Jean Guy LeBlanc, and Graciela Savoy de Giori

Abstract: Folate is a B-group vitamin that cannot be synthesized by humans and must be obtained exogenously. Although some species of lactic acid bacteria (LAB) can produce folates, little is known about the production of this vitamin by yogurt starter cultures. *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* strains were isolated from artisanal Argentinean yogurts and were grown in folate-free culture medium (FACM) and nonfat milk after which intracellular and extracellular folate production were evaluated. From the initial 92 isolated LAB strains, 4 *L. delbrueckii* subsp. *bulgaricus* and 32 *S. thermophilus* were able to grow in the absence of folate. *Lactobacillus delbrueckii* subsp. *bulgaricus* CRL 863 and *S. thermophilus* CRL 415 and CRL 803 produced the highest extracellular folate levels (from 22.3 to 135 μg/L) in FACM. In nonfat milk, these strains were able to increase the initial folate concentrations by almost 190%. This is the first report where native strains of *L. delbrueckii* subsp. *bulgaricus* were shown to produce natural folate. The LAB strains identified in this study could be used in developing novel fermented products bio-enriched in natural folates that could in turn be used as an alternative to fortification with the controversial synthetic chemical folic acid.

Key words: folate, lactic acid bacteria, lactobacilli, streptococci, vitamin fortification, fermented foods.

Résumé : Les folates, comme les autres vitamines du groupe B, ne sont pas synthétisées par les humains et doivent donc être obtenues de sources exogènes (aliments). Si bien certaines espèces de bactéries lactiques (BL) peuvent produire des folates, peut est connue sur la production de cette vitamine para les cultures initiatrices des aliments fermentés. *Lactobacillus delbrueckii* subsp. *bulgaricus* et *Streptococcus thermophilus*, isolées de yogourts Argentins ont été évalués dans un milieu de culture libre de folates (FACM) et dans du lait de vache. Des 92 souches évaluées, 4 *L. delbrueckii* subsp. *bulgaricus* et 32 *S. thermophilus* étaient capables de croitre en absence de folate. *Lactobacillus delbrueckii* subsp. *bulgaricus* CRL 863 et *S. thermophilus* CRL 415 et CRL 803 produisaient les plus élevé niveaux de folate extracellulaires (entre 22 à 135 μg/L) dans le FACM. Quand inoculées dans du lait écrémé, ces souches étaient aussi capables d'augmenter significativement (190 %) les niveaux de folates. Les souches identifiées dans cette étude seront évaluées dans le développement de produits fermentés naturellement enrichit en folates naturelles qui pourraient utilisés comme une alternative à la fortification des aliments avec l'acide folique, un composé synthétique qui n'existe pas dans la nature.

Mots-clés : folates, bactéries lactiques, lactobacillus, streptococcus, fortification, aliments fermentés.

Introduction

Human life could not exist without folate, since this B-group vitamin is involved in essential functions of cell metabolism, such as DNA replication, repair, and methylation; synthesis of nucleotides, vitamins, and some amino acids. Folate deficiency has been implicated in a wide variety of disorders from Alzheimer's to coronary heart diseases, osteoporosis, increased risk of breast and colorectal cancers, poor cognitive performance, hearing loss, and of course, neural tube defects (LeBlanc et al. 2007, 2010).

Since animals cannot produce folates, this essential vitamin must be obtained exogenously to prevent deficiencies. Although the beneficial effects of generalized fortification programs have been demonstrated, such as the decreased incidence of neural tube defects and neonatal mortality in countries such as Canada and the USA where folate fortification is mandatory since 1998 (Blencowe et al. 2010), many countries have not adopted a national fortification program because of possible unwanted side effects. The main concerns are based on the fact that vitamins are added at concentrations that allow people with low vitamin intake to reach their Recommended Daily Allowance (RDA) so as to prevent pathologies associated with deficiencies. However, at these levels of fortification, those with normal or elevated levels of vitamin ingestion would be subject to excessive intakes. In

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the case of folic acid (pteroylglutamic acid) fortification, excess intake of this synthetic chemical may mask the early hematological manifestations of vitamin B_{12} deficiency (Morris and Tangney 2007). Since natural folates, such as 5-methyltetrahydrofolate, that are normally found in foods and sometimes produced by microorganisms do not mask B_{12} deficiency (Scott 1999), this folate form would be a more efficient and secure alternative than supplementation with folic acid (Lamers et al. 2006).

The use of vitamin-producing microorganisms is thus a more natural and economically viable alternative to fortification with chemically synthesized pseudo-vitamins and would allow the production of foods with elevated concentrations of folates that are less likely to cause undesirable side effects. Lactic acid bacteria (LAB) represent a heterogeneous group of microorganisms that are naturally present in a wide range of ecological niches, such as foods and in the gastrointestinal and urogenital tract of animals, including humans. In addition to their important technological properties in food production, several studies have shown that LAB can confer beneficial properties to their hosts and certain strains are able to produce and (or) release specific beneficial compounds in foods.

