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Effect of indigenous lactic acid bacteria isolated from goat milk and cheeses on folate and riboflavin content of fermented goat milk

Fabiana Fernanda Pacheco Da Silva [a], Vanessa Biscola [a], Jean Guy LeBlanc [b, *, 1], Bernadette Dora Gombossy de Melo Franco [a, 1]

[a] Food Research Center, Faculty of Pharmaceutical Sciences, University of São Paulo, Avenida Professor Lineu Prestes 580, São Paulo, SP, Brazil
[b] Centro de Referencia para Lactobacilos (CERELA-CONICET), Chacabuco 145, Tucumán, Argentina

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ABSTRACT

The aim of the present study was to isolate riboflavin- and folate-producing lactic acid bacteria (LAB) from raw goat milk and cheeses, identify them and evaluate their capability to increase the content of these vitamins in fermented goat milk, envisaging potential application for development of novel bio-enriched goat milk products. From 179 LAB isolates obtained, 151 (84%) were capable to produce at least one of these vitamins. The average production of total folate and riboflavin in vitamin-free media was 138 ng/ml and 364 ng/ml, respectively. Based on RAPD-PCR and 16S rDNA sequencing, 19 different genetic profiles were obtained and 7 species were identified, with predominance of *Streptococcus thermophilus* (7), *Weissella paramensenteroides* (6), and *Lactococcus lactis* (4). Seven isolates that produced folate and riboflavin above the average were tested for vitamins production in UHT goat milk. Five isolates were capable to increase four to six fold the original amount of folate in the milk in 24 h. Folate content in milk fermented with *Lc. lactis* FP368 for 24 h was 313 ng/ml that could provide 19% of the recommended daily intake of this vitamin. In addition, *St. thermophilus* FP268 increased the folate concentration in the milk almost four fold in only 6 h.

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1. Introduction

There is an increasing tendency worldwide for the consumption of goat-milk derived products because of their organoleptic and nutritional properties (Park, Juárez, Ramos, & Haenlein, 2007; Schirru et al., 2012). The contribution of goat milk to local economies and nutrition of certain populations is indisputable in various countries, especially in certain regions of the Mediterranean, Middle Orient, Oriental Europe and South America (Ribeiro & Ribeiro, 2010). The physical-chemical characteristics of goat milk allow their use in a wide range of products besides fluid milk, that can be consumed either raw, pasteurized or UHT, such as cheese, butter, yogurt, ice-cream and sweets, amongst others.

Lactic acid bacteria (LAB) are a group of Gram-positive, catalase-negative microorganisms with similar metabolic and physiological characteristics. In general, LAB are classified by FDA as being "generally recognized as safe" or by the EFSA as having the "qualified presumption of safety" designation because of their long history of use in the elaboration of fermented foods. LAB are frequently used as starter or adjunct cultures because of their capacity to increase the safety of foods, provide texture and flavor, and produce beneficial compounds such as organic acids and vitamins (Carr, Chill, & Maida, 2002; Salvetti, Torriani, & Felis, 2012).

Although most LAB are auxotrophic for vitamins, there is increasing evidence that certain strains have the capacity to produce specific water soluble vitamins such as those found in the B-group (Capozzi, Russo, Dueñas, López, & Spano, 2012; LeBlanc et al., 2011, 2014, 2013; LeBlanc, Savoy de Giori, Smid, Hugenholtz, & Sesma, 2007; LeBlanc, Taranto, Molina, & Sesma, 2010). The most studied vitamins that are produced by LAB are folates and riboflavin because of their importance in human health and the frequency of deficiencies found in populations world-wide even in countries where obligatory fortification programs exist (ENNyS, 2007).

Riboflavin (vitamin B2) is an essential component of basal cellular metabolism and is the precursor of the enzymes flavin mononucleotide (FAD) and flavin adenine dinucleotide (FMN) that

* Corresponding author. CERELA-CONICET, Chacabuco 145, San Miguel de Tucumán, Tucumán, C.P. 4000, Argentina.
E-mail addresses: leblanc@cerela.org.ar, leblancjeanguy@gmail.com (J.G. LeBlanc).
[1] Authors participated equally in this work.

