Characterization of folate production and probiotic potential of *Streptococcus gallolyticus* subsp. *macedonicus* CRL415

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A R T I C L E   I N F O

A B S T R A C T

Mandatory fortification of foods with folic acid is being questioned by many scientists principally because of the potential adverse secondary effects associated with their excessive consumption. It has been shown that selected strains of lactic acid bacteria (LAB) are able to produce natural forms of folate and these could be included in foods to prevent deficiencies without causing adverse effects. The aim of this study was to evaluate folate production and *fol* gene expression by *Streptococcus gallolyticus* subsp. *macedonicus* (*S. macedonicus*) CRL415 under different growth conditions *in vitro* and to assess its potential probiotic application. Results showed that glucose as the principal carbon source, and incubation at 42 °C under controlled pH conditions (6.0) increased folate production, *fol* gene expression, and growth of *S. macedonicus* CRL415. This strain was able to produce elevated folate concentrations during milk fermentation without the need of prolonged incubation times and was able to resist conditions simulating the gastrointestinal tract. In addition, *S. macedonicus* was susceptible to all required antibiotics, and had a good adhesion level to intestinal cells *in vitro*, making it a promising candidate for biotechnological application as functional starter cultures in the dairy industry.

1. Introduction

Folate (vitamin B9) is involved in numerous essential functions such as amino acid metabolism, DNA replication, repair and methylation, and is thus essential for cell survival (Ball, 2005). As humans are unable to synthesize folate *de novo,* inadequate intakes can cause a wide variety of health disorders (Champier et al., 2012; Iyer and Tomar, 2009; LeBlanc et al., 2007). To address this problem, many countries have adopted mandatory fortification programs where folic acid is added to commonly consumed foods. However, a number of studies seem to raise doubts about the safe use of the chemically synthetized folic acid in foods (Iyer and Tomar, 2009; Laiño et al., 2013). In the last decade, the natural fortification of foods with vitamins through fermentation with food grade bacteria has been explored (LeBlanc et al., 2011; Capozzi et al., 2012; Patel et al., 2013).

Certain strains of bacteria can produce folate *de novo* through a pathway that consists of enzymes involved in the production of the essential parts of the tetrahydrofolate (THF) molecule, principally via a double-branched pathway involving the pterin and *p*ABA branches (Bekaert et al., 2008; Maguire et al., 2014). Three genes (*folB, folK,* and *folP*) encoding dihydroneopterin aldolase (DHNA), hydroxymethylpterin pyrophosphokinase (HPPK), and dihydropteroate synthase (DHPS), acting sequentially, are at the core of pterin branch in the folate pathway (Maguire et al., 2014). Some strains have also been shown to be able to produce *p*ABA *de novo*, but many others require this metabolite in order to produce the complete folate molecule (Pompei et al., 2007).

It has been reported that selected strains of lactic acid bacteria (LAB) are able to produce folate in growth medium as well as in milk, suggesting their application as functional starter cultures or co-cultures in fermented dairy products (Divya et al., 2012; Laiño et al., 2014; Rad et al., 2016). *Streptococcus gallolyticus* subsp. *macedonicus (*originally designated as *S. macedonicus* among the *Streptococcus thermophilus*-like microorganisms that belong to the *S. bovis/S. equinus* complex) is a LAB increasingly found in traditional cheeses worldwide, where the fermentation occurs through of a natural process (Callon et al., 2004; Poznanski et al., 2004). *S. macedonicus* strains are moderately acidifying and proteolytic, due to weak (phospho)-beta-galactosidase activity and extracellular proteolytic activity, respectively (Georgalaki et al., 2000, 2002; Lombardi et al., 2004). Certain strains can produce bacteriocins,

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such as the anti-clostridial lantibiotic macedocin, produced by *S. macedonicus* ACA-DC 198 (Anastasiou et al., 2009), and exopolysaccharides as heteropolysaccharide produced by *S. macedonicus* Sc136 (Lombardi et al., 2004; De Vuyst and Tsakalidou, 2008). These features make *S macedonicus* a potential candidate adjunct culture for dairy manufacturing.

Previously, it was demonstrated that *S. macedonicus* CRL415 was able to grow in folate-free culture medium (Laiño et al., 2012). However, folate production in different growth conditions and the expression of folate biosynthesis genes in *S. macedonicus* are lacking.

In the present study, folate production by *S. macedonicus* CRL415 under different growth conditions, and its effect on the expression of *fol* genes involved in the folate biosynthesis was evaluated. In addition, this strain was subjected to different *in vitro* analyses to assess its probiotic potential.

2. Materials and methods

2.1. Microorganisms, culture media and growth conditions

Streptococcus gallolyticus subsp. *macedonicus* (*S. macedonicus*) CRL415, previously selected for its folate-producing ability (Laiño et al., 2012), belongs to the Culture Collection of Centro de Referencia para Lactobacilos (CERELA, San Miguel de Tucumán, Argentina).

The strain was grown in mLAPTg (Laiño et al., 2017) broth culture, or in a Chemically Defined Medium (CDM, Hébert et al., 2004) without folic acid (CDM-WF) at 42 °C for 24 h (Laiño et al., 2012). All amino acids, vitamins, purines, pyrimidines, and inorganic salts were of analytical grade (Sigma Chemical Co., St. Louis, MO, USA). The media and stock solutions were sterilized by filtration through a cellulose acetate membrane (0.22-μm-pore size; Sartorius AG, Göttingen, Germany).