Many industrially important LAB such as *Lactococcus lactis* and *Streptococcus thermophilus* have the ability to synthesize folate and have been the subject of a very recent review (LeBlanc et al. 2011). LAB's ability to synthesize folate explains why some fermented dairy products, including yogurt, contain higher amounts of folate than nonfermented milks. However, the ability of microbial cultures to produce or utilize folate varies considerably, since it is a strain-dependent trait. Most authors claim that *S. thermophilus* normally produce folates (Iyer et al. 2010), whereas *Lactobacillus delbrueckii* subsp. *bulgaricus* is a folate consumer (Kneifel et al. 1991; LeBlanc et al. 2011; Rao et al. 1984), so the selection of an adequate combination of strains is essential to develop fermented foods with increased vitamin concentrations.

Since there are currently no reports of folate production by *L. delbrueckii* subsp. *bulgaricus*, one of the aims of the present study was to evaluate this property in strains of this species (and in *S. thermophilus*) that were isolated from artisanal yogurts of the northwestern region of Argentina. Furthermore, since there are no published studies on the kinetics of folate production by LAB, another objective of this work was to evaluate the folate production by the isolated strains at different time points in folate-free culture media and in milk. These strains could be used to produce yogurts with elevated folate concentrations.

Materials and methods

Microorganisms and growth conditions

A total of 92 strains of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* obtained from the culture collection of CERELA (CRL) were assayed for folate production. These strains were isolated from artisanal yogurts produced in the northwestern region of Argentina. Lactobacilli were grown without agitation in De Man – Rogosa – Sharpe (MRS) broth culture media (De Man et al. 1960) at 37 °C for 16 h, whereas streptococci were grown for 16 h at 42 °C without agitation in LAPTg broth culture media containing (*m*/*v*) 1.5% peptone, 1% tryptone, 1% yeast extract, 1% glucose, and 0.1% Tween 80.

Selection of folate-producing strains

Strains of *L. delbrueckii* subsp. *bulgaricus* ($n = 41$) and *S. thermophilus* ($n = 51$) were assayed for folate production. After activation in the above-mentioned conditions, these LAB were washed 3 times with saline solution (0.85% *m*/*v* NaCl), resuspended in this solution at the original culture volume, and used to inoculate at 4% (*v*/*v*) folate-free culture medium (Folic Acid Casei Medium (FACM), Difco, Becton, Dickinson, and Co., Sparks, Maryland) that were then incubated without agitation at 37 °C for 18 h. After growth, this washing–resuspension procedure was repeated, and the resulting LAB solution was used to inoculate at 2% (*v*/*v*) fresh FACM. This last step was repeated 7 times with the cultures showing good growth (observed by increased turbidity); strains that did not grow in FACM were not used in further studies. After the last incubation, 2 samples were taken to determine the concentration of extracellular and intracellular folates.

A sample (500 µL) of LAB-containing FACM was taken, and 500 µL of protecting buffer (0.1 mol/L phosphate buffer, pH 6.8, containing 1.5% (*m*/*v*) ascorbic acid to prevent vitamin oxidation and degradation) was added and mixed, followed by immediate centrifugation for 5 min at 5000*g*. The supernatant was collected (extracellular folate sample) and the pellet was resuspended in 500 µL of protecting buffer (intracellular folate sample). Both samples were then boiled (100 °C) for 5 min, centrifuged for 6 min at 10 000*g*, and stored at –70 °C until used for folate determinations.

Folate determination

Folate determination was performed using a modified microbiological assay (Horne and Patterson 1988) using *Lactobacillus casei* subsp. *rhamnosus* NCIMB 10463 as the indicator strain (O'Brien et al. 2001), which is naturally resistant to chloramphenicol (up to 500 µg/mL). The strain, stored at –70 °C, was inoculated in fresh MRS and incubated 24 h at 37 °C before use. After growth, 1 mL aliquot was taken and washed 3 times with saline solution, resuspended in the original volume, and used to inoculate at 2% (*v*/*v*) fresh FACM and then incubated for 24 h at 37 °C. This last step was repeated and the second culture was used to perform the folate determination (this procedure was performed to deplete folate reserves in the indicator strain).