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are electron acceptors and involved in many oxidation–reduction reactions (Powers, 2003). According to the World Health Organization, the recommended dietary allowance (RDA) for humans is between 0.9 and 1.6 mg (FAO/WHO, 2002). Although present in a wide variety of foods, deficiency of riboflavin (arriboflavinosis) or sub-clinical deficiencies are present in both developing and highly industrialized countries (O'Brien et al., 2001). Folates (vitamin B9) are involved in many metabolic functions such as replication, repair and methylation of DNA and synthesis of nucleotides, amino acids and vitamins (LeBlanc et al., 2010). Because folate deficiencies can cause serious health problems, such as defects in the formation of the neural tube (NTD), megaloblastic anemias and are correlated with Alzheimer's and coronary diseases and colon and breast cancer, many countries have adopted mandatory fortification of staple foods with folic acid. However, many countries have not adopted national fortification programs because of possible unwanted side effects associated with an excess consumption of folic acid (pteroylglutamic acid) that can mask early hematological manifestations of vitamin B12 deficiency (Morris & Tangney, 2007). Since natural folates, such as 5-methyltetrahydrofolate, that are normally found in foods and sometimes produced by microorganisms do not mask B12 deficiency (Scott, 1999), this folate form would be a more efficient and secure alternative than supplementation with folic acid (Lamers, Prinz-Langenohl, Brämswig, & Pietrzik, 2006).

Previous studies have shown that the concentration of riboflavin and folates is frequently increased in certain fermented milk products such as yogurts, buttermilk and cheeses due to the production of these vitamins by indigenous LAB (LeBlanc et al., 2011). For this reason, these authors have proposed that the adequate selection of vitamin producing LAB could be an economically feasible alternative to chemical fortification and production of high vitamin containing products. The isolation of LAB from the ecological niche where these are to be used is logical since these microorganisms are adapted to the specific food matrix, increasing their capability to grow and produce beneficial compounds.

There are no published reports on the occurrence of vitamin-producing LAB in goat milk derived products. Thus the objective of the current study was to isolate folate and riboflavin producing LAB from raw goat milk and goat cheeses, identify them and test their capability to increase the content of these vitamins in fermented goat milk, envisaging their potential application for elaboration of novel goat milk products bioenriched with the natural form of these vitamins.

2. Materials and methods

2.1. Isolation of LAB from goat milk and cheeses

Raw goat milk samples (n = 47) were obtained from the School of Veterinary Medicine and Animal Science, University of Sao Paulo, Brazil, and from Sitio Rekantinho goat dairy farm, located in the city of Ibiuna, SP, Brazil. Goat cheese samples (n = 5) were obtained from commercial establishments in the city of Sao Paulo, Brazil.

To isolate potential vitamin producing lactic acid bacteria, the technique described by Schirru et al., 2012 was slightly modified. In the case of goat milk, samples were serially diluted with 0.1% (w/v) peptone water. For cheese samples, a 25 g portion was homogenized with 225 ml 0.1% (w/v) peptone water using a stomacher and serial dilutions were also made. Diluted samples were then plated on MRS (Oxoid, Basingstoke, UK) and M17 (Difco, NJ, USA) containing 10% (w/v) lactose agar plates and incubated at either 30, 37 or 42 °C during 48 h under anaerobic conditions, using Anaerogen sachets (Oxoid, Basingstoke, UK). Five colonies were randomly selected from each plate and inoculated in MRS broth at 37 °C during 24 h. These isolates were then plated again on MRS agar to ensure purity and presumable LAB isolates (Gram-positive, catalase-negative, non-motile rods and cocci) were selected for further characterization and identification. Isolates fulfilling these requirements were stored at −20 °C in glycerol (20% v/v) for further analysis.

