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# Milk fermented with selected strains of lactic acid bacteria is able to improve folate status of deficient rodents and also prevent folate deficiency

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## ARTICLE INFO

### Article history:

Received 10 February 2015

Received in revised form 27 April 2015

Accepted 29 April 2015

Available online

### Keywords:

Folate

Vitamin deficiency

Lactic acid bacteria

Rodent models

Homocysteine

## ABSTRACT

Folate deficiencies are common in many parts of the world. The use of folate producing food grade microorganisms has been proposed as a more natural alternative to fortification with the chemical folic acid. The aim of this study was to evaluate the effectiveness of a novel fermented milk elaborated with adequately selected folate producing lactic acid bacteria. This product was tested using both a rodent depletion–repletion model and a complete deficient model. The folate bio-enriched fermented milk was able to increase plasma folate concentrations and decrease homocysteine levels. This is the first report demonstrating that a naturally folate bio-enriched fermented milk, elaborated with folate-producing starter cultures, not only is effective to improve folate status but can also prevent folate deficiency. Consumers also would obviously benefit from this type of product because they could increase their folate intakes while consuming foods that form part of their normal diets.

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## 1. Introduction

Folates are typically present as the reduced forms, dihydrofolate (DHF) and tetrahydrofolate (THF). THF can be substituted with a variety of one-carbon units (including formyl and methyl

groups) that function as a coenzyme in metabolic reactions involving transfer of one-carbon moieties (Lucock, 2000; Smulders & Stehouwer, 2005). Folates are involved in the biosynthesis of purines and pyrimidines, essential for DNA synthesis; amino acid interconversion and for the provision of methyl groups in methylation reactions as synthesis of S-adenosyl

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<http://dx.doi.org/10.1016/j.jff.2015.04.055>

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methionine, major methyl donor for most methyltransferase reactions, and in building block of many proteins (Blakley & Benkovic, 1984; Hanson & Roje, 2001; Kwon et al., 2008; Quinlivan, Hanson, & Gregory, 2006).

The efficiency of DNA replication, repair, and methylation are affected by folate availability; therefore, large amounts of folate are required by rapidly proliferating cells such as enterocytes (Goh & Koren, 2008; Scholl & Johnson, 2000; Tamura & Picciano, 2006). Humans cannot synthesize folate. Therefore, an exogenous supply of folate is necessary to prevent nutritional deficiency. The World Health Organization (WHO) recommend a daily intake of folate of 400 mg in adults, and 600 mg in pregnant women (FAO/WHO, 2002). Though folates are widely distributed in foods, their deficiency still occurs frequently even in well-developed countries. Suboptimal intake of folate has been linked to cardiovascular diseases (Wald, Law, & Morris, 2002), neuropathy, birth defects (Basset, Quinlivan, Gregory, & Hanson, 2005; Scott, Rébeillé, & Fletcher, 2000), cancer (Figuereido et al., 2009; Kim, 1999; Novakovic, Stempak, Sohn, & Kim, 2006; Xu & Chen, 2009), among other pathologies. Also, it has been shown that low folate concentrations cause an increase in homocysteine levels (Dhonushe-Rutten et al., 2007; Gamble et al., 2005; Mattson & Shea, 2003), which in turn increases the risks of cardiovascular diseases (Boushey, Beresford, Omenn, & Motulsky, 1995; Wald et al., 2002).

Although megaloblastic anemia that occurs due to extreme folate deficiency resulting in faulty DNA synthesis and diminished cell production is not common in developed countries, sub-clinical folate deficiency, which is characterized by low blood folate concentration and high plasma homocysteine levels, can persist without any other hematological alteration and is present in populations throughout the world (Herbert et al., 1960; Morris, Jacques, Rosenberg, & Selhub, 2007; Wickramasinghe, 2006).

Due to the high incidence of pathologies associated with folate deficiency, many countries have introduced mandatory fortification of foods with folic acid (FA) (ANMAT, 2002; Blencowe, Cousens, Modell, & Lawn, 2010). Despite the documented beneficial effects of FA supplementation (Bentley, Weinstein, Willett, & Kuntz, 2009; Berry, Bailey, Mulinare, Bower, & Dary, 2010; Rabovskaja, Parkinson, & Goodall, 2013), fortification with this chemical may have adverse effects in subpopulations, such as masking vitamin B<sub>12</sub> deficiency, primarily in the elderly (Morris & Tangney, 2007; Schneider, Tangney, & Morris, 2006), causing alterations in the liver dihydrofolate reductase enzyme activity (Bailey & Ayling, 2009). There are also indications that folic acid may induce cancer in certain conditions. Contrary to FA, there are no reports of side effects after high intakes of bio-produced folates, such as THF, which can be produced by certain bacteria.