All frozen samples were thawed at room temperature (21 °C) in the absence of light and processed in light-reduced conditions. The samples were diluted with protection buffer and each sample (100 µL) was placed into 1 well of a 96-well sterile microplate (Deltalab, Argentina). The folate concentration of each sample was determined in triplicate. The reference strain (*L. casei* subsp. *rhamnosus* NCIMB 10463), grown in FACM as described above, was inoculated at 4% (*v*/*v*) in 10 mL of 2× FACM containing 20 µg/mL chloramphenicol (to decrease the potential of microbial contaminants), and a fraction (100 µL) was added to each well and mixed. Sterile plate covers were placed on the microtiter plates that were then incubated for 48 h statically at 37 °C. After this optimized incubation period, the optical density (OD) was read

at 580 nm using a microplate reader (VERSAmax tuneable microplate reader, Molecular Devices, USA).

In each microplate, a standard curve was realized using HPLC-grade folic acid (Fluka BioChemica, Sigma-Aldrich, Switzerland) instead of samples at different concentrations (between 0 and 1.0 ng/mL) diluted in the protection buffer. Samples were diluted (normally in a 1/40 relation in protection buffer) to obtain values within the range of the standard curve. To obtain the final folate concentrations, the values obtained from the standard curve were multiplied by the dilution factor and expressed as micrograms per litre.

Microbial identification

Strains were identified using phenotypic and biochemical tests that include Gram reaction and sugar fermentation patterns using API 50 CH strips, as specified by the manufacturer (API-BioMérieux, Marcy l'Etolie, France). In selected strains, total cellular DNA was isolated and oligonucleotide primers (PLB16, 5′-AGAGTTTGATCCTGGCTCAG-3′; and MLB16, 5′-GGCTGCTGGCACGTAGTTAG-3′) were used to amplify the variable (V1) region of the 16S ribosomal RNA gene, as described previously (Hébert et al. 2000). Polymerase chain reaction amplicons were sequenced at the CERELA–CONICET (Tucumán) and DNA homology searches performed online using the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

Folate production and growth in FACM

Selected bacteria were inoculated individually in FACM at an initial OD_{580} of 0.1 and incubated 24 h at 37 °C. Samples (2 mL) were taken every 2 h after inoculation to evaluate growth and folate production. pH was measured using a pH meter (AD1040 pH/mV and Temperature meter, Adwa, Hungary), OD_{580} was measured using a UV-visible spectrophotometer (Cecil 2021 Cecil Instruments, England), and colony forming units (CFU) were determined by plating serial dilutions of the samples (100 µL) at each time point on MRS agar (for *L. delbrueckii* subsp. *bulgaricus*) or LAPTg agar (for *S. thermophilus*). The plates were incubated 24–48 h at 37 °C for *L. delbrueckii* subsp. *bulgaricus* and 42 °C for *S. thermophilus*. The CFU per millilitre were obtained after counting the number of colonies grown on the plates, using the following formula:

$$\text{CFU/mL} = \frac{(\text{number of colonies} \times \text{dilution})}{0.1}$$

Folate production in fermented milk

Selected folate-producing bacteria were inoculated at 2% (*v*/*v*) in reconstituted non-fat powdered milk (Svelty Calcio Plus, Nestle, Argentina) and incubated at 37 and 42 °C for 24 h. Samples were obtained at different times (0, 6, and 24 h postinoculation) to evaluate bacterial growth and folate production as described above. For folate determination, 500 µL milk samples were mixed with 500 µL of protection buffer. The resulting mixture was boiled (100 °C) for 5 min to precipitate proteins and release folate from binding proteins present in milk and was then centrifuged 6 min at 10 000*g*. The supernatant was collected and stored at –70 °C until it was used for total folate determinations.

Statistical analysis

All values were expressed as means ± standard deviations (SD). Statistical analyses were performed with the software package SigmaPlot for Windows version 12.0 (Systat Software Inc., Chicago Illinois, USA) using ANOVA GLM, followed by a Tukey's posthoc test, and differences were considered statistically significant at $p \leq 0.05$.

Results

Screening of folate-producing strains

From a total of 92 tested strains, only 4 *L. delbrueckii* subsp. *bulgaricus* strains and 32 *S. thermophilus* strains could grow in the folate-free culture medium after 7 subcultures.

Strain CRL 863 was the highest extracellular folate producer from the *L. delbrueckii* subsp. *bulgaricus* species, whereas *S. thermophilus* CRL 415 and CRL 417 were the highest extracellular folate-producing strains (Tables 1 and 2).

With respect to the intracellular folates *L. delbrueckii* subsp. *bulgaricus* CRL 866 and *S. thermophilus* CRL 412 and CRL 808 retained the highest amount of vitamin (Tables 1 and 2). Because of these results, 1 strain of *L. delbrueckii* subsp. *bulgaricus* (CRL 863) and 2 strains of *S. thermophilus* (CRL 415 and CRL 803) were selected to study the kinetics of folate production in a vitamin-free culture medium based on folate production and quickness to adapt to the FACM (data not shown).