2.2. Selection of presumptive folate producing LAB

After activation of the isolates in MRS broth at 37 °C for 24 h, the cultures were washed three times with saline solution (0.85% w/v NaCl), resuspended in this solution to the original culture volume, and used to inoculate 4% v/v folate-free Folic Acid Casei Medium (FACM, Difco, NJ, USA). The cultures were incubated without agitation at 37 °C for 24–72 h, and submitted to the same washing-resuspension procedure. The resulting solution was used to inoculate at 2% v/v fresh FACM. The cultures presenting growth, indicated by increased visible turbidity, were submitted to this last step seven times. Those strains that did not grow in FACM were tested again for growth in FACM added of vitamin, and those presenting growth in this medium were discarded. After the 7th passage, two samples of the cultures were taken after 24 h incubation to determine the concentration of extra- and intra-cellular folate, as described previously (Juarez del Valle, Laiño, Savoy de Giori, & LeBlanc, 2014; Laiño, LeBlanc, & Savoy de Giori, 2012). Briefly, samples of LAB-containing FACM (500 μL) were mixed with equal amount of the protecting buffer (0.1 mol/L phosphate buffer, pH 6.8, containing 1.5% (m/v) ascorbic acid to prevent vitamin oxidation and degradation), and immediately centrifuged at 5000× *g* for 5 min (Sigma 1–14k, Ostrode, Germany). The supernatant corresponded to extracellular folate sample and the pellet, resuspended in 500 μL of protecting buffer, corresponded to intracellular folate sample. Both samples were then boiled (100 °C) for 5 min, centrifuged for 6 min at 10 000× *g* (Sigma 1–14k, Ostrode, Germany), and stored at −70 °C until used for folate determinations.

In culture media samples, total vitamin concentrations were calculated summing the intra- and the extra-cellular vitamins concentrations. It is important to clarify that extra-cellular vitamin concentrations (from the supernatant) might be a consequence of cellular lysis or leakage and not necessarily secretion of the vitamins to the culture media.

2.3. Selection of presumptive riboflavin producing LAB

The procedure was the same described for folate, except that cultures were grown in riboflavin-free Riboflavin Assay medium (RAM, Difco). After washing, the resulting culture suspensions were used to inoculate (4% v/v) fresh RAM, and those cultures showing increased turbidity were submitted to the incubation and washing-resuspension steps four times. The strains that did not grow in RAM were discarded. After the forth incubation, two samples of the cultures were taken to determine the concentration of extra- and intra-cellular riboflavin, as described previously (Juarez del Valle et al., 2014; Laiño et al., 2012). The concentration of riboflavin in the RAM medium was determined mixing 500 μL of the cultures with 500 μL of 1% v/v acetic acid. The mixtures were immediately centrifuged for 5 min at 10 000× *g*. The supernatants were collected for determination of extracellular riboflavin and the pellets were resuspended in 500 μL of 1% v/v acetic acid for determination of intracellular riboflavin. Both samples were heated at 100 °C for 5 min, centrifuged for 6 min at 10 000× *g*, and stored at −70 °C.

2.4. Genetic grouping and identification of the LAB isolates

All presumptive folate and riboflavin producing isolates were submitted to PCR for screening of those belonging to genus *Enterococcus*. Detection was based on the amplification of the *tuf* gene, that codes for the elongation factor Tu present only in this genus (Ke et al., 1999). Isolates that possessed the *tuf* gene were excluded from the study. The identification of the remaining isolates was done in a two-step process. First, they were submitted to random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) for genetic grouping, using the primers described previously (Van Reenen & Dicks, 1996). In the next step, one isolate from each genetic group was selected and submitted to identification to species level and identified by comparing the sequence of the 16S rDNA PCR amplification product with those published in the NIH database. Amplifications were done in 50 μL reaction volume, containing 1.4 μL of each primer (OPL 02: 5′-TGGGCGTCAA-3′, OPL 14: 5′-GTGACAGGCT-3′ and OPL 20 5′-TGGTGGACCA-3′), 2 μL of DNA (20 μg/μL), 25 μL of GoTaq® Green Master Mix 2× [(2× Green GoTaq® Reaction Buffer, pH 8,5; 400 μM dATP; 400 μM dGTP; 400 μM dCTP; 400 μM dTTP and 3 mM $MgCl_2$ (Promega, USA)]. The volume (50 μL) was completed with sterile Milli-Q water. Amplification was done in a Veriti® 96-Well Thermal Cycler (Applied Biosystems, USA), and consisted of an initial denaturation step at 94 °C for 5 min, followed by 45 cycles composed by denaturation at 94 °C for 1 min, annealing at 28C for 1 min and extension at 72 °C for 2 min. The amplified material was submitted to electrophoresis in 1.5% agarose gels in 0.5 TBE buffer at 70 V for 157 min. Amplified fragments were visualized using ethidium bromide (0.5 μg/ml) under UV light. PCR products were purified using a commercial kit (QIAquick PCR Purification Kit, Qiagen, Brazil) and sequenced at the Human Genome and Stem Cell Research Center (University of São Paulo, SP, Brazil) and compared to sequences in GenBank using BLAST (Basic Local Alignment Research Tool) (http://www.ncbi.nlm.nih.gov/GenBank/).