Because of their documented beneficial effects, certain bacteria have been designated as probiotic that have been defined by the FAO/WHO as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (Hill et al., 2014). Recent reviews and research articles have shown that probiotics can: decrease body adiposity by altering the gut microflora (Omar, Chan, Jones, Prakash, & Jones, 2013), play a crucial role in the health of the gut–liver axis preventing non-alcoholic fatty liver diseases

(Mohammadmoradi, Javidan, & Kordi, 2014), alter the expression of beneficial genes in the gastrointestinal tract of rats (Paturi et al., 2015), reduce uremic toxin levels both *in vitro* and *in vivo* (Fang et al., 2014), beneficially alter the innate immune system in various animal models (Patel, Shukla, & Goyal, 2015), enhance the antioxidant capacity of soymilk and prevent metabolic syndrome biological markers (Marazza, LeBlanc, de Giori, & Garro, 2013), etc. It has been proposed that folate production could be considered a probiotic property of bifidobacteria (Pompei et al., 2007). Among food-grade microorganisms, lactic acid bacteria (LAB) are known for folate production in varying amounts making these strains potentially probiotic (Hugenholtz, Hunik, Santos, & Smid, 2002; Hugenholtz, Sybesma et al., 2002; Iyer, Tomar, Kapila, Mani, & Singh, 2009; Laiño, Juárez del Valle, Savoy de Giori, & LeBlanc, 2013; Laiño, LeBlanc, & Savoy de Giori, 2012). Certain strains of *Streptococcus* (*S.*) *thermophilus*, an important dairy starter, have been reported to produce larger amounts of folate compared with other bacteria, most of which is excreted into milk during the fermentation process (Iyer, Tomar, Kapila et al., 2009; Mousavi et al., 2013), which consumes folate during its growth in milk. Yogurts contain higher folate concentrations than non-fermented milk (Holasova, Fiedlerova, Roubal, & Pechacova, 2004; Sybesma, Starrenburg, Tijsseling, Hoefnagel, & Hugenholtz, 2003) because of folate production by *S. thermophilus* (Rao, Reddy, Pulusani, & Cornwell, 1984); however, in a previous study it was shown that some *Lactobacillus delbrueckii* subsp. *bulgaricus* strains were able to increase the initial folate concentrations in milk by almost 190%, constituting the first evidence that this species can produce natural folates (Laiño et al., 2012). These strains were used to obtain a fermented milk naturally bio-enriched in folate (Laiño et al., 2013), one portion (225 mL) of which would contribute to 10% of the daily recommended intake for adults. Thus, the aim of this study was to evaluate the effectiveness of this novel fermented milk using different murine folate deficiency models.

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## 2. Materials and methods

### 2.1. Microorganisms and growth conditions

*L. bulgaricus* CRL871, *S. thermophilus* CRL803, and *Streptococcus macedonicus* CRL415 were previously selected due to their high folate-producing capabilities in milk (Laiño et al., 2012), whereas *L. bulgaricus* CRL861 and *S. thermophilus* CRL1764 were used as folate consuming strains. All microorganisms were obtained from the culture collection (CRL) of the Centro de Referencia para Lactobacilos (CERELA-CONICET, Tucumán, Argentina). Before experimental use, cultures were propagated (2%, v/v) twice in sterile de Man–Rogosa–Shape (MRS) broth (De Man, Rogosa, & Sharpe, 1960) for lactobacilli, and LAPTg broth, containing (w/v) 1.5% peptone, 1% tryptone, 1% yeast extract, 1% glucose and 0.1% Tween 80 (Raibaud, Caulet, Galpin, & Mocquot, 1961) for streptococci, and incubated at 37 °C (lactobacilli) and 42 °C (streptococci) for 16 h. All bacteria were harvested by centrifugation at 5000 × *g* for 10 min, washed twice, and resuspended in sterile saline solution (0.15 M NaCl) to the same original volume. This cell suspension was used as the inoculum.

## 2.2. Fermented milk manufacture

Reconstituted non-fat powdered milk (250 mL) (Svelty Calcio Plus, Nestlé, Argentina) was heat treated at 87 °C for 30 min, cooled to 37 °C, and inoculated with 2% of previously selected folate producing starter cultures (*S. thermophilus* CRL803, *S. macedonicus* CRL415, and *L. bulgaricus* CRL871, cocci/bacilli ratio = 2:1, w/w), and incubated at 42 °C for 6 h. This fermented milk, with elevated concentrations of folates ( $187 \pm 7 \mu\text{g/L}$ ) will be referred as Folate enriched fermented milk (FEFM). Control fermented milk was obtained by inoculating 2% of folate consuming strains (*S. thermophilus* CRL1764 and *L. bulgaricus* CRL861, cocci/bacilli ratio = 2:1, w/w), and incubated at 42 °C for 6 h, and denominated Control fermented milk (CFM) ( $75 \pm 4 \mu\text{g/L}$ ).