Folate production in FACM

Lactobacillus delbrueckii subsp. *bulgaricus* CRL 863 showed the highest production of both intracellular and extracellular folate after 6 h of growth. In both sample types the strain presented a progressive increase in folate production up until 6 h, which was followed by a slight decrease at 10 h and then remaining constant until 24 h for extracellular folates but slightly decreasing for the intracellular forms (Figs. 1A and 1B). *Streptococcus thermophilus* CRL 803 produced the highest levels of intracellular and extracellular folates at 6 h. Although intracellular folate levels remained constant between 6 and 24 h of growth, a slight decrease was observed in extracellular folate concentrations in the 24 h sample. *Streptococcus thermophilus* CRL 803 showed the same kinetics of vitamin production as *L. delbrueckii* subsp. *bulgaricus* CRL 863. The other *S. thermophilus* strain, CRL 415, showed a progressive increase of intracellular and extracellular folate levels throughout the incubation period, reaching the highest production after 24 h for intracellular folate and at 10 h for extracellular folate, showing a completely different production kinetic from the other 2 studied LAB (Figs. 1A and 1B).

Growth parameters

Lactobacillus delbrueckii subsp. *bulgaricus* CRL 863 showed a continuous and progressive decrease of pH until reaching its lowest value (pH 5.2 ± 0.1) after 24 h (Fig. 2). *Streptococcus thermophilus* CRL 415 presented the same behavior and reached similar pH values, while CRL 803 showed significantly lower values (Fig. 2). The CFU/mL values showed that the *L. delbrueckii* subsp. *bulgaricus* CRL 863 reached the stationary growth phase at 8 h of growth, whereas *S. thermophilus* CRL 803 and CRL 415 grew continuously even after 24 h of incubation (Fig. 2). These results

Table 1. Growth of *Lactobacillus delbrueckii* subsp. *bulgaricus* strains in folate-free culture medium and folate production.

		Folate concn.[c] (μg/L)	
Strain[a]	Growth[b]	Extracellular	Intracellular
142	–	ND	ND
401	–	ND	ND
407	–	ND	ND
420	–	ND	ND
421	–	ND	ND
423	–	ND	ND
426	–	ND	ND
427	–	ND	ND
447	–	ND	ND
449	–	ND	ND
466	–	ND	ND
468	–	ND	ND
487	–	ND	ND
494	–	ND	ND
495	–	ND	ND
537	–	ND	ND
538	–	ND	ND
540	–	ND	ND
541	–	ND	ND
543	–	ND	ND
544	–	ND	ND
551	–	ND	ND
555	–	ND	ND
559	–	ND	ND
565	–	ND	ND
631	–	ND	ND
656	–	ND	ND
657	–	ND	ND
850	–	ND	ND
854	–	ND	ND
859	–	ND	ND
861	–	ND	ND
862	–	ND	ND
863	+	86.2 (0.3)	8.6 (0.1)
864	–	ND	ND
865	–	ND	ND
866	+	3.6 (0.3)	16.2 (0.8)
868	–	ND	ND
869	–	ND	ND
871	+	14.7 (0.4)	11.4 (0.1)
872	+	16.5 (0.3)	11.6 (0.6)

[a]Strain reference numbers are from Centro de Referencia para Lactobacilos (CRL).

[b]Presence (+) or absence (–) of growth in Folate Acid Casei Medium (folate-free medium).

[c]Values are the means ± standard deviation. ND indicates that no folates were detected.

were consistent with those of the OD_{580} value that increased continuously even beyond 24 h (data not shown).

Folate production in fermented milk

At 37 °C, *L. delbrueckii* subsp. *bulgaricus* CRL 863 increased folate levels significantly by 40% and 190% after 24 h of growth compared with its basal value and after 6 h

Table 2. Growth of *Streptococcus thermophilus* in folate-free culture medium and folate production.

