



## Evaluation of the bioavailability and intestinal effects of milk fermented by folate producing lactic acid bacteria in a depletion/repletion mice model

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### ABSTRACT

This study aimed to evaluate the bioavailability and effects on the intestinal mucosa of a bioenriched fermented milk (BFM) prepared with folate-producing lactic acid bacteria, using a depletion/repletion BALB/c mice model. Five *Streptococcus thermophilus* strains were combined with *Lactobacillus plantarum* 16cv and the combination that resulted in the best folate production was used for preparation of BFM. The folate content in BFM, prepared under controlled conditions (pH 6.0, 42 °C, 70 rpm, 24 h), was  $321.1 \pm 14.1$  ng/mL. Results for mice fed with BFM were compared to those fed with other diets. The BFM group presented an increase in hemoglobin, hematocrit and red blood cells, and the villi height/crypt depth ratio was similar to that of animals receiving milk supplemented with commercial folic acid. The strains were sensitive to most tested antibiotics and lacked virulence genes, indicating that consumption of BFM may be a promising alternative to increase intake of folate.

### 1. Introduction

The importance of vitamins to maintain proper functioning of the organism is well known. Folate (vitamin B9) is particularly relevant, playing an important role in one-carbon metabolism and DNA synthesis and methylation, acting as major coenzymes in these metabolic pathways (Bailey et al., 2015). Besides preventing neural tube defects (NTD) (Blencowe, Cousens, Modell, & Lawn, 2010) and reducing homocysteine levels (Bloom & Smulders, 2011), new epidemiological investigations suggest the association between type 2 diabetes mellitus (T2DM) and low serum folate levels. T2DM patients can have blood folate levels as low as 25% of that in healthy individuals (Choi et al., 2014; Nilsson et al., 2015). Additionally, folates act in cellular metabolism, maintaining genomic stability: high sugar concentration is nongenotoxic in normal folate concentrations but is genotoxic when folate is deficient, providing new insights into the regulation of folates and carbohydrates (Guo et al., 2019; Catala et al., 2019). In addition, some psychological disorders, such as depression, can be related with unbalanced folate levels in the body. Significantly lower levels of folate were found in individuals with depression than in individuals without depression (Bender, Hagan, & Kingston, 2017). Thus, the appropriate intake of folate is crucial for normal development, growth and health

homeostasis, especially for pregnant and women of childbearing age.

Although widely distributed in foods, folates are under-consumed due to several factors, mainly low concentration in food products, low stability, losses during processing and restrictive diets (Albuquerque et al., 2016). Mandatory fortification programs, adopted in several countries to fight folate deficiency and its health consequences, have been effective in reducing NTD (Blencowe et al., 2010). However, fortification has been the focus of concerns due to possible undesirable side effects caused by high folic acid intake, mainly masking vitamin B<sub>12</sub> deficiency and affecting the activity of certain liver enzymes. Consequently, some countries have not adopted mandatory fortification (Saubade, Hemery, Guyot, & Humblot, 2016; Bailey, Rampersaud, & Kauwell, 2003).

In this scenario, natural folate enhancement has gained special attention. The natural synthesis of this vitamin by some lactic acid bacteria (LAB) can enhance folate levels in foods, especially in milk (Kariluoto et al., 2010; Purwandhani, Utamin, Millati, & Rahayu, 2018; Laiño, LeBlanc, & Savoy de Giori, 2012; Albuquerque, Bedani, LeBlanc, & Saad, 2017; Pacheco et al., 2016). Furthermore, milk is a convenient food matrix for growth of LAB that are capable to produce vitamins. Additionally, folates produced by LAB can bind to milk proteins, increasing their stability and bioavailability and helping in the delivery of

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this vitamin to the body (Verwei et al., 2003).

The bioavailability of natural folates can be impaired by the polyglutamate chain in its structure, which does not exist in the chemical structure of folic acid (McKillop et al., 2006; LeBlanc, Savoy de Giori, Smid, Hugenholtz, & Sesma, 2007). In addition, folic acid must be converted into its bioactive form by reductases in the intestinal mucosa and liver. There is a lot of divergence about the bioavailability of folates versus folic acid in the literature, varying from 10% to 98% depending on the evaluation methodology (Scaglione & Panzavolta, 2014).

Since folate deficiency is common around the world, and folate fortification programs are controversial, it is necessary to find alternatives to supply this vitamin to target populations using foods. Laiño, Zelaya, Juárez del Valle, Savoy de Giori, and LeBlanc (2015) reported that selected strains of LAB produced folate in milk, and that milk fermented with these strains was able to improve folate status in experimental animals and also prevent folate deficiency. Using a rodent depletion-repletion model, these authors observed that the folate bio-enriched fermented milk increased the folate concentration in the plasma of mice and decreased the levels of homocysteine. Levit, Savoy de Giori, and de Moreno de LeBlanc & LeBlanc (2018) observed an anti-inflammatory potential in the use of folate producing strains in mice with intestinal mucositis. These studies opened a promising technological option for the delivery of folate to consumers in their normal diet, but more studies are required for better understanding the bioavailability of folates produced by selected microbial strains in fermented food products and for the evaluation of their effects to the intestinal mucosa.

The objectives of this study were to test if folate production by LAB in milk can be enhanced when co-cultures are used, as suggested in other studies conducted by our group (Albuquerque et al., 2017), and evaluate the bioavailability and the effects on the intestinal mucosa of milk fermented with these beneficial microorganisms using an animal model.

## 2. Materials and methods

### 2.1. Strains and growth conditions

The study was conducted with six folate-producing LAB strains isolated in Brazil from goat dairy products (Pacheco et al., 2016): five *Streptococcus thermophilus* (34v, 170v, 268v, 361v and 341pc) and one *Lactobacillus plantarum* (16cv). For use, *St. thermophilus* was grown in LAPTg broth, formulated with (w/v) 1.5% peptone, 1% tryptone, 1% yeast extract, 1% glucose and 0.1% Tween 80, and *Lb. plantarum* was grown in Man-Rogosa-Sharpe broth (MRS) (BD Difco™, Detroit, MI, USA), both at 37 °C for 24 h. Strains were stored at –20 °C, in media supplemented with 20% glycerol.

### 2.2. Screening for production of folate in bovine milk

The capability of the strains to produce folate in bovine milk was screened using 10% reconstituted skimmed milk powder (Molico™, Nestlé, Brazil), pasteurized at 75 °C for 15 s (Thermomix Vorwerk™, Wuppertal, Germany). The strains were activated separately in LAPTg or MRS broths for *St. thermophilus* or *Lb. plantarum*, respectively, at 37 °C for 18 h. One mL of each culture was centrifuged at 10,000g for 5 min, and the pellets resuspended in 1 mL of sterile saline. Prepared bacterial suspensions were used to inoculate (2% v/v) 150 mL of milk, which was incubated at 37 °C without agitation up to 24 h. Aliquots of fermented milk were withdrawn at 0, 6, 9, 12 and 24 h for determination of folate, count of viable bacteria (CFU/mL) and pH measurement.

### 2.3. Counts of viable bacteria

Counts of viable bacteria in the milk samples (biomass

determination) were performed by the drop plate method, according to Naghili et al. (2013), using MRS and M17 agar (both from BD Difco™, Detroit, MI, USA), supplemented with 10% lactose for *Lb. plantarum* and *St. thermophilus*, respectively, and incubation at 37 °C for 24 h.