2.5. Elaboration of fermented milk

Seven vitamins producing isolates were selected and used to produce fermented goat milk that would be naturally enriched with folates or riboflavin. The strains were activated in MRS broth, incubated at 37 °C for 18 h, washed 3 times with saline solution and used to inoculate 100 ml of Ultra High Temperature (UHT) treated goat milk at an initial concentration of approximately 1.0×10^5 colony forming units (CFU) per ml. The inoculated milk was incubated at 37 °C without agitation for 24 h, and samples were taken at times 0, 6, 8 and 24 h, in order to determine viable cell counts (CFU/ml), pH values, and folate and riboflavin concentrations. Viable cells counts were performed by serial dilutions and plating in MRS agar and incubation at 37 °C for 48 h under anaerobiosis (Anaerogen 2.5L Sachets, Basingstoke, Oxoid). The pH value was determined using a digital pH meter (PHS− 3BW, Bell Engineering, USA).

2.6. Vitamin quantification in the fermented goat milk

For determination of the concentration of folate in the milk, 500 μl of samples were mixed with the protection buffer as described above, boiled for 5 min at 100 °C, centrifuged at 10 000× *g* (Sigma 1–14k, Ostrode, Germany) for 10 min and the supernatants submitted to a tri-enzymatic treatment, to release folates sequestered by milk proteins (Iyer, Tomar, Singh, & Sharma, 2009). In the first step, 500 μl of supernatants were mixed with 100 μl of amylase from *Aspergillus oryzae* (4 mg/ml from Sigma–Aldrich) and incubated for 2 h at 37 °C, followed by inactivation by boiling during 5 min. After cooling, protease (100 μl) from *Streptomyces griseaus* (4 mg/ml from Sigma–Aldrich) was added and the samples were incubated for extra 2.5 h at 37 °C. After inactivation by boiling during 5 min and centrifugation at 500× *g* for 10 min, the supernatants were transferred to a new tubes and 100 μl of human plasma (Sigma–Aldrich) were added to deconjugate the glutamic chains of folates. Samples were incubated at 37 °C for 3 h, boiled for 5 min and centrifuged at 10 000× *g* for 5 min. Supernatants were conserved at −20 °C until used for vitamins quantification.

Folate concentration in the fermented milk samples was determined using the microbiological assay described by Laiño et al., 2012, using *Lactobacillus* (*L.*) *rhamnosus* NCIMB 10463 as the indicator organism. Briefly, samples of different concentrations of HPLC-grade folic acid (Fluka BioChemica, Sigma–Aldrich, Switzerland) were placed with the indicator strain and incubated statically during 48 h at 37 °C in 96-well sterile microplates containing the folate-free medium (Difco, USA). The optical density was read at 595 nm (OD595) using a microplate reader (Multiskan™ FC Microplate Photometer, Thermo Scientific™, Waltham, USA). The folate concentration of the samples was determined by comparing the OD obtained for treated samples with that obtained for the standard curve prepared using commercial folic acid.

Riboflavin concentrations were determined in the same manner but using *L. rhamnosus* ATCC 7469 as the indicator strain, grown in the riboflavin-free medium.

2.7. Statistical analysis

The results were obtained from three independent experiments and each data point was measured in triplicate ($n = 9$). 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 IL, USA) using ANOVA GLM followed by a Tukey's test. Differences were considered statistically significant at $p < 0.05$.