Working cultures of *S. macedonicus* CRL415 were prepared from frozen stock culture to 10 mL of LAPTg broth (Laiño et al., 2017), and incubating for 16 h at 42 °C. To eliminate carryover nutrients, cells were harvested by centrifugation at $8.000 \times g$ for 15 min, washed three times with saline solution (0.85% w/v NaCl), and resuspended in this solution to the original culture volume. This cell suspension was used to inoculate the CDM-WF or mLAPTg at an $OD_{580} = 0.1$ [10^5 colony-forming units (CFU) per mL].

2.2. Evaluation of carbon source, pABA, and environmental pH in folate production

Influence of several carbon sources (glucose, lactose, and sucrose) at different concentrations (10, 20, and 30 g L^{-1}), and *p*ABA (10, 150 or 1500 μM) were evaluated in CDM-WF. The culture medium was always prepared immediately before use.

The influence of environmental pH (controlled at 5.0 or 6.0 or free pH) was performed in a 2.0 L BioFlo C32 fermentor (New Brunswick Scientific Co., Edison, N.J.) containing 1.5 mL mLAPTg broth culture. Agitation of 100 rpm was applied, temperature was maintained at 42 °C, and pH was automatically controlled at 5.0 or 6.0 with sterile 3 N NaOH. Fermentation proceeded for 24 h and samples were aseptically withdrawn from fermentation vessel at different times (0, 2, 4, 6, 8, 10, 12, and 24 h) to evaluate cell growth (OD_{580}); cell viability [log (CFU per mL) using the plate dilution method in LAPTg agar plates incubated at 37 °C for 48 h] and folate production. pH of sample at each time point was measured.

Fermentations with free pH (without pH control) were carried out in the same conditions, with an initial pH value in the medium of 6.5 ± 0.2.

2.3. Folate production in fermented milk

The strain was inoculated at 2% (v/v) in reconstituted non-fat powdered milk (Svelty Calcio Plus, Nestle, Argentina) and incubated at 42 °C for 24 h. Samples were obtained at different times (0, 6 and 24 h) to evaluate bacterial growth and folate production.

2.4. Folate determination

Samples were processed as described by Laiño et al. (2017). Briefly, samples (1 mL) of LAB-containing CDM-WF, aseptically withdrawn at different incubation times, were mixed with 1 mL of protection buffer (0.1 mol L^{-1} phosphate buffer, pH 6.8, containing 1.5% (w/v) ascorbic acid to prevent vitamin oxidation and degradation) to evaluate folate production, and immediately cooled on ice bath. Two samples from resulting mixture were considered; one of them (1 mL) was boiled (100 °C) for 5 min, followed by immediate centrifugation (10,000 g for 6 min) and the supernatant (total folate sample) was collected. The second sample (1 mL) was centrifuged (5000 g for 5 min) to obtain the supernatant (extracellular folate sample), and pellet was resuspended to original volume (1 mL) with buffer protection (intracellular folate sample), boiled (100 °C) for 5 min, followed by immediate centrifugation (10,000 g for 6 min) and the supernatant was collected. All supernatants were stored at −70 °C until use for folate determination.

A trienzymatic treatment, as described previously, was performed in all samples (Laiño et al., 2012). The enzymes α-amylase from *Aspergillus oryzae* and protease from *Streptomyces griseus* (Sigma Chemical, St. Louis, MO, USA) were dissolved distilled-deionized water at concentrations of 4 mg mL^{-1} and filter-sterilized (0.22 μm). Rat plasma (INSIBIO-CONICET-UNT, Tucumán, Argentina) was used as a source of folate deconjugase enzyme as described previously (Aiso and Tamura, 1998).

Folate was quantified by a modified microbiological assay according to Laiño et al. (2012) using *L. casei* subsp. *rhamnosus* NCIMB 10463, a folate auxotroph as the indicator strain. In every case, non-inoculated samples analyzed simultaneously, were used as controls.

The samples were diluted with protection buffer and each sample (100 mL) was placed into one well of a 96 well sterile microplate (Deltalab, Argentina). The folate concentration of each sample was determined in triplicate. The reference strain (*L. rhamnosus* NCIMB 10463) grown in FACM as described above, was inoculated at 4% v/v in 10 mL of 2FACM containing 20 mg mL^{-1} chloramphenicol (to decrease the potential of microbial contaminants), and a fraction (100 mL) was added to each well and mixed. Sterile plate covers were placed on the microtiter plates that were then incubated 48 h statically at 37 °C. After this optimized incubation period, the optical density (OD) was read at 580 nm using a microplate reader (VERSAMax Tunable Microplate Reader, Molecular Devices, USA). In each microplate, a standard curve was realized using HPLC grade folic acid (Fluka Biochemika, Switzerland) instead of samples at different concentrations (between 0 and 1.0 mg L^{-1}) diluted in the protection buffer. Samples were diluted (normally in a 1/80 relation in protection buffer), in order to obtain values within the range of the standard curve. To obtain the final folate concentrations, the values obtained from the standard curve were multiplied by the dilution factor and expressed as mg L^{-1}.