### 2.4. pH measurements

The pH of the milk samples was determined with a potentiometer (Bel Engineering, Milano, Italy).

### 2.5. Folate determination

Folate determinations were conducted in triplicates, using the microbiological assay method and the folate-dependent and chloramphenicol-resistant *Lb. casei* subsp. *rhamnosus* NCIB 10,463 strain as indicator (Laiño, Juárez del Valle, Savoy de Giori, & LeBlanc, 2013). All testing materials were mixed with equal volume of a protective buffer [sodium acetate solution (0.82%) and ascorbic acid (1%)] and heated at 100 °C for 5 min in a thermoblock (Accublock™, Labnet, NJ, USA).

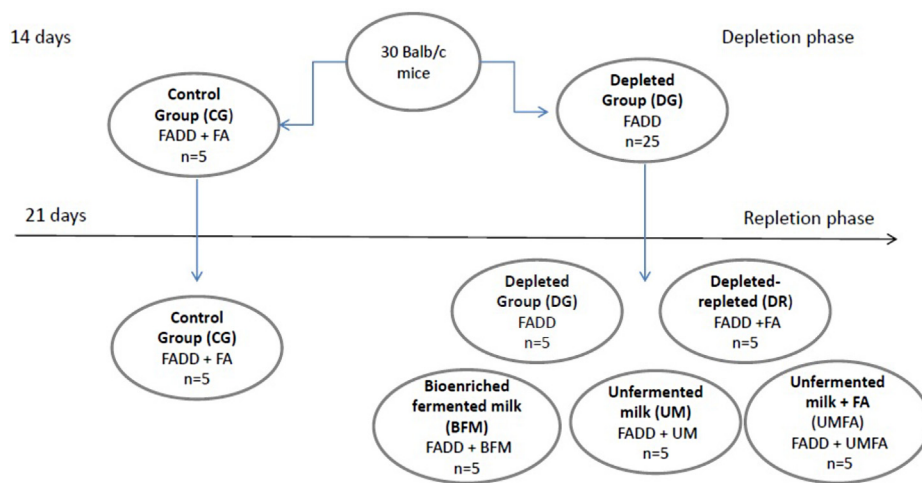
After cooling to room temperature, the mixtures were centrifuged at 10,000g for 5 min and the supernatants stored at –20 °C until use. Prior to folate quantification, samples were submitted to a stepwise tri-enzymatic treatment with  $\alpha$ -amylase from *Aspergillus oryzae* and protease from *Streptomyces griseus* for release of folate adhered to carbohydrates and milk proteins, and with human plasma folate deconjugase for release of folate from polyglutamate chains (Iyer, Tomar, Singh, & Sharma, 2009; Laiño et al., 2015). The enzymes were from Sigma Aldrich, St. Louis, MO, USA and the concentration was 4 mg/mL. Production of folate in the milk by the tested strains was determined subtracting the concentration of folate measured at time 0 and 24 h ( $\Delta$  folate ng/mL).

### 2.6. Genes encoding folate biosynthesis

Search for the genes involved in the folate production pathway was carried out according to Meucci et al. (2018). The six genes, tested by PCR, were *folP* (dihydropteroatesynthase); *folE* (GTP cyclohydrolase I); *folK* (2-amino-4-hydroxy-6-hydroxymethyl-dihydropteridine diphosphokinase, HPPK); *folQ* (dihydroneopterin triphosphate pyrophosphohydrolase); *pabB* (para-aminobenzoate synthetase component I ADCS) and *pabC* (4-amino-4-deoxychorismate lyase, ADCL). Genes *folE* and *folP* were detected using the same primer (*folP-E*); genes *folQ* and *folK* were detected using the same primer (*folK-Q*). The primer sets generate amplicons of 1850 bp (*pabC*: 5'–CGG ACA AGC ATA ATG AAT ACT CGG AAT–3' and 5'–GGA TTG ATA ACC GCT TCT ATT GCC GA–3'), 1666 bp (*pabB*: 5'–CCT CAA TTC ATA CAA CCC TCT CAC A–3' and 5'–CAG ACA AAT CTT CAC TCA CGC CAT AA–5'), 1350 bp (*folP-E*: 5'–GAG ATA GTC TTA ACG ACA TCA CGA TT–3' and 5'–GCA GTC TAT CAA TTA TTG GAA GCT TT–5') and 790 bp (*folK-Q*: 5'–CAC TAG TGT CTA TTG ACT CAA ATA TTT T–3' and 5'–CGT TTT TAT GGC TAT CAC GGG GCT–3'). The amplified products were separated by electrophoresis in 1.0% (w/v) agarose gels in 0.5x TAE buffer. Gels were stained in 0.5x TAE buffer containing SYBR™ Safe DNA Gel Stain (Thermo Fisher Scientific, Waltham, MA, USA).

### 2.7. Screening test for production of folate in milk by co-cultures

The capability of the strains to produce folate in bovine milk when combined in pairs was tested using 10% reconstituted skimmed milk powder (Molico™, Nestlé, Brazil), pasteurized at 75 °C for 15 s (Thermomix Vorwerk™, Wuppertal, Germany). Each *St. thermophilus* strain was combined with the *Lb. plantarum* 16cv, resulting in five combinations: A (*St. thermophilus* 34v and *Lb. plantarum* 16cv); B (*St. thermophilus* 170v and *Lb. plantarum* 16cv); C (*St. thermophilus* 268v and *Lb. plantarum* 16cv); D (*St. thermophilus* 361v and *Lb. plantarum* 16cv) and E (*St. thermophilus* 341pc and *Lb. plantarum* 16cv). Possible



**Fig. 1.** Summary of the depletion-repletion experimental protocol. FADD = Folic Acid Deficient Diet, FA = Folic Acid, CG = Control Group, DG = Depleted group, DR = Depleted Repleted group, BFM = Biofermented Milk Group, UM = Unfermented milk group and UMFA = Unfermented Milk with Added Folic Acid (see Materials and Methods for more details).

incompatibility between strains when in co-culture was checked by the well diffusion method, according to [Laiño et al. \(2012\)](#). The cultures were prepared as described previously and added to 150 mL of milk (5% v/v each). Milk was incubated at 37 °C without agitation up to 24 h and aliquots were withdrawn at 0 and 24 h for folate determination, counts of viable bacteria (CFU/mL) and pH measurements. Production of folate in the milk by the combination of bacteria was determined subtracting the folate concentration measured at time 0 from that measured at 24 h ( $\Delta$  folate ng/mL). Experiments were conducted in triplicates and the combination of strains that resulted in higher folate production in the screening tests (*St. thermophilus* 34v and *Lb. plantarum* 16cv) was selected for the production of the bioenriched fermented milk (BFM) used in the bioavailability and intestinal effects tests.

## 2.8. Selection of the best fermentation conditions for folate production in milk

Before preparation of the fermented milk (BFM) used in the bioavailability and intestinal effects tests, four fermentation conditions were compared: **a.** 37 °C without agitation or pH control, in an erlenmeyer flask; **b.** 42 °C without agitation or pH control, in an erlenmeyer flask; **c.** 42 °C with agitation (70 rpm) and pH controlled at 6.0, in a 1.5 L Infors HT™ bioreactor (Basel, Switzerland); and **d.** 42 °C with agitation (70 rpm) and without pH control, in an erlenmeyer flask. Based on previous results ([Laiño et al., 2017](#)), pH 6.0 was chosen as it was shown to improve folate production by the surveyed strains. For the tests, 10% reconstituted pasteurized skimmed milk powder was added of the cultures of *St. thermophilus* 34v and *Lb. plantarum* 16cv (5% v/v), prepared as described before and incubated in conditions **a**, **b**, **c** and **d** for 24 h, when the production of folate was determined, using non-inoculated 10% reconstituted skimmed milk powder for comparison. Aliquots for folate determination were withdrawn at times 0 and 24 h. Experiments were carried out in duplicates.