3. Results and discussion

Despite having been isolated from a wide range of ecological niches, such as fermented dairy products (Laino, Juarez del Valle, Savoy de Giori, & LeBlanc, 2014; Laiño et al., 2012), oat bran and rye sourdough (Kariluoto et al., 2006, 2010; Korhola et al., 2014), idli (Iyer, Singhal, & Ananthanarayan, 2013), other cereal products (Capozzi et al., 2011, 2012; Russo et al., 2014) and human gastrointestinal tract (LeBlanc et al., 2013), this is the first report on vitamin B-Group producing LAB strains isolated from goat milk and cheeses. In this study, 151 among 179 Gram positive and catalase negative isolates obtained from raw goat milk and goat cheeses were capable to grow in culture media in the absence of folate and/or riboflavin. Among these isolates, 72 came from goat milk and 79 from cheeses. As performed in the above mentioned previous trials, growth of the isolates was defined as an increase in turbidity in the vitamin-free medium. OD of the cultures was not measured at this stage, because the strains were just checked for capability to grow in the media. Even though previous trials have shown that different species and even different strains of the same species increased OD_{580nm} at different values (unpublished results), increased OD not necessarily correlated with increased vitamin production. In order to confirm that the isolates did not grow in these media due to the lack of vitamins, pure folate or riboflavin were added to the media. After the addition of the exogenous vitamin to the media, all strains were able to grow (data not shown) demonstrating that they did not have the capability to produce vitamins in sufficient amounts to support their own growth.

The observed frequency of presumptive folate producers (84%) is much higher than the 39% reported previously (Laino, LeBlanc, &

Savoy de Giori, 2012) among LAB isolated from yoghurt. In terms of riboflavin, only 8% (n = 15) of the isolates presented presumptive capacity to grow in the absence of this vitamin (4 from goat milk and 11 from cheese). This frequency is lower than observed previously for isolates from cow's milk, where 23% of the strains were able to produce vitamin B2 (Juarez del Valle et al., 2014).

Despite possessing potential health-promoting and technological properties, many members of the genus *Enterococcus* have detrimental characteristics such as antibiotic resistance and virulence factors and there are even examples of fermented food products containing enterococci that had to be withdrawn from the market due to consumers' awareness of possible health risk (Gomes, Franco, & Martinis, 2010). For this reason, this study included a screening test to exclude the folate and riboflavin producing isolates that presented the *Enterococcus*-specific *tuf* gene. With this test, 43 isolates that were presumptively capable to produce folate and 7 isolates that were presumptively capable to produce riboflavin were excluded from the study.

Based on the RAPD-PCR profiles, 19 genetically distinct groups were obtained. The 16S rDNA identification showed that the most frequent folate and riboflavin producing isolates were *Streptococcus thermophilus* (7 strains), *Weissella paramesenteroides* (6 strains) and *Lactococcus lactis* subsp *lactis* (4 strains) (Table 1). *Lactobacillus helveticus*, *Streptococcus luteniensis*, *Streptococcus infantarius* subsp. *infantarius and Bacillus* sp. were also detected (one isolate each). Two genetic groups contained two different species.

The following step was to confirm if the 21 strains were able to produce significant amounts of folate or riboflavin since at this point it was only shown that they did not need these vitamins for growth in the vitamin-free media. Result presented in Table 1 indicate that all strains produced folates *de novo* (since no folate derivatives are present in the folate free medium), at concentrations between 23 and 389 ng/ml. These values are higher than those reported by other authors for cow's milk strains, which produced 14 ng/ml (Gangadharan, Sivaramakrishnan, Pandey, & Madhavan Nampoothiri, 2010) to 95 ng/ml (Laino et al., 2012). Also, these results confirmed that folate production is a strain dependent trait (LeBlanc et al., 2010; Lin & Young, 2000), as strains belonging to a same species differed significantly in the amount of vitamin they produced, from "not detected" up to 253 ng/ml, as occurred for *Lc. lactis* (Table 1). Similar results were obtained by Sybesma, Starrenburg, Tijsseling, Hoefnagel, & Hugenholtz, 2003 for different strains of *Lc. lactis* (57–291 ng/ml), *Lb. helveticus* (2–89 ng/ml) and *St. thermophilus* (29–202 ng/ml) and by Pompei et al., 2007 for *Bifidobacterium* sp. (70–110 ng/ml). It is important to state that the samples for vitamin analysis were taken after 24 h incubation, when the strains were in the stationary growth period. Further studies would be required to analyze vitamin production in function of strain growth stage and to determine the folate concentration in function of biomass.