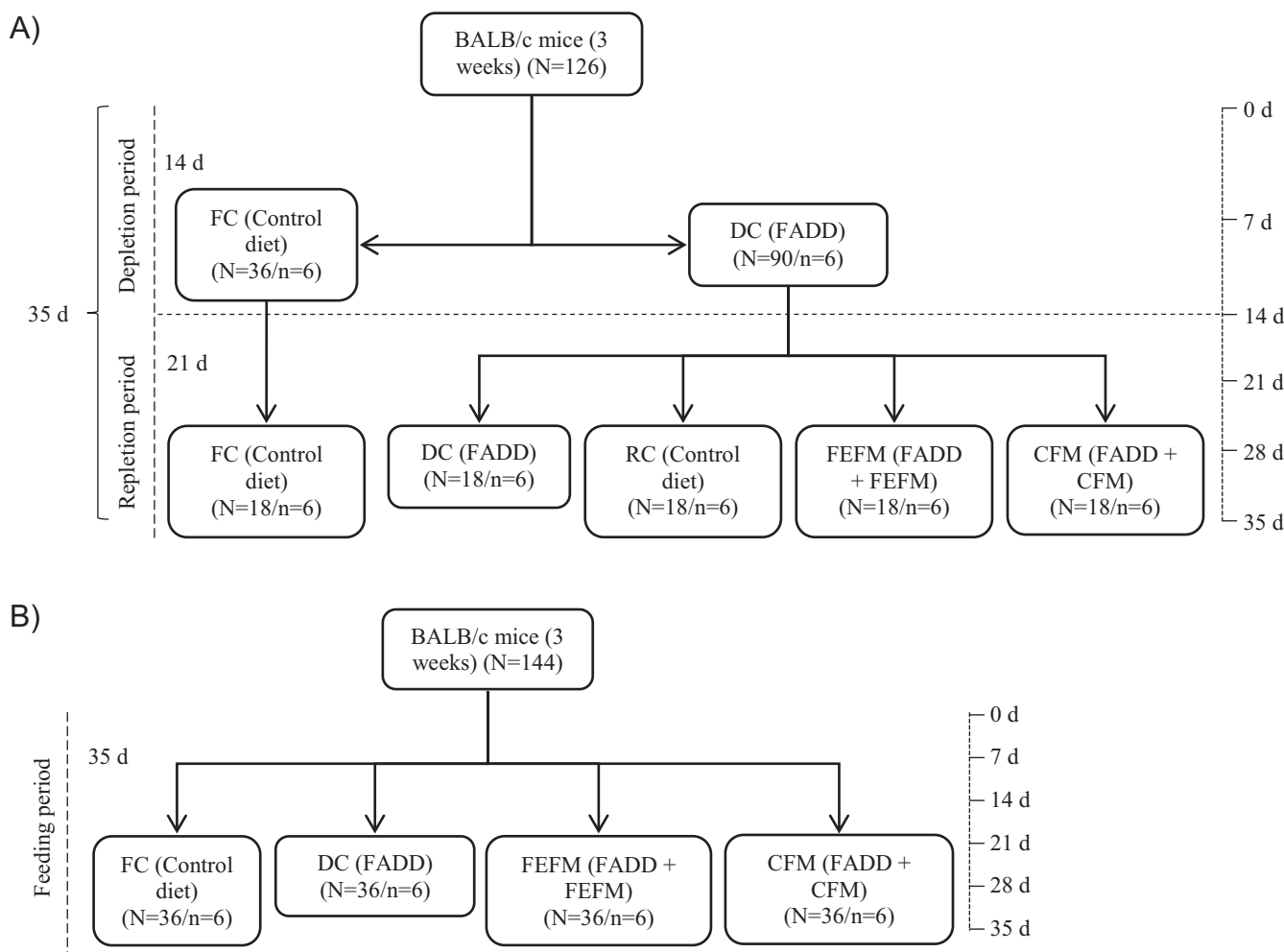
Fermentations were stopped by rapidly cooling the incubated milk in an ice bath, and stored at 4 °C before being used. Both, FEFM and CFM were prepared freshly every day and used directly in the animal trials.

## 2.3. Experimental design

The overall experimental protocol is summarized in Fig. 1. Weaned BALB/c mice, one of the most widely used strains, weighing  $10.0 \pm 0.5 \text{ g}$  were obtained from the inbred closed colony maintained at CERELA (Argentina). Animals remained under controlled environmental conditions (temperature  $22 \pm 2 \text{ }^\circ\text{C}$ , humidity  $55 \pm 2\%$ ) with 12 h light/dark cycles and were allowed free access to food and water throughout the study. Certified folic acid free defined composition diet (FADD) (Cat. N° 517812, Dyets, Bethlehem, PA, USA), and FADD with 2 mg of FA per kg of diet (Control diet) (Cat. N° 517802, Dyets) were used in this study. Diet composition is listed in Table 1.

### 2.3.1. Study 1: depletion/repletion protocol

One hundred twenty six (126) weaned BALB/c mice were randomly selected for the study, and divided into two groups of thirty-six (36) and ninety (90) mice of equal mean weights (Fig. 1A). The first group of mice ( $n = 36$ ) was fed with Control



**Fig. 1** – Feeding protocols with control diet containing 2 mg folic acid/kg of diet and certified folic acid free defined composition diet (FADD). **(A)** Study 1: Protocol of depletion/repletion model and **(B)** Study 2: protocol of prevention model. **FC:** Folate control group; **DC:** Deficient control group; **RC:** Repleted control group, mice fed with control diet containing 2 mg folic acid/kg of diet; **FEFM:** Folate enriched fermented milk group, mice fed with FADD + folate enriched fermented milk (FEFM); **CFM:** Control fermented milk group, mice fed with FADD + control fermented milk (CFM). **N =** total number of animals per group. **n =** number of animals/day/group. Time is expressed as days (d).

**Table 1 – Diets composition.**

Ingredient	Folate-free diet <sup>a</sup>	Control diet <sup>b</sup>
	Grams per kg	
L-Alanine	3.5	3.5
L-Arginine (free base)	11.2	11.2
L-Asparagine.H <sub>2</sub> O	6.82	6.82
L-Aspartic acid	3.5	3.5
L-Cystine	3.5	3.5
L-Glutamic acid	35	35
Glycine	23.3	23.3
L-Histidine (free base)	3.3	3.3
L-Isoleucine	8.2	8.2
L-Leucine	11.1	11.1
L-Lysine HCl	18	18
L-Methionine	8.2	8.2
L-Phenylalanine	11.6	11.6
L-Proline	3.5	3.5
L-Serine	3.5	3.5
L-Threonine	8.2	8.2
L-Tryptophan	1.74	1.74
L-Tyrosine	3.5	3.5
L-Valine	8.2	8.2
Dextrin	397	397
Sucrose	196.64	196.64
Cellulose	50	50
Corn oil (Stab. With 0.15% BHT)	100	100
Salt mix #210020	50	50
Vitamin mix #317759 (Folate free)	10	10
Choline chloride	2	2
Sodium acetate	8.1	8.1
Folic acid/Sucrose premix (5 mg/g)	–	0.4

Diets based on Clifford/Koury Folate deficient L-Amino acid rodent diet.