2.5. Real time PCR analysis for relative quantification of fol genes expression

The expression of the following genes was investigated: *folE* (GTP cyclohydrolase I), *folQ* (dihydroneopterin triphosphate pyrophosphohydrolase), *folK* (2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase), *folP* (dihydropteroate synthase), *folC1* (folylpolyglutamate synthase), *folC2* (dihydrofolate synthase) and *folA* (dihydrofolate reductase).

Total RNA was isolated from cell pellets, obtained from CDM-WF with glucose 20 g L^{-1} and 10 μM *p*ABA, containing approximately 1×10^8 CFU per mL from 6 h-, 8 h- and 10 h-incubation samples at 42 °C. RNA isolation was performed using the NucleoSpin1 RNA II kit (Macherey-Nagel GmbH & Co. KG; Düren, Germany) according to the

Table 1
List of oligonucleotides used in this study.

Primer			Sequence
folE	RT	Fwr	5' -TGG GGA TAA GGT GAC AGG AC- 3'
folE	RT	Rev	5' -CCT CGA GGA TTA AGG GCT TC- 3'
folQ	RT	Fwr	5' -CAG GGC TTC ACA AAG TGA CA- 3'
folQ	RT	Rev	5' -TAG CAC CAG CTA AAC GCT CA- 3'
folK	RT	Fwr	5' -GCT TGC TTG AGA CGG AAG TT- 3'
folK	RT	Rev	5' -CCT GAC AAG CAC TCA GCA AG- 3'
folP	RT	Fwr	5' -CGG AAT TTG TAA CCG AGG AA- 3'
folP	RT	Rev	5' -CTG GCA GTG GCT GTC TTG TA- 3'
folC1	RT	Fwr	5' -ATC ATT GAA GCA GGG ATT GG- 3'
folC1	RT	Rev	5' -GCT ATC GCC CAA GCT TTC AG-3'
folC2	RT	Fwr	5' -GGT CTT GGG CAA TAC TCA T- 3'
folC2	RT	Rev	5' -ATT GCT ATT TGG GCG GAA G- 3'
folA	RT	Fwr	5' -ATT GCT ATT TGG GCG GAA G- 3'
folA	RT	Rev	5' -CAT GCC ATC AAA GGT CAC AC- 3'
gyrB	RT	Fwr	5' -CTC CAA AGA AGG CTT GCA TC- 3'
gyrB	RT	Rev	5' -CCA CGA CCA TCA TCA ACA AC- 3'
recA	RT	Fwr	5' -TGT AGG ACT TCA AGC GCG TA- 3'
recA	RT	Rev	5' -TCC ACC TGG GGT AGT CTC TG- 3'

manufacturer's instructions. Total RNA was quantified using Qubit™ RNA Assay Kit (Invitrogen, Carlsbad, CA, USA) with Qubit™ 2.0 fluorometer (Invitrogen, Carlsbad, CA, USA).

Primers were designed using the following free online tools: Primer3 (v.0.4.0), Primer-BLAST, In-silico PCR using the corresponding gene sequences of *S. macedonicus* ACA-DC198 from the Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/), and GenBank (http://www.ncbi.nlm.nih.gov/genbank/) databases as templates. The primers used are described in Table 1. The housekeeping genes *recA,* and *gyrB* were used as internal standard for expression analysis.

All qPCR assays were performed using SensiFast™ SYBR & Fluorescein One-Step Kit (Bioline, Tauton, MA, USA). Changes in gene expression were quantified in a thermocycler with a coupled Bio-Rad iQ5 Multicolor RT-qPCR Detection System (Bio-Rad, California, USA).

The first step of the qPCR assay consisted of an initial hold at 45 °C for 10 min, 95 °C for 2 min, 95 °C for 5 s, 60 °C for 10 s, followed by 40 cycles at 72 °C for 5 s, 95 °C for 1 min, 55 °C for 1 min followed by 81 cycles at 55 °C for 10 s. For each amplification run, the calculated threshold cycle of the 16S rRNA was used for normalization. The formation of nonspecific products was excluded by using the melting curve function of the Bio-Rad iQ5 Optical System Software, Standard Edition v2.0.148.60623.

Two independent qPCR assays were performed for each condition (batch fermentation at constant pH 6.0, and 6, 8 and 10 h of incubation). The relative expression of *fol* genes in different conditions was estimated according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The condition of 6, 8, and 10 h of incubation in batch fermentation at free-pH was used as control. Values reported are the fold changes between each condition and the control (given the value 1) and were normalized to internal control gene *recA* expression.

The relative transcription levels were expressed as means ± standard deviations (SD) fold changes. Genes were significantly down- or up-regulated if their relative expression level was found to be at least 2-fold lower or higher than reference condition.

2.6. Tolerance to simulated gastric juice

Overnight cultures of the strain (10 mL) were pelleted by centrifugation, washed twice with phosphate buffer (0.1 M, pH 7.0), resuspended in saline solution, and immediately added to the same volume of "gastric" solution [0.6% (w/v) pepsin (Merck, Germany), 1% (w/v) NaCl]. Samples were incubated with agitation at 37 °C. Gastric environment was reproduced by progressive acidification (addition of 1 M HCl) from the initial pH value of 5.0 to 2.1 in 90 min, according to

Marteau et al. (1997) and Blanquet et al. (2004). Cell counts on LAPTg agar were performed at different time (0, 30, 60, 90 min of incubation). After 90 min, an aliquot of each cell suspension was taken, pelleted by centrifugation and the cells were resuspended in phosphate buffer (0.1 M, pH 8.0) containing 0.3% (w/v) and 0.1% (w/v) of bile and pancreatin (Sigma-Aldrich de Argentina S.R.L), respectively, and kept for further 60 min at 37 °C. Cell counts in the appropriate agar medium were performed. Washed cells resuspended in phosphate buffer and subjected to the same conditions as treated samples were used as controls. Viable cells (log CFU per mL) were calculated after 30, 60, 90 min and 150 min.