As for riboflavin production, only 8 strains were capable to produce this vitamin in riboflavin free medium, in concentrations between 173 and 532 ng/ml (Table 1). These concentrations are significantly higher than those described by Juarez del Valle et al., 2014, for *Lb. fermentum*, *St. thermophilus*, *Lb. plantarum* and *Lb. paracasei* (between 190 and 260 ng/ml). However, these concentrations are lower than those reported by Burgess, Smid, Rutten, & van Sinderen, 2006 for *Leuconostoc mesenteroides* (from 120 to 500 ng/ml), by Burgess, O'Connell-Motherway, Sybesma, Hugenholtz, & van Sinderen, 2004, for *Lc. lactis* (900 ng/ml), by Capozzi et al., 2011, for *Lactobacillus plantarum* (from 488 to 642 ng/ml) and by Russo et al., 2014 for *Lb. fermentum* (from 150 to 1200 ng/ml). The higher amounts of riboflavin detected by these authors can be explained by the fact that the strains were grown in the presence of roseoflavin, as the native strains were poor riboflavin producers. Roseoflavin induces mutations in the promoter region of the riboflavin biosynthesis cluster and consequently the amount of vitamin in the medium increases. In a study by Jayashree, Jayaraman, & Kalaichelvan, 2010, it was shown that a strain of *Lb. fermentum* was able to produce a much higher amount

Table 1
Identification of strains showing capacity to produce folates and riboflavin in folate and riboflavin deficient culture media.

Group[a]	Isolate	Identification[b]	Source[c]	Folate[d] (ng/ml)			Riboflavin[e](ng/ml)		
				Extra	Intra	Total	Extra	Intra	Total
A	34	*Streptococcus thermophilus*	M	107 ± 5	7 ± 1	114 ± 6	367 ± 6	165 ± 2	532 ± 8
A	170	*Streptococcus thermophilus*	M	190 ± 26	121 ± 9	311 ± 35	±	±	nd[f]
B	36	*Lactococcus lactis* subsp. *lactis*	M	74 ± 5	98 ± 5	172 ± 10	±	±	nd
C	179	*Weissella paramesenteroides*	M	102 ± 3	111 ± 4	213 ± 7	±	±	nd
D	190	*Weissella paramesenteroides*	M	134 ± 6	93 ± 7	227 ± 13	193 ± 4	86 ± 1	279 ± 5
E	348	*Bacillus* sp.	C	21 ± 1	149 ± 7	169 ± 8	364 ± 6	65 ± 1	429 ± 6
F	352	*Lactobacillus helveticus*	C	3 ± 1	20 ± 1	23 ± 1	97 ± 2	76 ± 3	173 ± 5
G	361	*Streptococcus thermophilus*	C	269 ± 11	25 ± 1	294 ± 12	±	±	nd
H	360	*Streptococcus thermoplhilus*	C	60 ± 2	107 ± 2	167 ± 4	±	±	nd
I	366	*Weissella paramesenteroides*	C	97 ± 10	292 ± 20	389 ± 30	±	±	nd
J	368	*Lactococcus lactis* subsp. *lactis*	C	19 ± 1	224 ± 2	243 ± 3	408 ± 2	115 ± 1	523 ± 3
K	472	*Streptococcus lutetiensis*	C	46 ± 1	30 ± 1	76 ± 1	293 ± 5	215 ± 6	508 ± 11
L	474	*Streptococcus infantarius*	C	26 ± 2	45 ± 4	71 ± 6	247 ± 4	191 ± 1	437 ± 5
M	334	*Streptococcus thermophilus*	C	191 ± 16	20 ± 3	211 ± 19	±	±	nd
M	341	*Streptococcus thermophilus*	C	52 ± 1	57 ± 5	109 ± 6	256 ± 3	195 ± 1	451 ± 4
N	340	*Lactococcus lactis* subsp. *lactis*	C	33 ± 2	150 ± 14	183 ± 16	±	±	nd
O	260	*Weissella paramesenteroides*	M	135 ± 4	128 ± 10	263 ± 14	±	±	nd
P	242	*Weissella paramesenteroides*	M	4 ± 1	146 ± 17	150 ± 18	±	±	nd
Q	244	*Weissella paramesenteroides*	M	143 ± 4	355 ± 10	498 ± 14	±	±	nd
R	268	*Streptococcus thermophilus*	M	53 ± 2	175 ± 16	228 ± 18	±	±	nd
S	343	*Lactococcus lactis* subsp. *lactis*	C	71 ± 6	182 ± 10	253 ± 16	±	±	nd

[a] As determined by RAPD-PCR.
[b] As determined by16s rDNA sequencing and BLAST analysis.
[c] M = raw goat milk, C = goat cheese.
[d] Folate concentrations in samples from FACM medium (Extracellular (Extra), Intracellular (Intra) and Total).
[e] Riboflavin concentrations in samples from RAM medium (Extracellular (Extra), Intracellular (Intra) and Total).
[f] nd = not detected.

of riboflavin (2.29 μg/ml (Jayashree et al., 2010); however, the method used for measurement of riboflavin concentration differed form that normally used, and overestimates the vitamin concentration because other compounds in the culture media could also absorb at 444 nm.