<sup>a</sup> Cat. N° 517812 (Dyets, Bethlehem, USA).

<sup>b</sup> Cat. N° 517802 (Dyets, Bethlehem, USA).

diet (folic control (FC group)) for 35 days (d). The remaining mice were fed with FADD for 14 d (depletion period) maintaining a group of mice (n = 36, depletion control, DC) fed with FADD throughout the study period (35 d). The 54 remaining mice were divided into three experimental groups, each containing 18 mice (depleted-repleted groups). These animals were fed with: Control diet (repletion control, RC group); FADD supplemented with FEFM (FEFM group), or FADD supplemented with CFM (CFM group). The last three experimental groups were fed for 21 days (repletion period) from day 15 of the depletion period.

Animal growth (live weight), food, and water intake were determined on a bi-daily basis. Blood and organ samples were withdrawn every 7 days throughout the experiment (35 days).

### 2.3.2. Study 2: prevention protocol

One hundred forty-four (144) weaned BALB/c mice were divided into four groups of thirty-six (36) mice of equal mean weights (Fig. 1B). One group of mice was fed with Control diet (folic control, FC), while other mice were fed with: FADD (depletion control, DC); FADD supplemented with FEFM (FEFM group), and FADD supplemented with control fermented milk (CFM group).

FEFM, CFM and diets were supplied *ad libitum* for 35 d.

Animal growth (live weight), food, and water intake were determined on a bi-daily basis. Blood and organ samples were withdrawn every 7 days throughout the experiment (35 days).

All animal protocols were pre-approved by the Animal Protection Committee of CERELA (CRL-BIOT-LT-20142A) and followed the latest recommendations of the Federation of European Laboratory Animal Science Associations and the Asociación Argentina para la Ciencia y Tecnología de Animales de Laboratorio. All experiments comply with the current laws of Argentina.

### 2.4. Blood and organ samples collection

When required, animals were anesthetized with an i.p. injection of weight ketamine (Holliday, Scott S.A., Buenos Aires, Argentina) – xylazine (Rompun, Bayer S.A., Buenos Aires, Argentina) to obtain a final concentration of 100 µg and 5 µg/kg body weight, respectively. Animals were bled by cardiac puncture. Blood was transferred into tubes with anticoagulant (ethylenediaminetetraacetic acid (EDTA) in a final concentration of 1.5 mg/mL of blood).

Blood smears were prepared immediately from samples taken with anticoagulant and were stained with May–Grünwald–Giemsa (Biopur Quimica, Buenos Aires, Argentina). These same samples were used for hematological studies. Red blood cells (RBC), total and differential leukocyte counts, hemoglobin (Hb), hematocrit (Htc), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC) and mean corpuscular hemoglobin (MCH) were performed using a Sysmex KX-21N Hematology Analyzer (Sysmex Corporation, Tokyo, Japan).

For preparation of whole-blood samples for erythrocytes folate analysis, an aliquot of blood (100 µL) containing anticoagulant was diluted in 9 volumes distilled water (900 µL) and incubated for 2 h at 37 °C to allow serum conjugase enzyme to convert folate polyglutamates released from the lysed erythrocytes to the assayable monoglutamate forms. These lysates were then heated at 100 °C, 5 min, and centrifuged (13,000 × g, 10 min).

For plasma samples, remaining blood containing anticoagulant was separated by centrifugation (1500 × g for 10 minutes) and diluted with assay buffer (0.1 M phosphate buffer, pH 6.8, containing 1.5% (w/v) ascorbic acid to prevent vitamin oxidation and degradation) as described previously (LeBlanc et al., 2010).

For organ samples, aliquots (0.5 g) of freshly excised organs (liver, spleen and kidneys which were weighed in order to determine organ weight ratios) were added to 9 volumes (w/v) of assay buffer, and homogenized. Samples were further processed as described previously (LeBlanc et al., 2010).

### 2.5. Folate determination

Folate determination was performed by a modified microbiological assay using *Lactobacillus casei* subsp. *raimosus* NCIMB 10463 as indicator strain as was previously described (Laiño et al., 2012).

A trienzymatic treatment as described previously by Iyer, Tomar, Kapila et al. (2009) and Iyer, Tomar, Singh, and Sharma (2009) was used before vitamin determination. α-amylase from *Aspergillus oryzae*, and protease from *Streptomyces griseus* (Sigma Chemical, St. Louis, MO, USA) were dissolved in distilled-deionized water at concentrations of 4 mg/mL and



filter-sterilized (0.22  $\mu\text{m}$ ). The endogenous folate in each enzyme was determined after folate conjugase treatment. Although the protease did not contain any measurable folate by microbiological assay,  $\alpha$ -amylase contained approximately 1.54 ng of folate per mg of solid enzyme. This endogenous folate in  $\alpha$ -amylase was subtracted for the final calculation of food folate content. Rat plasma, obtained from rats provided by Instituto de Estudios Biológicos (INSIBIO-CONICET-Universidad Nacional de Tucumán, Tucumán, Argentina), was used as a source of folate deconjugase enzyme as described previously (Aiso & Tamura, 1998).

Folate concentration was expressed as  $\mu\text{g/L}$  using a standard curve of folic acid HPLC grade (Fluka BioChemica, Buchs, Switzerland).