		Folate concn.[c] (μg/L)	
Strain[a]	Growth[b]	Extracellular	Intracellular
395	–	ND	ND
396	–	ND	ND
412	+	22.9 (1.1)	28.6 (0.9)
414	+	21.1 (1.1)	16.2 (0.4)
415	+	76.6 (7.0)	15.9 (0.2)
417	+	72.7 (0.3)	8.8 (0.3)
418	+	19.1 (1.0)	14.6 (0.3)
419	+	17.1 (0.9)	19.6 (0.9)
630	–	ND	ND
638	+	20.2 (1.0)	17.2 (0.9)
723	+	8.8 (0.4)	22.5 (0.5)
728	–	ND	ND
729	–	ND	ND
734	+	11.7 (0.3)	7.6 (0.2)
737	+	27.5 (0.4)	13.3 (0.1)
738	+	4.3 (0.2)	0.0 (0.0)
801	–	ND	ND
802	+	14.0 (0.7)	17.6 (0.5)
803	+	22.4 (0.4)	12.2 (0.1)
804	–	ND	ND
805	–	ND	ND
806	+	20.0 (0.8)	7.6 (0.4)
807	+	23.0 (0.3)	0.0 (0.0)
808	+	25.7 (0.7)	54.7 (1.2)
809	+	19.2 (0.4)	7.5 (0.2)
810	+	14.5 (0.8)	11.9 (0.9)
811	+	14.7 (0.3)	16.4 (0.1)
812	+	11.1 (0.2)	10.7 (0.2)
813	+	32.3 (0.4)	8.8 (0.1)
814	+	5.4 (0.3)	9.7 (0.3)
815	–	ND	ND
816	+	11.2 (0.2)	10.3 (0.3)
817	+	11.0 (0.6)	0.0 (0.0)
818	+	10.8 (0.5)	11.8 (0.4)
819	+	7.3 (0.4)	12.3 (0.5)
820	+	13.2 (0.4)	11.0 (0.2)
821	+	16.6 (0.7)	10.6 (0.5)
986	+	30.4 (1.5)	15.3 (0.3)
1184	–	ND	ND
1185	–	ND	ND
1186	–	ND	ND
1187	+	19.7 (0.8)	16.5 (0.4)
1188	–	ND	ND
1190	+	6.8 (0.3)	10.1 (0.5)
1191	–	ND	ND
1192	+	13.5 (0.3)	10.8 (0.3)
1193	–	ND	ND
1764	–	ND	ND
1765	–	ND	ND
1766	–	ND	ND
1767	–	ND	ND

[a]Strain reference numbers are from Centro de Referencia para Lactobacilos (CRL).

[b]Presence (+) or absence (–) of growth in Folate Acid Casei Medium (folate-free medium).

[c]Values are the means ± (standard deviation). ND indicates that no folates were detected.

Fig. 1. Intracellular (A) and extracellular (B) folate production in folate-free culture medium. Data are expressed as the mean ± standard deviation (SD). Different letters above bars indicate that the means are significantly different ($p < 0.05$).

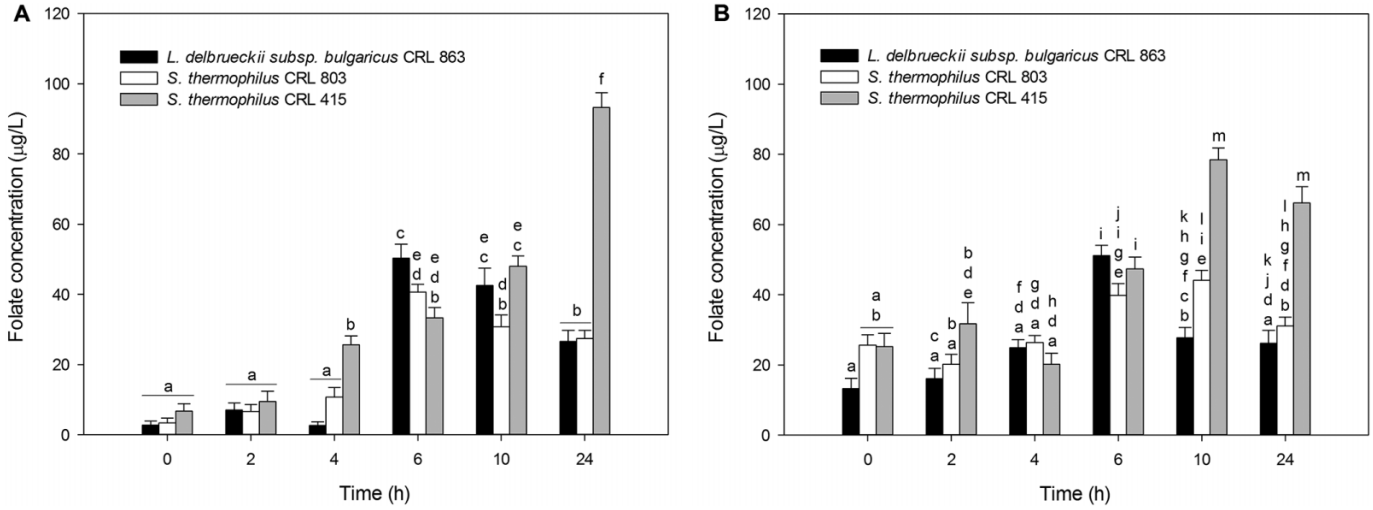
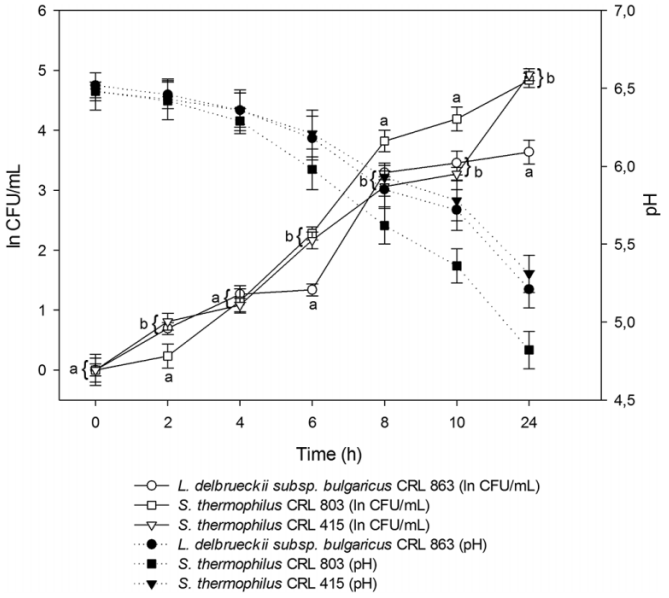