2.7. Bile resistance

The ability of the strain to grow in the presence of 0.3%, 0.5% and 1.0% (w/v) of bile was determined according to the method of Vinderola and Reinheimer (2003). The results were expressed as the percentage of growth compared to the control.

2.8. Antimicrobial susceptibility testing

The susceptibility to antimicrobials of bacterial strain was performed according to the European Food Safety Authority Report for assessment of bacterial susceptibility to antimicrobials of human and veterinary importance (EFSA, 2012) using SSM (Tosi et al., 2007) as medium.

Overnight cultures of the strain (10 mL) in LAPTg medium were centrifuged, and washed twice with phosphate buffer (0.1 M, pH 7.0). The inoculum was adjusted with SSM medium to a turbidity equivalent to 1 McFarland standard ($\approx 1 \times 10^8$ CFU per mL).

The following antimicrobial agents were used: ampicillin (A-9518), vancomycin (V-2002), clindamycin hydrochloride (C-5269), chloramphenicol (C-0378), erythromycin (E-6376), gentamicin sulfate (48760), kanamycin sulfate (K-4000), streptomycin sulfate (S-6501), tetracycline (T-3383). All the antibiotics were obtained from Sigma (St. Louis, Mo., USA) except gentamicin sulfate that were obtained from Fluka (Buchs SG, Switzerland). Each of the antibiotic powders was carefully weighed, dissolved, diluted in appropriate diluents and filter sterilized. SSM medium was supplemented with each one of these antibiotics at the concentration established, as microbiological cut-off values, by European Food Safety Authority (EFSA, 2012) for to determine possible presence of antimicrobial resistance in microbial products by ampicillin ($2 \, mg \, L^{-1}$), vancomycin ($4 \, mg \, L^{-1}$), clindamycin hydrochloride ($2 \, mg \, L^{-1}$), chloramphenicol ($4 \, mg \, L^{-1}$), erythromycin ($2 \, mg \, L^{-1}$), gentamicin sulfate ($32 \, mg \, L^{-1}$), kanamycin sulfate ($64 \, mg \, L^{-1}$), streptomycin sulfate ($64 \, mg \, L^{-1}$), or tetracycline ($4 \, mg \, L^{-1}$).

2.9. Adhesion assay of S. macedonicus CRL415 to intestinal epithelial cells

Human colonic adenocarcinoma Caco-2 cells (ATCC HTB37) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing high glucose and GlutaMAX™ (Gibco, Gran Island, New York, USA) supplemented with 10% (w/v) fetal bovine serum (FBS; NATOCOR, Córdoba, Argentina), 100 IU mL^{-1} penicillin, 100 µg mL^{-1} streptomycin, and 0.25 µg mL^{-1} amphotericin B (Gibco, Gran Island, New York, USA). The cells were grown in 25 cm^2 vented tissue culture flasks at 37 °C in presence of 5% CO$_2$ until 70–80% cell confluence.

The cells (passage 53) were seeded in a 24-well plate (5×10^4 cells per mL) and incubated for 48 h until attachment to the bottom of the well, reaching 1×10^5 cells per mL. Before the experiment, *S. macedonicus* CRL415 was cultured in LAPTg for 16 h at 37 °C. After incubation, the microbial culture was centrifuged and washed twice with PBS solution. Bacterial cells (10^8 CFU per mL) were suspended in 1 mL of DMEM medium without antibiotics and fetal bovine serum. The medium in the microplate was discarded and the cells were washed

three times with PBS to eliminate the antibiotic and antifungal. The cells were then co-cultured with *S. macedonicus* CRL415 (cell/bacteria ratio = 1/10 (10 μL of the bacterium and 990 μL of DMEM medium). After 2 h at 37 °C, the cells were washed three times with PBS to eliminate non-adhering bacteria. To release attached bacterial cells, the Caco-2 monolayer was treated with a solution of 1% Triton X-100 detergent (Sigma, USA). The lysis was carried out on ice for 10 min. Then, the lysates were centrifuged; the supernatant, washed twice with PBS, was suspended in 1 mL of 0.85% (w/v) NaCl, inoculated, and the number of adhered bacteria quantified by serial dilution and plating on LAPTg agar medium.

Bacterial adhesion was calculated as percentage of adherent bacteria in relation to the total number of bacteria added. Experiments were performed in duplicates and repeated three times.

2.10. Statistical analysis

Results were obtained from three independent experiments and each data point was measured in triplicate (n = 9). Data are presented as mean ± standard deviations (SD). Statistical analyses were performed with Sigma Plot 12.0 (Systat Software Inc., Chicago IL, USA) using ANOVA general linear models followed by Tukey's post hoc test, and differences between groups were significant at a P-value < 0.05.