## 2.6. Homocysteine determination

Plasma homocysteine (Hcy) concentration was determined using the commercial Homocysteine EIA Reagent kit (Axis-Shield, Dundee, Scotland, UK) according to manufacturer's instructions. Protein-bound Hcy is reduced to free Hcy and enzymatically converted to S-adenosyl-L-homocysteine (SAH) in a separate procedure prior to the immunoassay.

## 2.7. Bacterial translocation

Microbial translocation to extra-gut organs was determined as follows. Briefly, liver, spleen, and kidney were aseptically removed, weighed and homogenized in 5.0 mL sterile 0.1% (w/v) peptone solution. Serial dilutions of each homogenate were plated in triplicate in the following media, MRS agar for enumeration of lactobacilli, McConkey agar for analysis of enterobacteria, and brain-heart infusion (BHI) agar for enumeration of anaerobic and aerobic bacteria. Plates were incubated for 48 h at 37 °C under aerobic and anaerobic conditions and number of colony forming units (CFU) were counted and expressed as CFU/g of organs.

## 2.8. Statistical analyses

The experimental protocol was performed in triplicate and no interactions between the 3 independent trials were observed. All values were expressed as means  $\pm$  standard deviations (SD). Statistical analyses were performed using Sigma Plot software (Systat Software Inc., Chicago, IL, USA). A factorial design (replicates-treatment) was used for the experiments. Comparisons were done with an analysis of variance (ANOVA) general linear model followed by Tukey's *post hoc* test, and a *p* value <0.05 was considered significant.

# 3. Results

## 3.1. Plasma, whole blood and tissues folate concentrations

### 3.1.1. Study 1

The first phase of this experiment involved the induction of moderate folate deficiency by feeding mice with folic acid

deficient diet (FADD) for 14 days. Plasma (Fig. 2A) and kidney folate concentrations (Supplementary Table S1A) were found to be sensitive indicators of folate status. Mice fed with FADD had significantly lower ( $p < 0.05$ ) plasma, whole blood (Fig. 2C) (88 and 11.2% of decrease were observed respectively), and kidney folate concentrations (data not shown) at the end of the depletion phase (8.5 and 3-fold decreases were observed respectively) than those of mice fed with control diet (FC group). At the end of repletion period (35 days), significant differences between experimental groups were observed. Animals that received the milk fermented with folate-producing strains (FEFM) showed a significant increase in plasma (5-fold) (Fig. 2A) and whole blood (Fig. 2C) folate concentrations compared with depleted mice, but slightly lower than those that receive the control diet (RC showed a 7-fold increase) in plasma folate concentration, and similar in whole blood folate level. The administration of the control fermented milk (CFM) also improved plasma levels of folate but values were significantly lower than those that received FEFM.

Regarding the concentration of folate in organs, only FADD supplemented with 2 mg of folic acid (RC) administrated to depleted animals was able to partially restore kidney folate concentrations while the other diets had no effect on the recovery of tissue folate levels during repletion phase (Supplementary Table S1A).

### 3.1.2. Study 2

Diets administered to animal groups after 35 days influenced plasma folate status in mice (Fig. 2B). No significant differences were observed in plasma folate levels of animals fed with FEFM compared to those receiving Control diet (FC group), whereas significantly lower concentrations were observed in CFM group compared to FEFM group (Fig. 2B).

Furthermore, depleted animals showed an approximate reduction of one-half fold in total folate levels in spleen and kidney (no significant differences in folate concentrations were observed in liver) (Supplementary Table S1B).

## 3.2. Plasma homocysteine levels

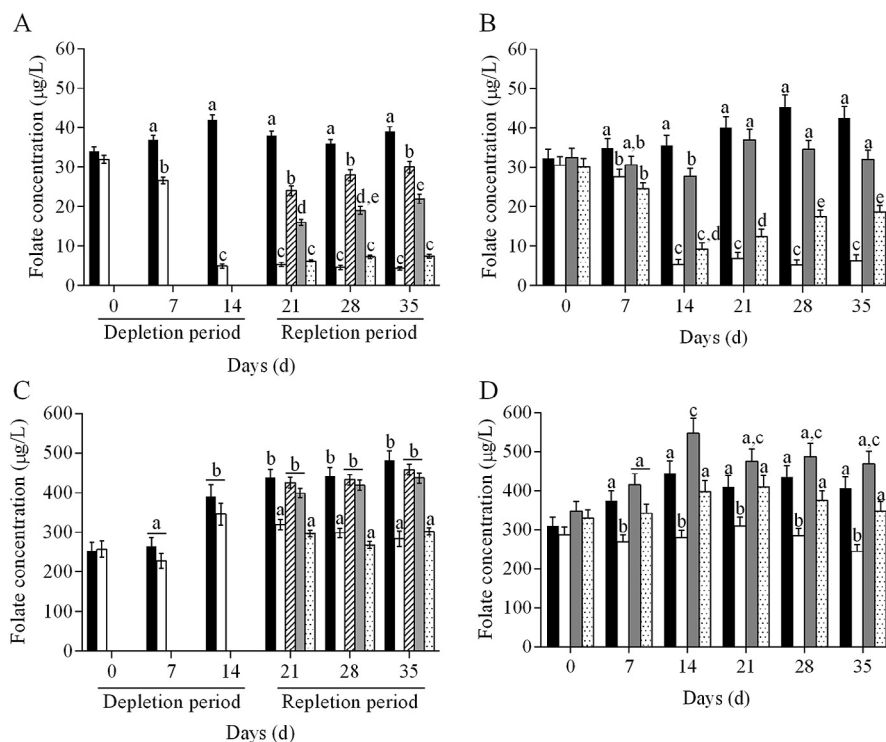
### 3.2.1. Study 1

Plasma homocysteine concentrations were unchanged in the FC group with values below 10  $\mu\text{M}$  (Fig. 3A) during both depletion (14 d) and repletion (21 days) phases (Fig. 3A). Following 14 d of consumption of FADD (DC group), plasma homocysteine concentrations were significantly elevated, and increased significantly (8-fold relative to FC group) after 35 d on the diet.