Fig. 2. Growth in folate-free culture medium, in CFU/mL and pH. Data are expressed as the mean ± standard deviation (SD). Different letters at each time point indicate that the means are significantly different ($p < 0.05$).



of growth, respectively (Fig. 3A). In the case of *S. thermophilus* CRL 803, folate levels significantly increased by 32% and 39.5% at 6 and 24 h compared with its initial value, respectively (Fig. 3A), whereas *S. thermophilus* CRL 415 showed a slight but significant increase in the vitamin level at 6 h (29%), which was maintained until the end of the experiment (24 h, Fig. 3A), compared with its value at the start of the fermentation.

At 42 °C, *L. delbrueckii* subsp. *bulgaricus* CRL 863 significantly increased folate concentrations (more than 78%) after 6 h of fermentation compared with its initial value, but folate levels decreased in the 24 h sample (Fig. 3B).

For *S. thermophilus* CRL 803, at 6 h the folate concentrations increased by 99% and by 175% at 24 h compared with the starting level (Fig. 3B), whereas the amount of folate produced after fermentation by *S. thermophilus* CRL 415 significantly increased by 137% after 6 h of fermentation compared with its basal value; this level of increase was maintained until 24 h of incubation (Fig. 3B).

Lactobacillus delbrueckii subsp. *bulgaricus* CRL 863 at 37 and 42 °C clotted the milk at 6 h; however, it reached its lowest pH value (3.81 ± 0.05) after 24 h at both temperatures.

Streptococcus thermophilus CRL 803 decreased pH progressively until reaching its lowest value (3.9 ± 0.2) at 24 h at 37 and 42 °C. Even though *S. thermophilus* CRL 415 showed a similar behavior at both temperatures, the pH values were lower at 42 °C, with milk clotting at only 6 h after fermentation at 42 °C but after 6 h at 37 °C.