## 2.9. Preparation of fermented milk (BFM) for the bioavailability and intestinal effects tests

Based on results of the best fermentation conditions for folate production in milk, preparation of BFM for the bioavailability and intestinal effects tests was performed in the 1.5 L Infors HT™ bioreactor (Basel, Switzerland). *St. thermophilus* 34v and *Lb. plantarum* 16cv were activated separately as described before, centrifuged at 10,000g for 5 min, washed with sterile saline (0.85% NaCl) and resuspended in the same volume of sterile saline. The cultures were added (5% v/v) to 150 mL of 10% reconstituted pasteurized skimmed milk powder (prepared as described before), and incubated at 42 °C for 24 h (“seed

milk”). The “seed milk” was transferred to the bioreactor containing 1 350 mL of 10% reconstituted pasteurized skimmed milk powder. Fermentation was performed at 42 °C for 24 h, at pH controlled at 6.0 and agitation at low speed (70 rpm) to minimize air entrapping and foam formation, but enough to guarantee homogeneity and mixing with the pH control solution (10 M NaOH). No purge air or other gas was applied. The BFM milk was divided in 50 mL samples and frozen at −20 °C. The other types of milk were produced on the same day and frozen at −20 °C. Every 7 days of storage, samples of each type of milk were thawed in a refrigerator and monitored to ensure that folate concentrations remained constant.

## 2.10. Bioavailability assays

The bioavailability of folate produced in the fermented milk was measured according to [Laiño et al. \(2015\)](#), using BALB/c mice and the depletion-repletion model (14 days depletion and 21 days repletion). The mice were obtained from the inbred closed colony maintained at the Centro de Referencia para Lactobacilos (CERELA, San Miguel de Tucuman, Argentina). Animals remained under controlled environmental conditions (temperature 22 ± 2 °C, humidity 55 ± 2%) with 12 h light/dark cycles. Experimental protocols were evaluated and approved by CERELA's Animal Research Ethics Committee (CRL-BIOT-LT-2014-1A), following the recommendations of the Federation of European Laboratory Animal Science Associations and Asociación Argentina for Science and Laboratory Animal Technology.

### 2.10.1. Experimental protocol

The experimental protocol, summarized in [Fig. 1](#), consisted of two phases: a depletion phase of 14 days followed by a repletion phase of 21 days. In the depletion phase, three week old male weaned BALB/c mice, weighing approximately 12 g, were randomly distributed in two groups: **a.** control group (five mice) fed with FADD (Folic Acid Deficient Diet) (AIN-93G Purified Rodent Diet with Vitamin Free Casein Cat. No. 116765, Dyets, Bethlehem, PA, USA) supplemented with 4 mg/kg folic acid (Sigma Aldrich, Germany) and **b.** depleted group (25 mice) fed with FADD only. The composition of FADD is described in [Laiño et al. \(2015\)](#).

In the repletion phase, the control group (**a**) continued to be fed with FADD supplemented with folic acid for 21 days and the depleted group (**b**) was subdivided into five new groups, as follows:

- DG group: fed with FADD only, for 21 days;
- DR group: fed with FADD supplemented with 4 mg/kg folic acid (Sigma Aldrich), for 21 days;
- BFM group: fed with FADD and 50 mL of milk fermented by *St.*

**Table 1**  
PCR conditions for virulence genes.

Gene	Primer Sequence (5'-3')	Amplicon size (bp)	Reactions
<i>efa A</i>	F: GCCAATTGGGACAGACCCTC R: CGCCTTCTGTTCCCTTTGGC	688	20 $\mu$ L, 94 °C for 4 min, 35 cycles at 94 °C for 1 min, 57 °C for 1 min, 72 °C for 1 min, 72 °C for 7 min
<i>asa 1</i>	F: GCACGCTATTACGAACTATGA R: TAAGAAAGAACATCACCCAGA	375	20 $\mu$ L, 94 °C for 4 min, 35 cycles at 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min, 72 °C for 7 min
<i>esp</i>	F: AGATTTCATCTTTGATTCITG R: AATTGATTCITTTAGCATCTGG	510	20 $\mu$ L, 94 °C for 4 min, 35 cycles at 94 °C for 1 min, 43 °C for 1 min, 72 °C for 1 min, 72 °C for 7 min
<i>tdc</i>	F: GAYATNATNGGNATNGGNYTNGAYCAR R: CCRTARTCNNGNATAGCRAARTCNNGTRTG	924	20 $\mu$ L, 94 °C for 4 min, 35 cycles at 94 °C for 1 min, 57 °C for 1 min, 72 °C for 1 min, 72 °C for 7 min
<i>hdc1</i>	F: AGATGGTATTGTTTCTTATG R: AGACCATACACCATAACCTT	534	20 $\mu$ L, 94 °C for 4 min, 35 cycles at 94 °C for 30 s, 40 °C for 30 s, 72 °C for 2 min, 72 °C for 7 min
<i>odc</i>	F: GTNTTYAAYGNGAYAAARCANTAYTTYGT R: ATNGARTTNAGTTCRCAYTTYTCNGG	1446	20 $\mu$ L, 94 °C for 4 min, 35 cycles at 94 °C for 30 s, 54 °C for 30 s, 72 °C for 2 min, 72 °C for 7 min
<i>cylA</i>	F: ACTCGGGGATGATAGGC R: GCTGCTAAAGCTGCGCTT	688	20 $\mu$ L, 94 °C for 4 min, 35 cycles at 94 °C for 1 min, 52 °C for 1 min, 72 °C for 1 min, 72 °C for 7 min
<i>gel E</i>	F: TATGACAAATGCTTTTGGGAT R: AGATGCACCCGAAATAATATA	231	20 $\mu$ L, 94 °C for 4 min, 35 cycles at 94 °C for 1 min, 47 °C for 1 min, 72 °C for 1 min, 72 °C for 7 min
<i>cob</i>	F: AACATTGAGCAAAACAAAGC R: TTGTCATAAAGAGTGGTCAT	1405	20 $\mu$ L, 94 °C for 2 min, 44 °C for 2 min, 72 °C for 2 min, 35 cycles at 94 °C for 15 s, 44 °C for 15 s, 72 °C for 15 s, 72 °C for 5 min
<i>cpd</i>	F: TGGTGGGTATTTTCAATTC T R: T TACGGCTCTGGCTACTA	782	20 $\mu$ L, 94 °C for 2 min, at 44 °C for 2 min, 72 °C for 2 min, 35 cycles at 94 °C for 15 s, 44 °C for 15 s, 72 °C for 15 s and 72 °C for 5 min

*thermophilus* 34v and *Lb. plantarum* 16cv, at 42 °C for 24 h and at controlled pH 6.0, for 21 days;

- UM group: fed with FADD and 50 mL of unfermented milk, for 21 days;
- UMFA group: fed with FADD and 50 mL of unfermented milk supplemented with folic acid (Sigma Aldrich) in a concentration similar to that detected in the fermented milk (300 ng/mL), for 21 days.