3. Results

3.1. Influence of carbon source, pABA and pH on the microbial growth and folate production by S. macedonicus CRL415

The influence of carbon source on growth and folate production was studied in CDM-WF using glucose, lactose, or sucrose as carbon source. The concentration of each carbon source was 10, 20, and 30 g L^{-1}, and the temperature of incubation was 42 °C. Growth was not affected by the carbohydrate source used (Table 2). In the presence of lactose or sucrose, lower total folate synthesis was observed (73 ± 4 and 75 ± 2 μg L^{-1}, respectively), whereas glucose was the most favorable sugar sources for folate production (89 ± 4 μg L^{-1}) at 10 h of incubation. When a lower glucose concentration was used (10 g L^{-1}), this affected both bacterial growth and folate synthesis, while no further increase in folate production was observed when the concentration of this carbohydrate was increased to 30 g L^{-1} (data not shown). For this reason, a carbohydrate concentration of 20 g L^{-1} was used in the culture medium throughout this study.

The presence of 10 μM *p*ABA assured the growth and folate production by *S. macedonicus* CRL415 (Table 3) after 10 h of incubation. However, increasing *p*ABA concentration did not improve growth and folate synthesis. Minor folate production was observed in the absence of *p*ABA.

Based on these results, the influence of pH (pH 6.0, 5.0, and no pH control) on bacterial growth and folate production by *S. macedonicus* CRL415 was determined in batch cultures performed with glucose (20 g L^{-1}) as the carbon source, and *p*ABA (10 μM) at 42 °C for 24 h.

A clear influence of pH on folate production was observed (Fig. 1). The maximum total folate production, reached at 12 h-incubation, under controlled pH conditions was significantly higher at pH 6.0

Table 3
Influence of *p*ABA on growth and folate production by *S. macedonicus* CRL415 in CDM-WF[a] at 10 h.

*p*ABA (μM)	Cell number log (CFU mL^{-1})	Folate (μg L^{-1})			Total Folate (mg CFU^{-1})
		Extracellular	Intracellular	Total	
0	8.94	7 ± 2	4.7 ± 0.8	12 ± 3	1.3 ± 0.3
10	9.07	63 ± 3	10 ± 1	73 ± 4	8.0 ± 0.4
150	8.91	63 ± 4	15 ± 1	77 ± 5	8.6 ± 0.6
1500	8.89	65 ± 3	5.0 ± 0.7	70 ± 4	7.9 ± 0.4

[a] Chemically defined medium without folate.
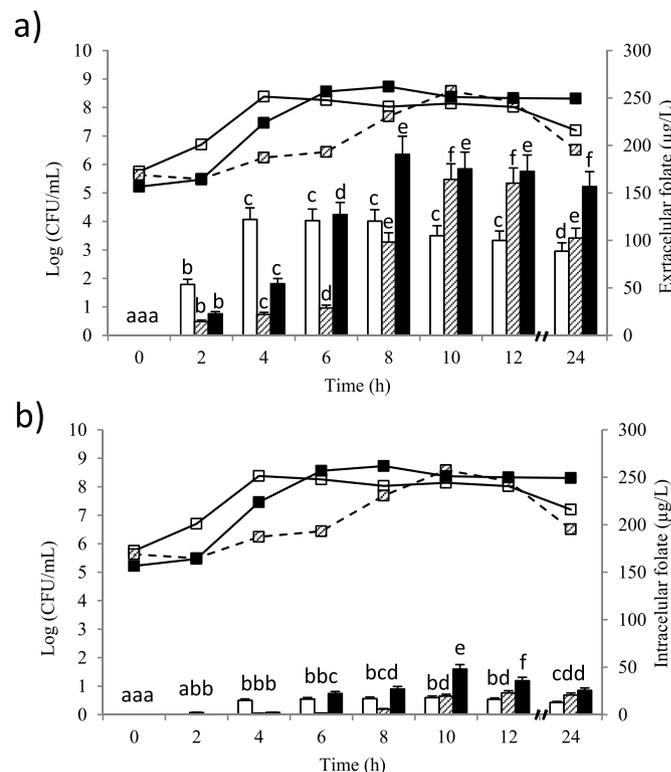
Fig. 1. Growth [log (CFU per mL)] and extracellular (a) and intracellular (b) folate production (μg L^{-1}) by *S. macedonicus* CRL415 in mLAPTg in different pH conditions. Growth (symbols) and folate (bars) at free-pH (empty), pH 5.0 (stripped) and pH 6.0 (black). Results are mean ± SD from experiments conducted in triplicate. [a-f] Means from the same fermentation conditions without common letter are significantly different ($p < 0.05$).

(210 ± 1 μg L^{-1}) and at pH 5.0 (163 ± 1 μg L^{-1}) compared to free pH fermentations (116 ± 3 μg L^{-1}, this condition was used as control). Controlled pH conditions favored the synthesis of this vitamin, the total amount of folate produced by *S. macedonicus* CRL415 raised *ca.* 2-fold when the pH was increased from 5.0 to 6.0 respect to free-pH. The folates were produced mainly during the exponential growth phase. Under controlled growth conditions at pH 6.0, vitamin production

Table 2
Influence of carbohydrates on growth and folate production by *S. macedonicus* CRL415 in CDM-WF[a] at 10 h of incubation.