What is also important to note is that the 8 strains that produced riboflavin were also capable to produce folate, a phenomenon that has only been described in one previous study (Cardenas et al., 2015).

Five *St. thermophilus* (FP 34, 170, 268, 341 and 361) and two *Lc. lactis* (FP343 and 368) isolates were evaluated for their potential to increase the vitamin concentration in fermented goat milk. These isolates were selected because of their folate producing capability and because *St. thermophilus* and *Lc. lactis* are normally used in the elaboration of fermented foods. All tested isolates were able to grow in goat milk and decreased the pH of the milk and all increased the initial concentration of folate (40 ng/ml) to concentrations between 92 and 313 ng/ml (Table 2). *St. thermophilus* FP268 increased the folate concentrations almost five fold in only 6 h (Fig. 1), whereas the other strains increased folate concentrations after 8 h or 24 h. These results make this strain very interesting from a technological/economic point of view because this lower incubation time, if translated to scaling-up protocols, could imply significant reductions in costs of the manufacture of a fermented milk product with increased natural folates. A similar behavior was observed for *St. macedonicus* CRL415 (previously identified as *St. thermophilus*) that showed a significant increase in folate concentrations in cow's milk after only 6 h of incubation (Laiño et al., 2012). The variations in folate production is in accordance to previous works that have shown that production is not a species specific trait but rather a strain specific one (Gangadharan et al., 2010; LeBlanc et al., 2007, 2011; Lin & Young, 2000; Pompei et al., 2007).

According to the World Health Organization, the recommended dietary allowance (RDA) of folate for an average adult is 400 μg per day (FAO/WHO, 2002). This organization also established that a food can only be considered a good source of folate when it provides more than 10% of the RDA. Considering a one cup (237 ml) daily intake, the milk fermented with *St. thermophilus* FP268 would provide 19% of the RDA, making it an excellent source of folates.

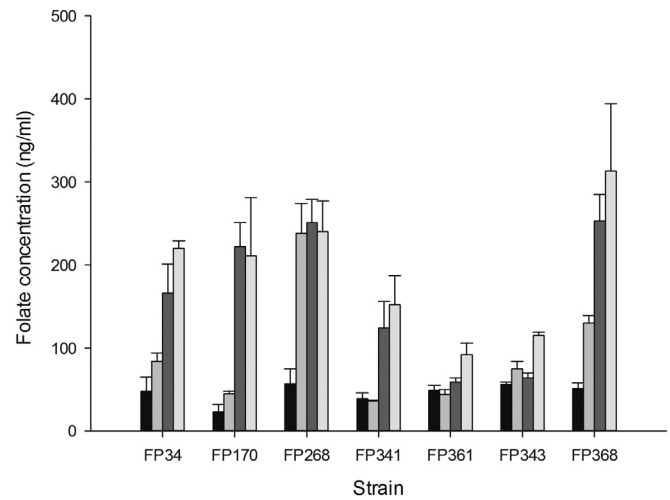

Fig. 1. Folate concentration in UHT goat milk incubated at 37 °C after 0 h (black boxes), 6 h (light gray boxes), 8 h (dark gray boxes) and 24 h (white boxes) after inoculation with strains *Streptococcus thermophilus* FP34, FP170, FP268, FP341 or FP361 or *Lactococcus lactis* FP343 or FP368. Results are presented as means ± SD.

In terms of riboflavin production in goat milk, all eight isolates that were able to produce this vitamin (Table 1) in culture media were evaluated. Despite able to grow in goat milk, production of riboflavin by all isolates in goat milk was minimal, as no detectable differences in riboflavin concentrations (27 ± 3 μg/ml) were observed after 24 h at 37 °C. Similar results were observed in strains isolated from cow's milk that possessed riboflavin producing

Table 2
Growth and folate production by the selected lactic acid bacteria in UHT goat milk.