FADD (supplemented or not) was offered *ad libitum*, and the amount consumed was measured every day. All mice received water *ad libitum*, except those that received milk (groups BFM, UM and UMFA).

### 2.10.2. Blood and organ samples collection

On the 35th day, the mice were anesthetized with an intraperitoneal injection of ketamine (Holliday Scott, BA, Argentina) mixed with xylazine (Rompun, Bayer AG, Leverkusen, Germany) to a final concentration of 100  $\mu$ g/kg and 5  $\mu$ g/kg mouse body weight, respectively. Animals were sacrificed by cardiac puncture and the blood was transferred to tubes containing 0.5 M EDTA. Blood samples were incubated for 30 min at 37 °C, and the plasma was separated by centrifugation at 1.500g for 10 min.

The liver, spleen, kidneys and intestines of the animals were removed on the 35th day and transferred to tubes where 9 volumes of 0.1 M phosphate buffer pH 7.0 added of 1.5% (w/v) ascorbic acid were added. The mixtures were homogenized (Homogeniser MSE, Torfaen, UK), heated at 100 °C for 5 min and centrifuged at 13,000g for 15 min. The supernatants were removed and kept frozen at -20 °C until folate determination. After removal of intestines content with sterile saline (0.85%), the empty intestines were cut into three parts and transferred separately to tubes containing 10% formaldehyde for histological analysis.

### 2.10.3. Hemogram and erythrocyte folate quantification

Sera were submitted to determination of total red blood cells (RBC), leukocytes, lymphocytes, neutrophils, hemoglobin (Hb), hematocrit (Htc), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelets, eosinophils and monocytes. Tests were performed at Clinical and Microbiological Analysis Laboratory, San Miguel de Tucuman, Argentina.

For erythrocyte folate quantification, 100  $\mu$ L of blood was diluted in 900  $\mu$ L of distilled water and incubated at 37 °C for 2 h for release of folate from polyglutamate chains by enzymatic reaction of blood

deconjugase. The mixture was heated in a thermoblock at 100 °C for 5 min, centrifuged at 13.000g for 10 min and the supernatant transferred to another tube, which was frozen at -20 °C until folate determination. Plasma samples were diluted 10x with a protective buffer (0.1 M phosphate buffer pH 7.0 added of 1.5% w/v ascorbic acid), heated in a thermoblock at 100 °C for 5 min and centrifuged at 10.000g for 5 min and the supernatant was frozen at -20 °C.

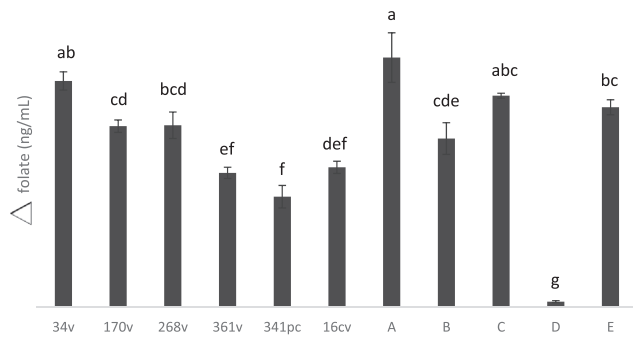
### 2.11. Measurement of small intestine villi length and crypts depth

Measurements of small intestine villi length and crypts depth were performed using an optical microscope (Carl Zeiss-Axio Scope-A1, Germany), with a 100x magnification lens. The images were analyzed using AxioVision Release 4.8 software, calculating the villus length/crypt depth ratio (5 measurements per animal).

### 2.12. Safety of *St. thermophilus* 34v and *Lb. plantarum* 16cv

*St. thermophilus* 34v and *Lb. plantarum* 16cv were tested for presence of virulence genes using PCR simplex as described by Moraes et al. (2017): enterococcal surface protein (*esp*), cytolysin (*cyl A*), gelatinase (*gel E*), aggregation substance (*asa1*), endocarditis antigen (*efa A*), sex pheromones (*ccf*, *cob* and *cpd*), histidinedecarboxylases (*hdc1* and *hdc2*), tyrosine decarboxylase (*tdc*), ornithine decarboxylase (*odc*), *epf*STR (extracellular factor), *mrp*STR (muramidase-released protein), *sly*STR (suilysin), *hyl*STR (hyaluronidase), *arc*STR (arginine deaminase) and *bay046*STR (protective antigen) (Maneerat et al., 2013). The PCR conditions are presented in Table 1. The amplicons were separated by 1%, 1.5% or 2% agarose gel electrophoresis using 100 bp and 1 kb DNA ladders (ThermoFisher, Waltham, USA) as molecular markers. The runs were performed at 100 V for 40 min in 0.5X TBE buffer (89 nM Tris-Borate and 2 nM EDTA, pH 8.0). The gels were stained with SYBR™ Safe DNA Gel Stain (1  $\mu$ L/10 mL, ThermoFisher, Waltham, USA) and the amplicons were visualized under ultraviolet light at 320 nm (Molecular Imager® Gel Doc™ XR + Imaging System, Bio-Rad, USA).

The strains were also tested for antibiotic resistance using the disc diffusion method (CLSI, 2007). Eleven Sensifar™ antibiotic discs (CEFAR, Sao Paulo, Brazil) were used: ampicillin + sulfactam, cefuroxime, ceftiofur, cefotaxime, ciprofloxacin, cotrimoxazole, enrofloxacin, gentamicin, levofloxacin, ofloxacin and tetracycline. Interpretation of results (resistant, sensitive or intermediate) was based on instructions of the manufacturer.



**Fig. 2.** Production of folate (ng/mL) in skimmed milk, fermented for 24 h at 37 °C by *St. thermophilus* strains (34v, 170v, 268v, 361v and 341pc) and *Lb. plantarum* 16cv, isolated and in combinations A, B, C, D and E. Results are expressed as  $\Delta$  folate (mean  $\pm$  SD), considering the initial amount of folate in the skimmed milk. Means (N = 3) with different letters (<sup>a-c</sup>) differ significantly ( $p < 0.05$ ). Combinations: A = *St. thermophilus* 34v and *Lb. plantarum* 16cv; B = *St. thermophilus* 170v and *Lb. plantarum* 16cv; C = *St. thermophilus* 268v and *Lb. plantarum* 16cv; D = *St. thermophilus* 361v and *Lb. plantarum* 16cv and E = *St. thermophilus* 341pc and *Lb. plantarum* 16cv.

### 2.13. Statistical analyses

The results were expressed as means  $\pm$  standard deviation and submitted to ANOVA GLM followed by Tukey post-hoc test, using Minitab 17 software. Differences were considered significant when  $p \leq 0.05$ .

## 3. Results

The amounts of folates produced by the tested strains, isolated and in combinations, in reconstituted skimmed milk powder fermented for 24 h at 37 °C, without pH control, are presented in Fig. 2, expressed as the difference between the concentration of folate measured at time 0 and 24 h. The best folate producing strain was *St. thermophilus* 34v (208  $\pm$  8 ng/mL), followed by *St. thermophilus* 268v (167  $\pm$  12 ng/mL). In the same fermentation conditions, *Lb. plantarum* 16cv produced 128  $\pm$  6 ng/mL. There was no association between growth of the strains in the milk, determined by counts of viable cells, and the resulting folate content.