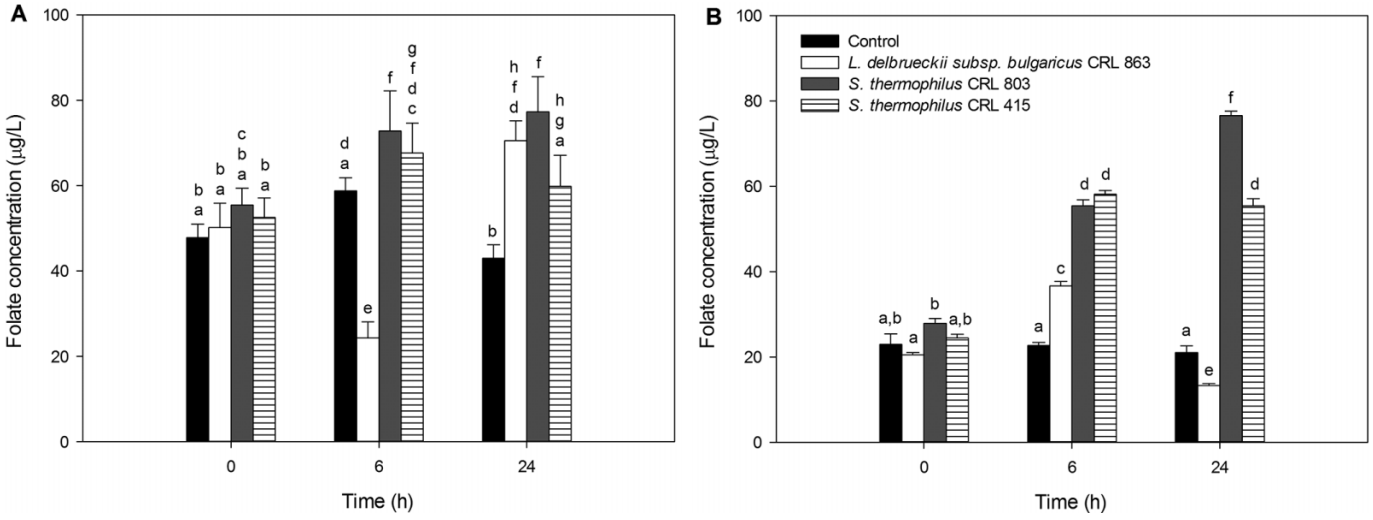
Discussion

Certain strains of LAB have been shown to be able to synthesize natural folates. Not only do some industrially important species such as *Lactococcus lactis* and *S. thermophilus* have the ability to produce this B group vitamin but other LAB such as *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus reuteri*, *Leuconostoc lactis*, *Propionibacterium*, and *Bifidobacterium longum* also have this trait (Crittenden et al. 2003; Gangadharan et al. 2010; LeBlanc et al. 2011; Lin and Young 2000; Pompei et al. 2007). However, it is important to clarify that even though certain species have the potential to produce folates, this is a strain-dependent trait, and thus, the adequate selection of strains is necessary to find those that produce important amounts of this essential vitamin.

Lactobacillus delbrueckii subsp. *bulgaricus* and *S. thermophilus* are LAB species that are commonly used as starter cultures for the elaboration of a variety of fermented dairy products such as yogurts, cheeses, and other fermented milk products. In this study, 92 different strains of these species were tested for their ability to produce folates not only in the absence of the vitamin (in a folate-free culture media) but also in its presence (in milk).

As stated in the introduction, many *S. thermophilus* strains have been shown to produce folates; however, no published

Fig. 3. Folate production in fermented milk at 37 °C (A) and 42 °C (B). Data are expressed as the mean ± standard deviation (SD). Different letters above the bars indicate that the means are significantly different ($p < 0.05$).



studies have identified strains of *L. delbrueckii* subsp. *bulgaricus* that can increase folate levels in culture media or in foods.

From the 51 strains of *S. thermophilus* that were tested, 32 were able to grow in the absence of folate, a somewhat expected result, since it has previously been shown that many strains from this species can produce folates (Iyer et al. 2010). A surprising result was that 4 strains of *L. delbrueckii* subsp. *bulgaricus* (from the 41 that were tested) were able to grow in the same experimental condition (without the exogenous presence of folate). Although growth in the absence of folate does not prove that these bacteria produce it, because some strains might not need folate for growth, it is a good indicator and an efficient tool to select potential folate-producing LAB.

Folate production by the 36 LAB strains that grew in the absence of folates was determined, and all of these strains were able to increase folate concentrations but in a strain-dependent manner, i.e., some strains produced high concentrations of the vitamin, whereas others produced barely detectable amounts (Tables 1 and 2). With these results, we were able to confirm that the 4 *L. delbrueckii* subsp. *bulgaricus* strains that grew in the absence of folates did in fact produce this essential vitamin. To our knowledge, this is the first report of strains of *L. delbrueckii* subsp. *bulgaricus* that can produce folate. To confirm the identity of the folate-producing *L. delbrueckii* subsp. *bulgaricus* strains, the variable region of the 16S ribosomal RNA gene was amplified and sequenced showing a 100% identity with similar sequences in *L. delbrueckii* subspecies, including the newly sequenced *L. delbrueckii* subsp. *bulgaricus* PB 2003/044-T3-4. This result confirmed the identity of *L. delbrueckii* subsp. *bulgaricus* determined by phenotypic and biochemical (API 50 CH) methods. It has been suggested that *L. delbrueckii* subsp. *bulgaricus* type strain ATCC 11842 possesses all of the genes necessary for the de novo synthesis of folates (van de Guchte et al. 2006); however, the actual production of this vitamin has never been shown in this strain. Thus, it would be interesting (*i*) to confirm the presence of all the folate biosynthesis genes in *L. delbrueckii* subsp. *bulgaricus* CRL 863 to see if the genes show similarities with the ATCC 11842 strain and (*ii*) to determine if this latter strain is able to effectively produce folates.

From the results of folate production in FACM, 1 strain of *L. delbrueckii* subsp. *bulgaricus* (CRL 863) and 2 strains of *S. thermophilus* (CRL 803 and 415) were selected for further study because of their high vitamin production.

Many researchers have isolated and characterized LAB from different fermented products and studied their folate production but only at a single time point. To the best of our knowledge, there are no studies of folate production kinetics in culture media deficient in this vitamin. In this study we demonstrated that *L. delbrueckii* subsp. *bulgaricus* CRL 863 and *S. thermophilus* produced significantly high amounts of folates in FACM with a production peak at 6 h, whereas *S. thermophilus* CRL 415 showed the highest concentrations of folates after 24 h of growth.