Strain	Time (h)	Growth (log CFU/mL)	pH	Folate (μg/L)	Folate production[a] (μg/L)
Streptococcus thermophilus	0	5.3 ± 0.3	6.55	48 ± 18	172 ± 9
FP34	6	8.7 ± 0.3	5.36	84 ± 10	
	8	8.9 ± 0.1	4.89	166 ± 35	
	24	8.8 ± 0.3	4.15	220 ± 9	
Streptococcus thermophilus	0	5.1 ± 0.5	6.54	23 ± 9	188 ± 61
FP170	6	8.2 ± 0.7	5.95	45 ± 3	
	8	8.6 ± 0.2	5.33	222 ± 29	
	24	8.6 ± 0.1	4.28	211 ± 70	
Streptococcus thermophilus	0	5.3 ± 0.3	6.53	57 ± 18	183 ± 19
FP268	6	8.3 ± 0.2	5.36	238 ± 36	
	8	8.0 ± 0.5	4.87	251 ± 28	
	24	7.7 ± 0.6	4.13	240 ± 37	
Streptococcus thermophilus	0	5.1 ± 0.2	6.53	39 ± 7	113 ± 29
FP341	6	8.6 ± 0.4	5.37	36 ± 1	
	8	8.9 ± 0.3	4.89	124 ± 33	
	24	9.1 ± 0.4	4.15	152 ± 35	
Streptococcus thermophilus	0	5.6 ± 0.1	6.53	49 ± 6	43 ± 7
FP361	6	8.3 ± 0.7	5.67	44 ± 6	
	8	8.1 ± 0.9	5.03	59 ± 5	
	24	8.8 ± 0.2	4.21	92 ± 14	
Lactococcus lactis subsp. *lactis*	0	5.6 ± 0.1	6.54	56 ± 3	58 ± 1
FP343	6	9.0 ± 0.3	5.09	75 ± 9	
	8	8.7 ± 0.4	4.52	64 ± 6	
	24	7.8 ± 0.8	4.21	115 ± 4	
Lactococcus lactis subsp. *lactis*	0	5.6 ± 0.1	6.53	51 ± 7	262 ± 74
FP368	6	8.2 ± 0.3	5.20	130 ± 9	
	8	8.8 ± 0.5	4.74	253 ± 32	
	24	8.3 ± 0.7	4.07	313 ± 81	

[a] Value corresponds to the difference between folate concentrations after 24 h incubation compared to the initial concentration (T0).

capability but did not increase the concentration of this vitamin in milk above the elevated concentrations already present in the milk, which would suppress the genes involved in its biosynthesis (Juarez del Valle et al., 2014). However, these authors did show that the strains were able to significantly increase riboflavin concentrations in soymilk, that contains significantly lower concentrations of the vitamin, not affecting the expression of the riboflavin biosynthesis genes.

It must be highlighted that even though goat milk contains nearly the same amount of folate and riboflavin as cow's milk, most of these water soluble vitamins are lost during manufacturing of cheeses. For this reason, the use of vitamins producing LAB isolated from the same type of product could increase their concentration in cheeses, providing the health promoting benefits associated to the consumption of these vitamins.

4. Conclusions

This is the first complete study where folate and riboflavin producing LAB were isolated from goat milk and cheeses and tested for the capability of producing these vitamins in goat milk. In total, 179 LAB isolates were obtained. Among them, 151 (84%) were capable to produce at least one of these vitamins and were identified by RAPD-PCR and sequencing of 16S rDNA. Five strains (*St. thermophilus* FP 34, 170, 268 and 341 and *Lc. lactis* FP368) were capable to increase four to six fold the original amount of folate in goat milk. Folate content in milk fermented with *Lc. lactis* FP368 during 24 h was 313 ng/ml that could provide 19% of the recommended daily intake of this vitamin. In addition, *St. thermophilus* FP268 increased the folate concentration in the milk almost four fold in only 6 h. These results indicated that the strains identified in this study could be or great interest to the food industry as a tool for development of novel goat-milk derived products with elevated concentrations of natural folates. These products could be included in a conventional diet as an alternative to obligatory folic acid fortification of foods. Also, further studies are being conducted to evaluate if the strains identified in this study could provide other health promoting benefits or produce other beneficial compounds that could lead in the development of new value-added products.

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