The amount of folate produced in the fermented milk was strain or combination of strains dependent (Fig. 2). The co-culture of *St. thermophilus* 34v with *Lb. plantarum* 16cv (combination A) resulted in the highest amount of folate detected in the fermented milks ( $p > 0.05$ ), while co-culture of *St. thermophilus* 361v with *Lb. plantarum* 16cv (combination D) resulted in significantly lower production of folate than each strain individually ( $p \leq 0.05$ ).

Two behaviors were observed in the co-cultures: one in which the combination of strains resulted in increased folate production (combinations A, C and E) when compared to individual folate production, and the other in which the combination caused a reduction in the amount of folate produced (combination D). The best co-culture was the combination A (229  $\pm$  25 ng/mL), followed by combination C (195  $\pm$  2 ng/mL). In combination D, very little folate was produced (5.3  $\pm$  0.9 ng/mL). When the fermentation of the milk was carried by strains of combination A, performed in the bioreactor with controlled pH (6.0), for production of BFM used in the bioavailability and effects on the intestinal mucosa assays in the animal model, the measured amount of folate was higher: 321.1  $\pm$  14.1 ng/mL (Table 2).

The PCR screening tests indicated that all *St. thermophilus* strains were positive for the six key genes involved in folate biosynthesis, but *Lb. plantarum* 16cv presented amplicons only for *fol K* and *fol Q* genes.

**Table 2**

Amount of folate (ng/mL  $\pm$  SD) produced by the co-culture of *St. thermophilus* 34v and *Lb. plantarum* 16cv in milk after 24 h, according to the fermentation conditions.

Fermentation conditions	Production of folate
a	229.6 $\pm$ 25.6
b	246.1 $\pm$ 11.7
c	321.1 $\pm$ 14.1
d	237.7 $\pm$ 10.1

a. 37 °C without agitation or pH control, in an erlenmeyer flask; b. 42 °C without agitation or pH control, in an erlenmeyer flask; c. 42 °C with agitation (70 rpm) and pH controlled at 6.0, in a 1.5 L Infors HT™ bioreactor (Basel, Switzerland); and d. 42 °C with agitation (70 rpm) and without pH control, in an erlenmeyer flask. Folate measurements were done in triplicates.

### 3.1. Bioavailability assays

The average feed consumption by the mice was 3.4  $\pm$  0.2 g per day, with no significant differences in consumption between groups ( $p < 0.05$ ). The initial average weight was 12.2  $\pm$  0.6 g and reached 30.8  $\pm$  1.2 g at the end of the experiments, with no significant difference in weight between groups ( $p < 0.05$ ).

The hemogram (Table 3) indicated that the animals from the BFM group presented higher values ( $p > 0.05$ ) for hemoglobin, red blood cells and hematocrit than animals from the other groups, including the controls that were fed with milk supplemented with synthetic folic acid (UMFA) at the concentration contained in the selected fermented milk (300 ng/ml) or with FADD containing synthetic folic acid (DR). These results suggest the bio-efficacy of the folate produced by the co-culture of *St. thermophilus* 34v and *Lb. plantarum* 16cv (combination A) in the organism. The differences for the other hematological tests did not differ significantly ( $p < 0.05$ ).

There was no significant difference in the amount of folate detected in the plasma of the animals of the different groups ( $p < 0.05$ ). Regarding red blood cells (Fig. 3), the highest folate concentration was found in animals from the BFM group, which was significantly higher than in all other groups, including the control group ( $p < 0.05$ ). The folate content in the organs (Fig. 4) differed from that observed in the red blood cells. Animals of the control and UMFA groups presented higher folate concentration in the liver (Fig. 4A) than the other groups ( $p < 0.05$ ). Regarding the spleen (Fig. 4B), the group with the highest folate content was the control group (CG), followed by the BFM, DR and UMFA groups. The spleen folate concentration in the animals of the depleted group was statistically lower than in the other groups ( $p < 0.05$ ). In the kidneys (Fig. 4C), the lowest concentration was observed in mice from the depleted group (DG) and unfermented milk (UM), and highest in the control groups (CG), BFM and milk with folic acid (UMFA). However, these differences were not significant ( $p > 0.05$ ).

Except for the liver, where the UMFA group presented significantly higher folate content than the BFM group ( $p < 0.05$ ) and equal to the control group ( $p > 0.05$ ), folate amount in the other organs followed a trend: the values for the depleted group were lower than for all other groups, whereas the values for the animals of the BFM group were higher or equal to those for animals in the depleted-repleted group (DR), without reaching the amount in the animal of the control group (CG).

### 3.2. Measurements of small intestine villi length and crypts depth

Histological assays (Fig. 5) indicated that the lack of folate ingestion (DG group) damaged the intestinal mucosa of the animals. The villus length/crypt depth ratio (Fig. 5A) in animals of the experimental groups varied significantly: in the depleted group (DG), this ratio was

**Table 3**  
Hemogram of the animals of the six experimental groups.

Group*	RBC 10 <sup>6</sup> /μL	Leukocyte 10 <sup>3</sup> /μL	Lymphocyte 10 <sup>3</sup> /μL	Neutrophil 10 <sup>3</sup> /μL	Hb g/dL	Htc %	MCV fL	MCH pg	MCHC g/dL	Platelet 10 <sup>5</sup> /μL
CG	6.8 ± 0.6 <sup>ab</sup>	4.0 ± 0.8 <sup>a</sup>	3.1 ± 0.7 <sup>a</sup>	0.8 ± 0.15 <sup>ab</sup>	11.5 ± 0.4 <sup>ab</sup>	32.0 ± 3.0 <sup>ab</sup>	47.0 ± 1.5 <sup>a</sup>	16.9 ± 1.1 <sup>a</sup>	36.0 ± 2.2 <sup>a</sup>	2.2 ± 0.2 <sup>a</sup>
DG	6.6 ± 0.5 <sup>b</sup>	2.4 ± 0.3 <sup>ab</sup>	1.9 ± 0.3 <sup>ab</sup>	0.4 ± 0.01 <sup>b</sup>	10.8 ± 0.8 <sup>b</sup>	31.2 ± 2.8 <sup>ab</sup>	45.8 ± 0.7 <sup>a</sup>	16.3 ± 0.4 <sup>a</sup>	35.6 ± 1.5 <sup>a</sup>	2.0 ± 0.3 <sup>a</sup>
DR	6.9 ± 0.3 <sup>ab</sup>	2.4 ± 0.3 <sup>ab</sup>	2.0 ± 0.2 <sup>ab</sup>	0.4 ± 0.06 <sup>b</sup>	11.2 ± 0.4 <sup>b</sup>	31.3 ± 0.8 <sup>ab</sup>	45.9 ± 0.9 <sup>a</sup>	16.4 ± 0.5 <sup>a</sup>	35.7 ± 0.9 <sup>a</sup>	2.0 ± 0.2 <sup>a</sup>
BFM	7.4 ± 0.4 <sup>a</sup>	2.7 ± 0.8 <sup>ab</sup>	2.0 ± 0.6 <sup>ab</sup>	0.7 ± 0.02 <sup>ab</sup>	12.4 ± 0.7 <sup>a</sup>	33.4 ± 1.5 <sup>a</sup>	46.1 ± 1.8 <sup>a</sup>	16.8 ± 1.2 <sup>a</sup>	36.5 ± 1.1 <sup>a</sup>	2.0 ± 0.2 <sup>a</sup>
UM	6.6 ± 0.4 <sup>b</sup>	2.3 ± 0.8 <sup>b</sup>	1.8 ± 0.7 <sup>b</sup>	0.5 ± 0.01 <sup>b</sup>	11.2 ± 0.3 <sup>b</sup>	31.4 ± 2.1 <sup>ab</sup>	47.6 ± 1.1 <sup>a</sup>	17.2 ± 0.6 <sup>a</sup>	36.1 ± 1.3 <sup>a</sup>	2.1 ± 0.3 <sup>a</sup>
UMFA	5.9 ± 0.3 <sup>b</sup>	2.9 ± 1.4 <sup>ab</sup>	1.8 ± 0.8 <sup>ab</sup>	1.1 ± 0.01 <sup>a</sup>	10.2 ± 0.9 <sup>b</sup>	27.0 ± 1.5 <sup>b</sup>	46.6 ± 0.3 <sup>a</sup>	17.0 ± 0.7 <sup>a</sup>	36.6 ± 1.5 <sup>a</sup>	1.7 ± 0.2 <sup>a</sup>

Results are expressed as mean ± SD. Means (N = 5) with different letters differ significantly (p < 0.05).