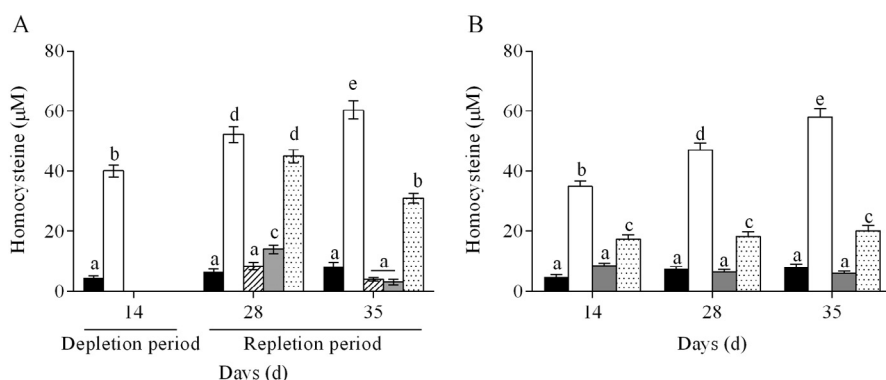
Supplementation with folic acid or FEFM to FADD (RC and FEM groups, respectively), and administrated to mice for 21 d (repletion phase) produced a significant reduction of plasma homocysteine levels (Fig. 3A) reaching similar values than those of control group (35 d). In both RC and FEFM groups, a great decrease in plasma homocysteine concentration with increasing folate repletion time was observed (Fig. 3A). Even though CFM group reduced the homocysteine levels, it was unable to restore homocysteine status similar to RC group (Fig. 3A).

### 3.2.2. Study 2

FADD supplemented with FEFM, and administrated to mice from the first day of prevention protocol, prevented increase



**Fig. 2 – Plasma (A, B) and whole blood (C, D) folate concentration (µg/L). (A, C) Study 1: Depletion/repletion model; and (B, D) Study 2: Prevention model. FADD: certified folic acid free defined composition diet; FC: Folate control group; DC: Deficient control group; RC: Repleted control group, mice fed with control diet containing 2 mg folic acid/kg of diet; FEFM: Folate enriched fermented milk group, mice fed with FADD + folate enriched fermented milk (FEFM); CFM: Control fermented milk group, mice fed with FADD + control fermented milk (CFM). Time is expressed as days (d). Results are expressed as the means ± standard deviation (SD) of n = 8. <sup>a-c</sup>Means with different letters differ significantly (p < 0.05). Black bars represent FC group, empty bars represent DC group, diagonally striped bars represent RC group, dark gray bars represent FEFM group, and dotted bars represent CFM group.**



**Fig. 3 – Plasma homocysteine concentration (µM). (A) Study 1: Depletion/repletion model, and (B) Study 2: Prevention model. FADD: certified folic acid free defined composition diet; FC: Folate control group; DC: Deficient control group; RC: Repleted control group, mice fed with control diet containing 2 mg folic acid/kg of diet; FEFM: Folate enriched fermented milk group, mice fed with FADD + folate enriched fermented milk (FEFM); CFM: Control fermented milk group, mice fed with FADD + control fermented milk (CFM). Time is expressed as days (d). Results are expressed as the means ± SD of n = 8. <sup>a-e</sup>Means with different letters differ significantly (p < 0.05). Black bars represent FC group, empty bars represent DC group, diagonally striped bars represent RC group, dark gray bars represent FEFM group, and dotted bars represent CFM group.**

of plasma homocysteine levels, reaching similar values to FC group after 35 d (Fig. 3B). The DC and CFM groups had higher plasma homocysteine concentrations (6.7 and 2.7-fold, respectively) compared to FC group (Fig. 3B).

### 3.3. Hematological studies

Results from the hematological assessment in a folate depletion/repletion mouse model or in a prevention model are provided in Table 2. There were no statistically significant differences between the different experimental groups as compared to control for most of the parameters measured in the two models evaluated. Only, the mean corpuscular volume (MCV) slightly increased in folate-depleted mice (CD) compared with control group in the model system of folate depletion/repletion and in the prevention model (Table 2). However, this parameter showed a transiently tendency to decrease in the RC group reaching the FC group values at the end of repletion phase, although the differences observed between the experimental groups (RC, FEFM and CFM) were not significant.

Blood smears from folate-deficient mice did not show anisocytosis (data not shown), or any other morphological modification in erythrocytes, leukocytes, or platelets (data not shown).

### 3.4. Growth parameters

Feeding efficiencies, growth rates, final growth weights, and relative organ weight were not significantly different among the

different experimental groups in the folate-deficiency/repletion and prevention models (data not shown).

### 3.5. Microbial translocation

No microbial growth in extra-gut organs was observed in any of the experimental groups (data not shown) which demonstrates that FEFM did not cause any adverse effects on the intestinal cell wall integrity.