Carbohydrate (20 g L^{-1})	Cell number log (CFU mL^{-1})	Folate (μg L^{-1})		Folate (mg CFU^{-1})	
		Extracellular	Intracellular	Extracellular	Intracellular
Glucose	8.0 ± 0.1	80 ± 4	9.1 ± 0.9	10.0 ± 0.9	1.1 ± 0.1
Lactose	7.9 ± 0.2	68 ± 3	4.9 ± 0.4	8.6 ± 0.4	0.6 ± 0.1
Sucrose	7.8 ± 0.2	70 ± 2	5.2 ± 0.5	9.0 ± 0.3	0.7 ± 0.1

[a] Chemically defined medium without folate.

started after about 4 h of fermentation increasing the excretion of folate into the medium with a maximum level ($191 \pm 1\,\mu g\,L^{-1}$) at 8 h-incubation (Fig. 1). This value was statistically different from those attained at the others studied growth conditions.

Lengthening the incubation time (from 8 to 24 h) led to increase intracellular folates concentration ($43 \pm 3\,\mu g\,L^{-1}$ at 24 h-fermentation) (Fig. 1), this value being 3.34-fold greater than that obtained at free-pH. As results, folate production by *S. macedonicus* CRL415 is growth-associated. For all experiments, folate production was first detected in the mid-exponential growth phase and increased rapidly until the end of the exponential growth phase (Fig. 1). After 8 h of fermentation, the microorganism reached the stationary growth phase at pH 6.0, whereas at pH 5.0, it was attained after 10 h. Similar behavior was observed in cell viability, which was higher at pH 6.0 [8.31 log (CFU per mL)] than at pH 5.0 and non-constant pH [6.51 log (CFU per mL) and 7.2 log (CFU per mL), respectively] after 24 h of incubation.

3.2. Expression of fol genes under controlled pH batch fermentation condition

The differential expression of *fol* genes as a function of environmental pH was determined by qPCR using 6 h- (log phase), 8 h- (late log phase) and 10 h- (stationary phase) cells grown in controlled pH 6.0 batch fermentations. The growth of *S. macedonicus* CRL415 under free pH conditions during 10 h-incubation was considered as the reference condition.

The analyses revealed that the expression of four genes (*folE, folQ, folK* and *folP*) related to folate synthesis increased more than 2-fold when the culture reached the late logarithmic growth phase under controlled pH conditions (Fig. 2) corresponding to the maximal folate production. The highest transcriptional response during folate production was found in *folK* (9.9 ± 0.7-fold expression). The expression level of *folP* gene was similar (4.6 ± 0.5-fold) in both the late log (8 h) and stationary (10 h) cells.

Regarding *folA* gene, over-expression was also observed 8 h after inoculation and at the end of fermentation time (10 h), but the relative expression levels were different (6.5 ± 0.50- and 4.59 ± 0.35-fold, respectively). In addition, an increasing relative expression of *folC1, folC2* and *folA* genes was exhibited at 10 h-fermentation.

3.3. Folate production in fermented milk

S. macedonicus CRL415, inoculated in milk and incubated at 42 °C, significantly increased folate concentration ($62 \pm 2\,\mu g\,L^{-1}$) after 6 h of fermentation, this value being 1.42-fold greater than its basal value. From this time point, the folate levels remained constant until the end of the fermentation. Regarding, the pH values decreased to 4.7–4.8 during the first 6 h (milk clotting was found), reaching values of pH 4.1–4.2 at the end of fermentation (data not shown).
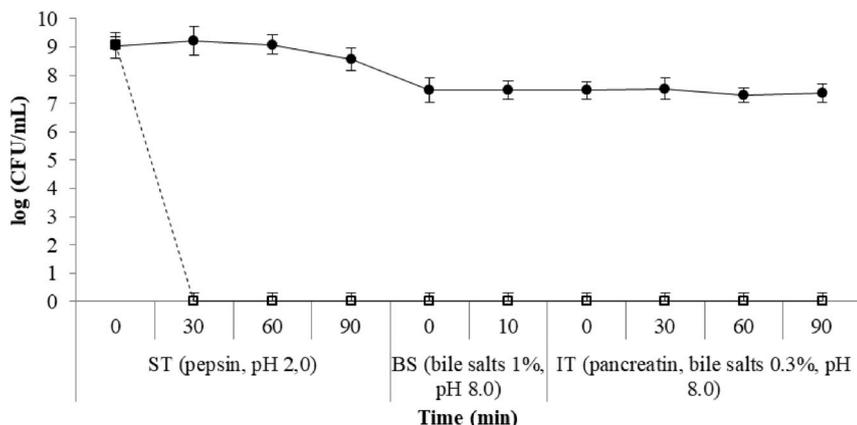
3.4. Antibiotic resistance

The strain was found sensitive to ampicillin ($\leq 2\,mg\,L^{-1}$) vancomycin ($4\,mg\,L^{-1}$), clindamycin ($2\,mg\,L^{-1}$), chloramphenicol ($4\,mg\,L^{-1}$), erythromycin ($2\,mg\,L^{-1}$), gentamicin ($32\,mg\,L^{-1}$), kanamycin ($64\,mg\,L^{-1}$), streptomycin ($64\,mg\,L^{-1}$) and tetracycline ($4\,mg\,L^{-1}$) (data not shown).

3.5. Adhesion assay of S. macedonicus CRL415 to intestinal epithelial cells

The adhesion ability of *S. macedonicus* CRL415 was evaluated *in vitro* using human intestinal cells (Caco-2). The strain showed an adhesion percentage to Caco-2 cells of 17.3% when it was incubated in a cell: bacteria ratio of 1:10 (data not shown).