RBC = Red Blood Cells, Hb = Hemoglobin, Htc = Hematocrit, MCV = Mean Corpuscular Volume, MCH = Mean Corpuscular Hemoglobin, MCHC = Mean Corpuscular Hemoglobin Concentration. \* CG = Control Group, DG = Depleted Group, DR = Depleted-Repleted, BFM = Bioenriched Fermented Milk, UM = Unfermented Milk, UMFA = Unfermented Milk with Folic Acid.

2.1, well below that found in the control (CG), BFM and folic acid supplemented milk (UMFA) groups (3.0–3.5), which did not differ significantly (p > 0.05). The photomicrographs (Fig. 5B) also evidenced the effect of folate on the villus length and crypt depth of the animals of the six experimental groups.

### 3.3. Safety of *St. thermophilus* 34v and *Lb. plantarum* 16cv

None of the strains was positive for the tested virulence genes. Both strains were sensitive to most of the tested antibiotics. *St. thermophilus* 34v was resistant to gentamicin and *Lb. plantarum* 16cv was resistant to enrofloxacin and levofloxacin.

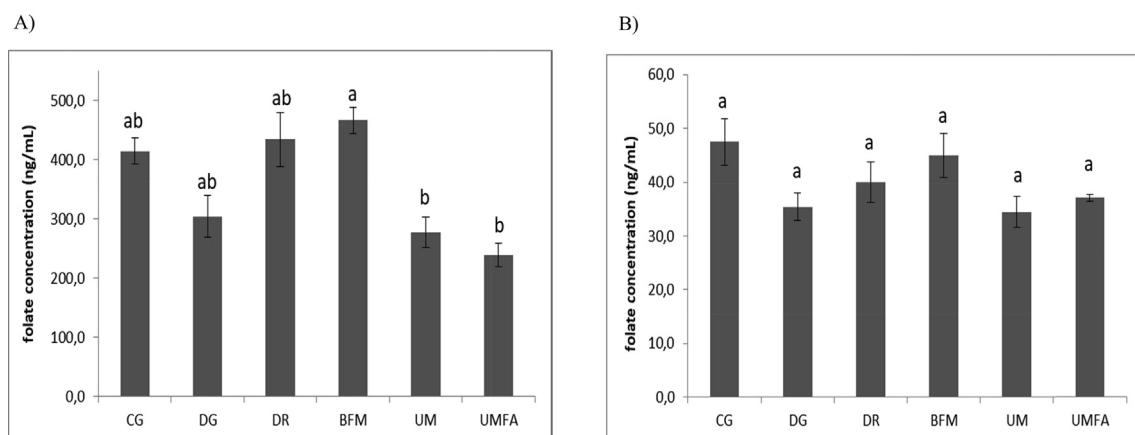
## 4. Discussion

The use of properly selected lactic acid bacteria strains can increase the folate levels in milk (Crittenden, Martinez, & Playne, 2003; Laiño et al., 2013). Since fermented dairy products are widely consumed worldwide, these products can be manufactured with these special strains to achieve an enhanced intake of the natural form of folate for consumers. Production of folate by LAB in milk is strain dependent, but *St. thermophilus* is considered ideal, as all published genomes indicate that the genome of this species has the six genes required for folate synthesis (Meucci et al., 2018; Laiño, Juárez del Valle, Hébert, Savoy de Giori & LeBlanc, 2017; Capozzi, Russo, Dueñas, López, & Spano, 2012), also observed in this study. In the *Lactobacillus* genus, strains may be both folate consumers or folate producers (Greppi, Hemery, Berrazaga, Almaksour, & Humblot, 2017). The species *Lb. plantarum* is considered a folate producer, but yields are strain-dependent, and influenced by the growth medium, as observed in this study. The amount of folate produced by *Lb. plantarum* 16cv in bovine milk at 37 °C was

129 ± 10 ng/mL, much higher than that reported by Purwandhani et al. (2018) for *Lb. plantarum* Dad-13 (29 ± 4 ng/mL) in skimmed milk fermented at 37 °C for 18 h, but much lower than that reported by Hugenschmidt, Schwenninger, Gnehm, and Lacroix (2010) for *Lb. plantarum* SM39 in supplemented whey permeate (397 ± 60 ng/mL) or Khalili, Rad, Khosroushahi, Khosravi, and Jafarzadeh (2019) for *Lb. plantarum* 5HN in yoghurt at the 14<sup>th</sup> day of storage (642 ± 47 ng/mL).

Considering that a symbiosis between *S. thermophilus* and *Lactobacillus* sp. may occur during milk fermentation, different combinations containing both genera were evaluated. In this study, it was shown that co-cultures can favor or impair the folate production, depending on the strains combination (Fig. 2). In combination D (*St. thermophilus* 361v + *Lb. plantarum* 16cv), very low amounts of folate was detected in the fermented milk. It was previously reported that folate producing microorganisms in specific conditions can become folate consumers, which could explain why there is a decrease in folate concentrations in this combination compared to when the strains are grown separately (Greppi et al., 2017). This behavior was also observed by Laiño et al. (2013), who reported that from fifteen combinations of *Lb. bulgaricus* and *St. thermophilus*, two resulted in lower vitamin production than each culture alone. On the other hand, the co-cultures in combinations A, C and E in milk resulted in higher folate content than when cultures were inoculated separately. Apparently, there was a symbiosis between the strains, reinforcing the findings of Albuquerque et al. (2017), who reported increased levels of folate in a fermented soybean product resulting from the combination of *St. thermophilus* TH-4 with *Lb. rhamnosus* LGG. Laiño et al. (2012) also observed that the combination of *St. thermophilus* CRL 803 and *Lb. delbrueckii* subsp. *bulgaricus* CRL 863, resulted in a fermented product considered a “good source of folate” (180 ng/mL).

It is relevant to consider the nutritional aspects of the bioenriched



**Fig. 3.** Folate concentration (ng/mL) in the red blood cells (A) and plasma (B) of mice of the six experimental groups. Results are expressed as mean (N = 15) ± SD. Means with different letters differ significantly (p < 0.05).