## 4. Discussion

Folate deficiency is associated with anemia and neural tube defects which constitute important public health problems. Additionally, low folate levels are linked with increased homocysteine concentration (Boushey et al., 1995; Homocysteine Lowering Trialists' Collaboration, 1998). In turn, low homocysteine levels have been associated with colorectal cancer (Cravo et al., 1994; Giovannucci et al., 1993), cognitive decline (Durga et al., 2007; Tucker, Qiao, Scott, Rosenberg, & Spiro, 2005), cardiovascular disease, among other undesirable effects. Folate supplementation may be particularly important in ensuring adequate folate status in vulnerable populations. Although folates are widely distributed in foods, their concentration is low in most products. Therefore, fermented foods bio-enriched with "natural" or "bio-produced" folates constitute a promising alternative for people to reach the recommended daily

**Table 2 – Hemogram.**

A											
Group	P <sup>a</sup>	Leukocyte	Lymphocyte	Neutrophil	RBC	Hb	Htc	MCV	MCH	MCHC	Platelet
		10 <sup>9</sup> cell/L	10 <sup>9</sup> cell/L	10 <sup>9</sup> cell/L	10 <sup>12</sup> cell/L	g/L	%	fL	pg	g/L	10 <sup>9</sup> /L
FC	Dp	6.0 ± 0.3	5.1 ± 0.2	0.71 ± 0.04	6.52 ± 0.51	128 ± 7	45 ± 3	54.4 ± 2.6	15.7 ± 0.6	289 ± 17	1124 ± 50
	Rp	6.7 ± 0.4	5.6 ± 0.3	1.01 ± 0.05	6.83 ± 0.45	129 ± 5	43 ± 2	55.2 ± 2.6	16.7 ± 0.3	308 ± 16	1352 ± 81
DC	Dp	6.4 ± 0.3	5.5 ± 0.2	0.89 ± 0.05	6.85 ± 0.29	124 ± 4	41 ± 3	55.8 ± 2.4	16.8 ± 0.5	298 ± 11	1183 ± 95
	Rp	6.6 ± 0.2	5.7 ± 0.4	0.91 ± 0.04	6.24 ± 0.34	126 ± 7	40 ± 2	60.7 ± 2.3	17.2 ± 0.8	287 ± 17	1210 ± 54
RC	Dp	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Rp	6.1 ± 0.4	5.3 ± 0.2	0.79 ± 0.04	6.65 ± 0.34	124 ± 7	41 ± 4	54.8 ± 2.4	16.1 ± 0.2	310 ± 19	1102 ± 21
FEFM	Dp	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Rp	6.2 ± 0.2	5.3 ± 0.4	0.81 ± 0.04	6.97 ± 0.46	132 ± 8	47 ± 4	55.2 ± 2.6	16.9 ± 0.6	294 ± 15	1284 ± 45
CFM	Dp	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Rp	6.5 ± 0.3	5.6 ± 0.3	0.89 ± 0.06	6.58 ± 0.44	126 ± 7	44 ± 6	54.4 ± 2.3	16.2 ± 0.2	298 ± 16	1159 ± 37
B											
Group		Leukocyte	Lymphocyte	Neutrophil	RBC	Hb	Htc	MCV	MCH	MCHC	Platelet
		10 <sup>9</sup> cell/L	10 <sup>9</sup> cell/L	10 <sup>9</sup> cell/L	10 <sup>12</sup> cell/L	g/L	%	fL	Pg	g/L	10 <sup>9</sup> /L
FC		6.7 ± 0.4	5.8 ± 0.2	0.72 ± 0.03	7.12 ± 0.47	128 ± 8	42 ± 3	54.7 ± 2.7	16.8 ± 0.5	307 ± 23	1230 ± 36
DC		6.6 ± 0.3	5.6 ± 0.3	0.82 ± 0.04	6.94 ± 0.46	125 ± 6	45 ± 3	60.4 ± 2.3	16.8 ± 0.5	281 ± 15	1263 ± 28
FEFM		6.1 ± 0.4	5.2 ± 0.3	0.79 ± 0.05	6.55 ± 0.43	124 ± 5	41 ± 3	54.8 ± 2.1	16.1 ± 0.2	310 ± 24	1240 ± 34
CFM		6.4 ± 0.2	5.5 ± 0.4	0.91 ± 0.04	6.72 ± 0.54	128 ± 6	45 ± 4	55.7 ± 2.7	16.2 ± 0.3	289 ± 18	1278 ± 21

(A) Study 1: Depletion/repletion model and (B) Study 2: Prevention model, after 35 d at the end of feeding period.

RBC: red blood cells; Hb: hemoglobin; Htc: hematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; FADD: certified folic acid free defined composition diet; FC: Folate control group; DC: Deficient control group; RC: Repleted control group, mice fed with control diet containing 2 mg folic acid/kg of diet; FEFM: Folate enriched fermented milk group, mice fed with FADD + folate enriched fermented milk (FEFM); CFM: Control fermented milk group, mice fed with FADD + control fermented milk (CFM).