3.6. In vitro resistance to bile and simulated gastric juice

S. macedonicus CRL415 showed a capability to grow (average value about 57%) in the presence of 0.3% (w/v) bile salts. However, growth percentages at the maximum concentration of bile assayed (1% w/v) were lower than 30%, which reflected low bile tolerance (Fig.S1 and Table S1). In addition, this microorganism showed a lack adaptation to simulated gastric juice at pH 2.0 (Fig. 3) since cell viability fell at least 8.0 log-units within the first 30 min of incubation at pH 2. When, this strain was used as starter in fermented milk, the survival was markedly improved (Fig. 3). After 90 min (pH 2.0), survivor counts did not differ significantly from the beginning with values higher than 8 log (CFU per mL). When this fermented milk was put in conditions that simulated the stomach duodenum passage [pH 8.0 with 0.3% (w/v) bile and 0.1% (w/v) pancreatin for a further 90 min at 37 °C], the survival rates did not change significantly, and the cell populations remained over 7 log (CFU per mL) (Fig. 3).

4. Discussion

Production of folate by LAB can lead to obtaining healthier food when these bacteria are used during food processing (Rad et al., 2016; Meucci et al., 2018). Among LAB, *S. macedonicus* is considered a multifunctional and promising specie for dairy fermentation (De Vuyst and Tsakalidou, 2008). Previously, it was shown that *S. macedonicus* CRL415 grew optimally at 42 °C and was able to synthetize folate *de novo* in a folate-free culture medium (Laíño et al., 2012). In the present study, the influence of growth conditions, especially the role of carbon source, *p*ABA and pH on bacterial growth and folate biosynthesis by *S. macedonicus* CRL415 were studied. The highest levels of folate were obtained in the presence of glucose whereas sucrose was a poor carbon source for cell growth and folate production. Similar observations were made for *S. thermophilus* BAA-250 where it was also shown that folate synthesis was growth-dependent (Mousavi et al., 2013).

Fig. 2. Relative expression of *fol* genes in *S. macedonicus* CRL415 grown in mLAPTg with constant pH of 6.0 and free-pH incubated at 42 °C after 6 (white bars), 8 (stripped bars) and 10 h (black bars) of incubation. Results are expressed as means ± SD. [a-c]Means with different letters differ significantly for the same gene evaluated at the different time points ($P < 0.05$).
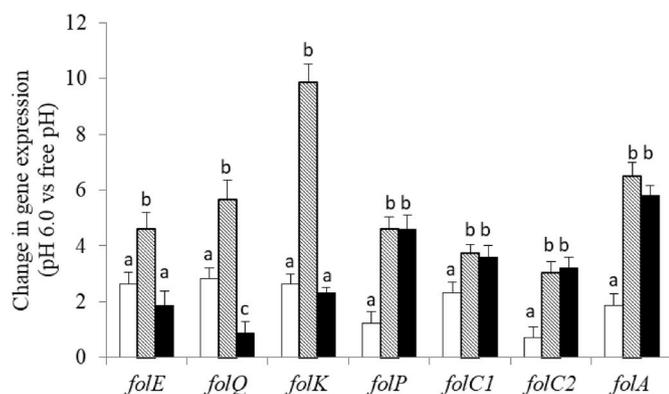
Fig. 3. Cell viability (log CFU ml^{-1}) of *S. macedonicus* CRL415 in saline solution (empty squares) and in milk fermented (filled circles) during 190 min of gastric transit. ST: stomach transit; BS: bile shock; IT: intestinal transit. Results are expressed as means ± SD.

*p*ABA is one of the building blocks of folate biosynthesis. It has been demonstrated that the addition of *p*ABA at concentrations between 1 and 100 μM can increase folate production by *Lactococcus lactis* (Sybesma et al., 2003). In our study, the addition of 10 μM *p*ABA largely stimulated folate production by *S. macedonicus* CRL415 without affecting cell growth, whereas no further improve of these parameters was observed when increasing *p*ABA concentration up to 1.5 mM. Our findings were consistent with those reported by Mousavi et al. (2013), who showed that a *p*ABA concentration higher than 10 μM had not significant effect on folate biosynthesis by *S. thermophilus* BAA-250. However, some authors clearly showed enhanced folate levels with increased *p*ABA concentrations (Sybesma et al., 2003; Pompei et al., 2007).

As an approach to improve folate production, fermentations with automatic pH control, at values of 6.0 and 5.0, and free-pH were carried out. Folate production by this strain was enhanced at a constant pH value of 6.0 compared to fermentations with non-controlled pH. Moreover, under controlled growth conditions in chemostat, the total amount of this vitamin increased more than 2-fold when the pH was raised from 5.0 to 6.0 respect to free-pH. This is in agreement with literature data reporting that a pH *ca.* 7.0, is the optimum pH of all folate biosynthesis enzymes in several microorganisms such as *S. thermophilus* CRL803 (Laíño et al., 2017), and strains St 563 and St 399 (Meucci et al., 2018). These results are in contrast to those found in *B. adolescentis* MB 239, where no statistically significant differences in folate biosynthesis was observed in function of pH and the carbon source (Pompei et al., 2007). Furthermore, Sybesma et al. (2003), studying the folate production in synthetic medium under controlled growth conditions, observed an influence of pH on the intra- and extracellular folate distribution. Our results also showed that the intra- and extracellular folate relation was influenced by the pH. *S. macedonicus* CRL415 grown at lower pH had a larger extracellular folate relation than cells cultured at pH 6.0. The difference in distribution could be explained by the different length of the polyglutamyl tail of the folates since one of the functions of the polyglutamyl tail is believed to be the retention of folate within the cell.