As stated before, folate production is a strain-dependent trait, and in this study, it was demonstrated that the vitamin production must be evaluated at different time points to know when the maximum folate production is obtained, information that is of vital importance if these strains are to be used in industrial processes. To understand if folate production was related to growth, OD, pH, and CFU/mL were evaluated at each time point. Based on these results, it was shown that the folate production is only directly associated with growth of *S. thermophilus* CRL 415 where the amount of vitamin gradually increased together with growth. In the other 2 LAB strains, vitamin production peaked at 6 h of incubation, whereas a different behavior was observed with bacterial growth where the strains grew exponentially until 10 h of incubation. These results demonstrate, yet again, that folate production is a strain-dependent trait and may not always be directly associated with the growth of the producing strain.

Although milk is considered one of the most nutritious foods, it is not considered a good source of folates. It has previously been reported that milk contains only between 20

and 50 μg/L of folate (LeBlanc et al. 2007); similar concentrations were detected in this study when folate levels were determined before inoculation with the selected LAB (data not shown). However, many dairy products such as yogurt are processed using microbial fermentations in which folate might be synthesized (LeBlanc et al. 2007).

All the 92 LAB strains used in this study belong to the bacterial species *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* that are used as starter cultures to produce fermented food, such as yogurt and other fermented dairy products. The folate production of the 3 selected LAB was evaluated in milk at different time points after incubation at 37 and 42 °C, temperatures that are normally used in the manufacture of commercial yogurts. The results showed that at 37 °C, *L. delbrueckii* subsp. *bulgaricus* CRL 863 significantly increased the folate levels about 40% after 24 h of incubation, demonstrating that instead of consuming the vitamin this LAB was able to produce it. As for the *S. thermophilus* strains, at 37 °C, CRL 803 and CRL 415 increased the folate levels by 39.5% and 29%, respectively, after 24 h of fermentation, compared with the initial values. When the bacteria were incubated at 42 °C, the behavior of the *L. delbrueckii* subsp. *bulgaricus* strain was very different than at 37 °C. After 6 h of fermentation, *L. delbrueckii* subsp. *bulgaricus* CRL 863 increased the folate values by about 78% and returned to the initial concentrations after 24 h. The *S. thermophilus* strains showed a similar dynamic of folate production at 42 °C: CRL 803 significantly increased vitamin levels by about 99% after 6 h and by 174% after 24 h compared with its basal value, and CRL 415 increased folate concentrations by 137% after 6 and 24 h of incubation. Considering that a serving of yogurt is 225 mL, according to the *Guide to Food Labeling and Advertising* (Canadian Food Inspection Agency 2011), 180 μg folate/L would need to be present to claim that this food is "a good source of folate", which is defined as a product that contains at least 40 μg of folate per serving (approved claim 21 CFR 101.79). In the conditions evaluated in our study, some individual strains were able to produce milk products with folate concentrations of >80 μg/L. The correct combination of strains, such as fermenting milk with *L. delbrueckii* subsp. *bulgaricus* CRL 863 and *S. thermophilus* CRL 803 and (or) CRL 415 in optimized conditions, might produce a product with sufficient folate amounts to make a legal nutritional claim. These studies are currently being performed in our laboratory.

What is important to point out is that the presence of folate in milk did not influence folate production by the selected strains. These results imply that folate production would not be downregulated in these LAB by the presence of exogenous folates. This is very important, since selected strains could increase the folate amounts in foods above what the microorganisms need for their own growth. In other studies, it has been reported that the overexpression of specific genes involved in folate biosynthesis increase vitamin production (Sybesma et al. 2003). However, certain folate intermediates have been shown to cause a negative feedback on specific enzymes involved in folate biosynthesis (such as folylpoly-γ-glutamate synthetase) (Andreassi Ii and Moran 2002). It is obvious that the absence of product inhibition by our selected folate-producing strains must be studied in more detail.

Conclusion

In this study it was demonstrated that certain starter cultures, isolated from artisanal yogurts of northwestern Argentina, were able to produce folates. This property was a strain-dependent trait and not directly associated with growth. Also, factors such as incubation time and temperature can influence the production of this important micronutrient. More importantly, this is the first report where it was demonstrated that some strains of *L. delbrueckii* subsp. *bulgaricus* were able to synthesize both intracellular and extracellular folates, whereas all previous studies showed that this species was a folate consumer.

Moreover, the selected LAB strains produced significant amounts of folate in milk even though the vitamin was already present. The right combination of folate-producing *L. delbrueckii* subsp. *bulgaricus* (described for the first time in this study) and *S. thermophilus* and the optimization of the fermentation conditions (for example incubation periods and temperature) could lead to the development of foods with very elevated concentrations of folates without the need of using genetic engineering techniques or chemical fortification. These novel bio-enriched foods could thus be introduced as an efficient tool to prevent folate deficiencies in countries where folate fortification programs do not exist.

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