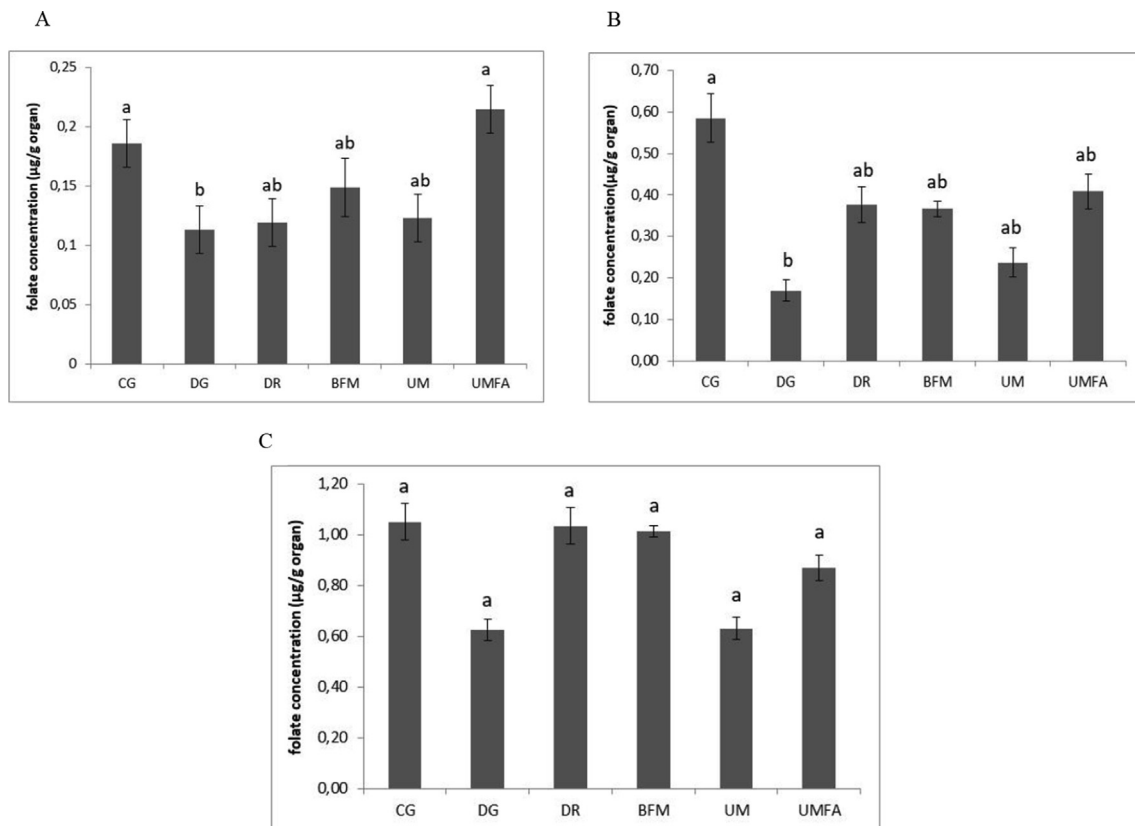


Fig. 4. Folate content ( $\mu\text{g/g}$  organ) in the liver (A), spleen (B) and kidneys (C) of mice of the six experimental groups. Results are expressed as mean ( $N = 15$ )  $\pm$  SD. Means with different letters differ significantly ( $p < 0.05$ ).

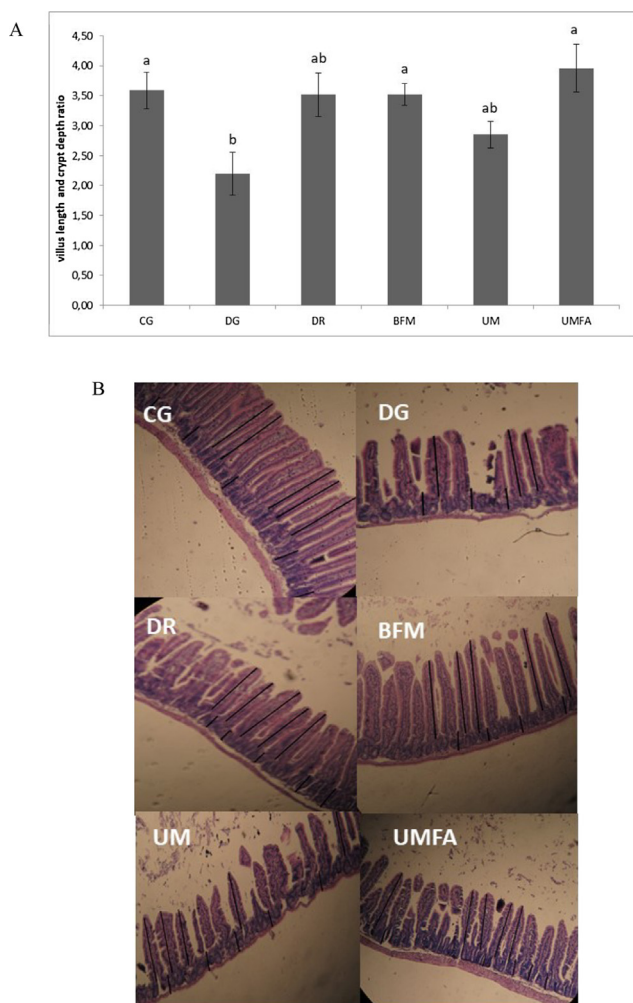
fermented milk obtained by the combination of strains *St. thermophilus* 34v and *Lb. plantarum* 16cv at pH 6.0 ( $321 \pm 14.1$  ng/mL). Based on the most recent recommendations in Europe (EFSA, 2014), a portion of 250 mL of BFM can provide 80  $\mu\text{g}$  of folate, which corresponds to 23% of the recommended daily intake of folate for adults (330  $\mu\text{g}$  per day). When FAO/WHO recommendations are considered (WHO, 2002) the same 250 mL portion of BFM would provide 20% of the recommended intake for adults (400  $\mu\text{g}$  per day) and 40% for children (200  $\mu\text{g}$  per day). These results reinforce that the BFM obtained in this study is an interesting alternative to prevent folate deficiencies in adults and children.

Bacterial growth conditions have a remarkable influence on folate production, as demonstrated in this study (Table 2), in agreement with other recent reports (Divya & Nampootheri, 2015; L aino et al., 2017, 2019; Meucci et al., 2018), reinforcing the concept that pH plays a fundamental role in vitamin production, with pH controlled 6.0 being the most appropriate. L aino et al. (2017) found that folate production was 2–3 times higher when fermentation occurred at pH 6 when compared to fermentation without pH control. These authors suggested that pH was important for the up-regulation of the expression of the *folE*, *folQ*, *folK* and *folP* genes involved in the synthesis of folate *de novo*. The influence of pH in folate synthesis can also have another reason: Liu et al. (2019) observed that intracellular acidity is a stress factor for *St. thermophilus*, which is counteracted with excessive consumption of nutrients and lower folate accumulation.