At both constant pH, 6.0 and 5.0, folate production by *S. macedonicus* CRL415 was cell growth-associated; although this vitamin was also produced during the mid-exponential growth phase, the maximum folate values were found at the beginning of the stationary phase. These results are consistent with previous studies carried out in other bacterial species (Pompei et al., 2007; Laíño et al., 2017; Meucci et al., 2018).

In addition to the physiological studies of folate production by *S. macedonicus* CRL415 at constant pH of 6.0 and at free pH, a complementary approach using qPCR revealed that six out of seven genes

involved in folate biosynthesis exhibited more than 2-fold relative expression in this strain at constant pH 6.0, confirming their role in the increase of folate production under this growth condition. Specifically, *folE, folQ, folK, folP,* and *folA* genes encoding enzymes that catalyze consecutive steps in *de novo* folate biosynthesis, were the highest regulated genes at the transcriptional level in CRL415 at 8 h of incubation. Recently, a different regulation of expression of *folK* and *folP* was shown in *S. thermophilus* strains that were grown in milk (Meucci et al., 2018). According to this study, there was a feedback inhibition since *folK* was expressed only in early phase of milk fermentation and declined afterwards (3 h), whereas *folP* was overexpressed up to 6 h when the maximum growth and folate production were attained. Our results showed that all *fol* genes were overexpressed, remaining an up-regulation of *folP* and *folA* even after 10 h of incubation. To achieve an increase of folate production by certain LAB strains, an overexpression of specific genes of the biosynthetic pathway has been suggested by Sybesma et al. (2003), Laíño et al. (2017) and recently by Meucci et al. (2018). *folK* and *folP* encode for two signature proteins of the folate pathway (*i.e.* the enzyme 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase and dihydropteroate synthase, respectively), which are essential for the folate *de novo* synthesis in many bacteria (de Crécy-Lagard et al., 2007). Our results demonstrated an overexpression of these genes after 8 h of incubation, when the maximum folate production was reached. Furthermore, the increased intracellular folate concentrations upon prolonged fermentation (10 h) could be associated with overexpression of *folC1, folC2* and *folA* genes as was revealed in this study. The resulting folylpolyglutamates are preferentially retained, helping to concentrate the folates intracellularly. To date, studies on genes involved in folate biosynthesis in *S. macedonicus* have not been reported.

In addition, *S. macedonicus* CRL415 was able to produce folate in milk increasing *ca.* 1.42-fold the level initially present in milk, after 6 h of fermentation These results are consistent with previous studies carried out in *S. thermophilus* strains (van Hylckama Vlieg and Hugenholtz, 2007; Laíño et al., 2012, 2013; Meucci et al., 2018). According to Iyer et al. (2011), who categorized *S. thermophilus* strains into three groups on the basis of the net amount of folate accumulated in milk, in our studies *S. macedonicus* CRL415 would be classified as high-producer.

The absence of transmissible antibiotic resistance is considered a key safety prerequisite for both FAO/WHO (2006) probiotic selection guidelines and the EFSA QPS system for food and feed additives (EFSA, 2012). *S. macedonicus* CRL415 was found sensitive to all the antibiotics tested.

A relevant property of candidate probiotics is the ability of bacteria to adhere to mucus and/or intestinal epithelial cells which is one of the mechanisms protecting the host organism from pathogen invasion and adhesion. *S. macedonicus* CRL415 had good adherence capacity which would help them to attach to the intestinal cells in the host and possibly colonize, and release or produce folate in the gastrointestinal tract. Similar results were reported for *S. thermophilus* BGVLJ1-44 and ACA-DC 170 when the adhesion properties were evaluated under similar assays conditions (Veljovic et al., 2017; Zoumpopoulou et al., 2017).

Fermented milks are considered as suitable vehicles for delivering probiotic bacteria to humans due to their ability in maintaining sufficient probiotic viability during shelf life. In this study, *S. macedonicus* CRL415 alone or in fermented milk was analyzed for its susceptibility to digestive tract conditions. This strain was sensitive to acid pH and bile salt. However, CRL415 was able to sustain the harsh gastrointestinal conditions when cells were administered in fermented milk. These results would be attributed to the food matrix which would protect this bacterium to gastrointestinal stresses (Bönisch et al., 2007).

5. Conclusion

This is the first report describing the production of folates by *S. macedonicus* and the influence of culture conditions (carbohydrate

source, temperature and pH) on the expression of *fol* genes in CRL415.

The ability of *S. macedonicus* CRL415 to produce folates reaching high levels during milk fermentation without the need of prolonged incubation times, and resistance showed in fermented milk to the simulated gastrointestinal hostile environment, antibiotic susceptibility and good adhesion level to the intestinal cells, makes *S. macedonicus* CRL415 a great and high value promising candidate for biotechnological application as functional starter cultures in dairy industry.

Conflicts of interest

The authors declare no competing interests.

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2018.10.015.

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