<sup>a</sup> P: feeding periods, Dp: at the end of depletion period (14 d), Rp: at the end of repletion period (35 d). ND: Not determined. Results are expressed as mean ± SD of n = 8.

intakes through the ingestion of foods that normally form part of their diets. Similar vitamin bio-enrichment strategies using lactic acid bacteria have been previously described by others (Burgess, Smid, & van Sinderen, 2009; Capozzi et al., 2011; Capozzi, Russo, Dueñas, López, & Spano, 2012; LeBlanc et al., 2006); however, the biological activity of the produced vitamins was not evaluated, except for one study where a vitamin B<sub>12</sub> strain was used to ferment soymilk and evaluated in an animal model (Molina, Médici, Font de Valdez, & Taranto, 2012). The present study was conducted to evaluate the effectiveness of a novel fermented milk, manufactured with folate-producing lactic acid bacteria in a folate depletion/repletion mouse model and also in a folate prevention model. Using the former model, the temporal changes in folate status during both the depletion and repletion phases were determined, with the primary goal of determining the potential for plasma folate concentrations to serve as biomarker for folate status. Our data support the use of 14 days as an adequate folate depletion period since it produced a marked folate deficiency state characterized by nadirs in plasma and kidney folate concentrations, and significantly high levels of plasma homocysteine compared with those fed a control diet. Plasma homocysteine is well documented by its sensitivity to folate status (House, Jacobs, Stead, Brosnan, & Brosnan, 1999), due to the role that this vitamin plays in homocysteine re-methylation to methionine through methionine synthase acting folate as methyl donor. Factors that perturb the steps in homocysteine metabolic pathways, such as folate deficiency, can cause an increase in cellular homocysteine levels and lead to its elevation in blood (Woo, Prathapasinghe, Siow, & Karmin, 2006).

In this study, the response variables, low plasma folate levels and hyperhomocysteinemia, are appropriate to justify the use of mice to study folate deficiencies.

Folate deficiency induced during the depletion phase did not result in the appearance of skin lesions or hair loss in folate-depleted mice even after 35 days of depletion. This phenomenon was not observed by other researchers in a folate-depletion and repletion rat model (Martin, 1999); however in this latter study animals had severe non-reversible folate deficiencies which are not the case in the current study. Also, only the mean corpuscular volume among blood parameters slightly increased as hematological response to folate deficiency in mice.

In addition, degree of folate deficiency allowed ensuring prompt recovery of mice during the repletion phase, minimizing the effects of a severe depletion. In our mouse model, folate and homocysteine levels where the biological markers that varied the most in response to the different folate intakes of the animals. Consequently, kidney folate levels were considered to be a poor indicator of folate status to distinguish between folate-depleted and repleted mice as were reported by other authors (Clifford, Heid, Müller, & Bills, 1990; O'Leary & Sheehy, 2001) in rat model systems.

In this study, plasma folate and homocysteine levels were considered appropriate indicators to measure even relatively subtle changes in folate status of mice during the repletion phase.

Daily intake of fermented milk enriched with bio-produced folates (190 µg/L) added to the folate deficient diet produced a significant increase (ca. 90%) of plasma folate levels, and homocysteine reduction ( $3.2 \pm 0.9$  µM) in mice experimental group

reached similar values as the control group, with folic acid concentration in control diet (2 mg of folic acid/kg of diet) being 10 times higher than in bio-enriched fermented milk. In comparison, a slight increase in plasma folate level was observed in the control fermented milk group, clearly indicating that there was no significant folate contribution by this product obtained with folate-consuming strains. Thus, folate generated by *S. thermophilus* CRL803, *S. macedonicus* CRL415 and *L. bulgaricus* CRL871 during milk fermentation had a beneficial effect on plasma folate and homocysteine levels. These results are in agreement with a study by LeBlanc et al. (2010) who studied the bioavailability of different folates produced by engineered *Lactococcus lactis* strains using a rodent depletion-repletion bioassay. They observed that the folates produced by the engineered strains were able to compensate the folate depletion in the diet and showed similar bioavailability in terms of increased folate concentration in organ and blood samples in animals receiving folate-producing strains compared with commercial folic acid normally used for food fortification. However, there are limited data related with naturally folate-enriched fermented products elaborated with folate-producing wild strains able to restore folate status in folate-depleted and repleted mice and prevent folate deficiency in mice.

The fermented milk evaluated in this study contains elevated amounts of folate produced by food-grade bacteria and was also able to prevent folate deficiency when administered as a supplement to folate-free diet. Collected data showed that the indicators, plasma folate and homocysteine levels, were similar in mice group fed with FEFM or control diet. Many authors demonstrated that bio-produced folates from microbial fermentation are more effective than folic acid due to the first one being easily absorbed and transported in the organism (Dudeja, Torania, & Said, 1997; Said et al., 2000; Zhao, Diop-Bove, Visentin, & Goldman, 2011). In addition, 5-methylTHF and 10-formylTHF are commonly the folate forms synthesized by bacteria and the most used for cellular metabolic reactions (Laiño, Savoy de Giori, & LeBlanc, 2013). Moreover, fermented milk is considered an adequate matrix among dairy products for folate enrichment because folate binding proteins of milk improve folate stability and the bioavailability of 5-methylTHF (Jones & Nixon, 2002). These observations would explain the effectiveness of oral administration of fermented milk rich in natural folate to prevent readily folate deficiency avoiding adverse health outcomes of the population associated with low folate status.

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## 5. Conclusions

To the best of our knowledge, this is the first report demonstrating that a naturally folate bio-enriched fermented milk, elaborated with folate-producing starter cultures, is not only effective in improving folate status but can also prevent folate deficiency. Thus, folate bio-enriched fermented milk can be considered a viable, economic, useful, and effective alternative to folic acid fortification with no side effects. Consumers also would obviously benefit from this type of product because they could increase their folate intakes while consuming foods that form part of their normal diets.



## Acknowledgments

This work was financially supported by the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), and the Consejo de Investigaciones de la Universidad Nacional de Tucumán (CIUNT)

## Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.jff.2015.04.055.

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