The hematological indicators red blood cells (RBC), hemoglobin (Hb) and hematocrit (HTC) (Table 3) suggest that the intake of fermented milk enriched with the natural form of folate (BFM) was more bioeffective than the intake of the other types of fermented milk supplemented with folic acid at the same concentration as that in the BFM (300 ng/ml for the UMFA) and at excess concentrations (2 mg/ml) for the CG and DR groups. A recent literature review conducted by Bayes,

Agrawal, and Schloss (2019) on the bioavailability of different forms of folate, both in humans and animals, indicated the scarcity of data, and only three studies in twelve found a statistically significant difference between supplements with different forms of folate, whereas the bioavailability of the natural folate (5MTHF – 5 methyltetrahydrofolate) was greater than folic acid. One of these studies was conducted by Lamers, Prinz-jangenohl, and Bra (2006), assessing the efficacy of daily supplementation with the naturally occurring 5-MTHF compared with folic acid in healthy women of child-bearing age and found an increase in RBC and plasma folate concentrations significantly higher in the group receiving the natural form of folate (5-MTHF). Corroborating with those findings, in our study, the positive effect of the intake of the natural form of folates on RBC was also observed, whereas the highest folate concentration was found in animals from the BFM group, which was significantly higher than in all other groups, including the control group receiving folic acid supplemented milk ( $p < 0.05$ ) (Fig. 3). The BFM group presented the highest plasma concentration ( $45 \pm 4$  ng/mL) among the others groups that received folic acid, approaching that of the CG control group ( $48 \pm 4$  ng/mL), but these were not significantly different. This lack of significant difference in plasma could be due to the depletion period used in this study (14 days) which should be increased or due to the animal

Although in the present study the forms of folate produced by the tested LAB strains were not evaluated, previous studies have shown that the main natural form produced by LAB in milk and culture medium are 5-MTHF, tetrahydrofolate (THF) and 5-formyltetrahydrofolate (5-FmTHF) (Lin & Young, 2000; Sybesma, Starrenburg, Tijsseling, Hoefnagel, & Hugenholtz, 2003; Sanna et al., 2005) A study of Meucci et al. (2018) have shown that among these three vitamers (5-FmTHF, THF and 5-MTHF), the 5-MTHF was the main folate form produced by *St. thermophilus*. The mice hemograms of the group supplemented with the natural form of vitamin (BFM) presented significantly better results



**Fig. 5.** Intestinal villus length/crypt depth ratio (A) and photomicrographs (B) of mice of the six experimental groups. Means (N = 15) with different letters differ significantly ( $p < 0.05$ ).

that those supplemented with folic acid (DR and UMFA groups) indicating that the natural form of folate produced by the combination of *St. thermophilus* 361v and *Lb. plantarum* 16cv in milk, when administered with a folate-free diet, was effective in maintaining adequate folate levels in the body, preventing the deficiency of this vitamin, observed in the depleted group (DG). It can be concluded that the natural form of folate in milk fermented with the combination of *St. thermophilus* 361v and *Lb. plantarum* 16cv was effectively absorbed by the mice. In accordance, Lamers et al. (2006) stated that natural forms of folates, 5-MTHF specifically, would be considered a more efficient and safer alternative than the fortification of foods with folic acid. It must be stated here that unfermented milk (as does the UMFA) does contain natural folates (especially 5-MTHF) and that these are increased by LAB fermentation. Although we were able to increase natural folate concentrations, controls containing only folates produced by our strains would be necessary in order to confirm that natural folates are more bioavailable than folic acid.

No significant differences were observed in the folate concentrations in kidney, spleen or liver of animals from the BFM group when compared to other experimental groups (Fig. 4), as also reported in the study of Laiño et al. (2015). These findings confirm that these organs are poor indicators of folate status in animal models like the one used in this study probably because of their low growth rate that make the cells in these organs not be as dependent on folate intakes as are blood cells and cells of the intestinal tissues.

The results of this study (Fig. 5) showed that lack of folate intake can cause damages to the intestinal cells and that ingestion of natural folate produced by the combination of *St. thermophilus* 361v and *Lb. plantarum* 16cv has a beneficial effect on the mucosa, helping to maintain its integrity and consequently the intestinal health. The maintenance of mucosa intestinal integrity requires coordination of cell proliferation and differentiation process (Parker et al., 2017). Folate plays a key role in cell proliferation therefore folate deficiency reduces proliferation of various cell types. The lack of folate in cells promotes a nucleotide imbalance and slow DNA synthesis, which leads to an interruption in the cell cycle, more precisely in the S phase. In this line, the mucosa intestinal cells, that undergoes continual renewal are affected by the low intake of folate. Since these cells also have a rapid renovation, as soon as folate is added back to folate-deficient cells, the S phase impairment is reversed and the proliferation is restored. (Courtemanche, Elson-Schwab, Mashiyama, Kerry, & Ames, 2004). It is possible that due to this metabolism the mucosa intestinal was more sensitive to the folate depletion and repletion. To the best of our knowledge, this is the first study that evaluated the effect of administration of fermented milk containing natural folates produced by LAB in addition to those normally present in milk on intestinal histology. The anti-inflammatory potential of folic acid supplementation was reported by Burr, Hull, and Subramanian (2017), who observed a reduced risk of developing colorectal cancer in patients suffering from inflammatory bowel disease. Levit et al. (2018) also showed the beneficial effect of administering a folate-producing strain suspension in mice with mucositis. These authors propose that the additional folate intakes would stimulate the fast growing cells of the intestines that are affected in these inflammatory diseases and thus help in their treatment. However, no study has yet shown the effect of ingesting a naturally bioenriched milk with folate on animal intestinal cells in a depletion-repletion model.

The screening for virulence genes and antibiotic resistance is important to guarantee the safety of strains for food application. Even though the tested strains belong to species with Generally Recognized as Safe (GRAS) status, there are concerns about horizontal gene transfer (HGT) which may lead to acquisition of new virulence and resistance genes harbored on mobile genetic elements such as plasmids, integrons and transposons (Founou, Founou, & Essack, 2016). None of the strains in this study harbored the tested virulence genes and only resistance to gentamicin in *St. thermophilus* and levofloxacin and enrofloxacin in *Lb. plantarum* 16cv was detected. According to Mathur and Singh (2005), resistance to gentamicin in *St. thermophilus* strains, as observed here, is quite common and has been reported in other studies as well (Aslim & Beyatli, 2004). Sharma, Tomar, Sangwan, Goswami, and Singh (2015) reported a wide range of antibiotic resistance among *Lactobacillus* strains isolated from commercial probiotic products, while in this study the *Lb. plantarum* 16cv strain was resistant to levofloxacin and enrofloxacin only, among eleven tested antibiotics. The results in this study are consistent with these previous works and the selected strains can thus be considered safe for human consumption.

## 5. Conclusions

The proper selection of LAB strains and growth conditions play an essential role for obtaining high levels of folate in fermented milk. The administration of a fermented milk containing folate produced by selected LAB strains to mice was effective in maintaining adequate folate levels in the organism of the animals increasing the red blood cell counts, hemoglobin and hematocrit. The important function of folate in maintaining the integrity of the intestinal mucosa was demonstrated. These results indicate that consumption of fermented products containing natural folate, produced by selected LAB that lack virulence factors and are safe for human consumption is a promising alternative for increasing the intake of this vitamin by the population.



## CRedit authorship contribution statement

**Ana Clara C. Cucick:** Formal analysis, Investigation, Methodology, Writing - original draft, Writing - review & editing. **Katia Gianni:** Investigation, Methodology, Writing - original draft, Writing - review & editing. **Svetoslav D. Todorov:** Investigation, Methodology, Supervision, Writing - original draft, Writing - review & editing. **Alejandra de Moreno de LeBlanc:** Investigation, Methodology, Supervision, Writing - original draft, Writing - review & editing. **JeanGuy LeBlanc:** Funding acquisition, Investigation, Methodology, Supervision, Writing - original draft, Writing - review & editing. **Bernadette D.G.M. Franco:** Formal analysis, Funding acquisition, Project administration, Supervision, Visualization, Writing - review & editing.

## Declaration of Competing Interest

The authors declare no conflict of interest.

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