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Folate production and *fol* genes expression by the dairy starter culture *Streptococcus thermophilus* CRL803 in free and controlled pH batch fermentations

Jonathan Emiliano Laiño [a], Marianela Juárez del Valle [a], Elvira María Hébert [a], Graciela Savoy de Giori [a, b], Jean Guy LeBlanc [a, *]

[a] Centro de Referencia para Lactobacilos (CERELA-CONICET), Chacabuco 145, T4000ILC San Miguel de Tucumán, Tucumán, Argentina
[b] Cátedra de Microbiología Superior, Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, San Miguel de Tucumán, Tucumán, Argentina

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ABSTRACT

Folate production by *Streptococcus thermophilus* CRL 803 was examined during batch fermentations without pH control and at constant pH (6.0 and 5.0) at different incubation temperatures. Folate production was higher at 42 °C under both constant and non-constant pH fermentation conditions, the increase being more pronounced at constant pH 6.0. Folate production by *S. thermophilus* CRL 803 under different fermentation conditions was cell-growth-associated. These observations were supported by analysis at the transcriptional levels of *fol* genes in controlled pH of 6.0 and uncontrolled pH batch culture. At pH 6.0, the highest relative expression levels of *folE, folQ, folK* and *folP* genes (involved in *de novo* synthesis) were observed after 6 h of incubation.

This is the first study on the expression of *fol* mRNA and their relation with folate synthesis by *S. thermophilus*, which is influenced by culture conditions.

1. Introduction

Folate is involved in many metabolic reactions, including the biosynthesis of ribonucleotides which are the building blocks of DNA and RNA (Hanson & Roje, 2001; Sybesma, Starrenburg, Tijsseling, Hoefnagel, & Hugenholtz, 2003). Mammalian cells are unable to synthesize folate and it is necessary to assimilate this vitamin exogenously to avoid adverse health outcomes associated to its deficiency. Therefore, an optimal dietary intake of folate is important. An alternative approach, currently being carried out in many countries, is the mandatory fortification of foods of mass consumption with folic acid. However, the benefits and risks of folic acid supplementation remains widely debated. Many studies have shown that excess intake of folic acid, the chemically synthesized form of folates, can cause adverse effects in some individuals such as the masking of the early hematological manifestations of vitamin B_{12} deficiency (reviewed in LeBlanc et al., 2011; Laiño, Savoy de Giori, & LeBlanc, 2013). Since natural folates, found in foods and sometimes produced by micro-organisms as derivatives of the reduced form tetrahydrofolate (THF), do not mask vitamin B_{12} deficiency, these would be a more efficient and secure alternative than supplementation with folic acid (Lamers, Prinz-Langenohl, Bramswig, & Pietrzik, 2006). Certain lactic acid bacteria (LAB) are capable to produce folates being a strain-dependent trait (Crittenden, Martinez, & Playne, 2003; Lin & Young, 2000; Sybesma et al., 2003). The *fol* genes (*folE, folQ, folK, folP, folA* and *folC*), which code the enzymes responsible of folate synthesis, have been identified in some LAB such as *Lactococcus* (*Lc.*) *lactis,* and *Lactobacillus* (*L.*) *plantarum* (Kleerebezem et al., 2003; Li, Zhou, & Gu, 2016; Rossi, Amaretti, & Raimondi, 2011; Wegkamp, van Oorschot, de Vos, & Smid, 2007; Capozzi, Russo, Due, ñ, as, L, ó, pez, & Spano, 2012). Although published genomes of *Streptococcus* (*S.*) *thermophilus* demonstrate the presence of *fol* genes, not all members of this specie produce folates (Laiño, LeBlanc, & Savoy de Giori, 2012). Previously we demonstrated that *S. thermophilus* CRL803 was able to grow in folate-deficient medium and was shown to produce large amounts of folate on complex media such as yogurt (Laiño et al., 2012; Laiño, Juárez del Valle, Savoy de Giori, & LeBlanc,

* Corresponding author.
E-mail addresses: leblanc@cerela.org.ar, leblancjeanguy@gmail.com (J.G. LeBlanc).

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2013). Thus, the use of folate producing *S. thermophilus* as a strategy for the bio-fortification of fermented foods would represent an attractive alternative to mandatory folic acid fortification programs.

The aim of the present study was to obtain better insight into the folate production by *Streptococcus thermophilus* CRL803 in free and controlled pH batch fermentations using different incubation temperatures and evaluate the expression of *fol* genes.

2. Materials and methods

2.1. Microorganisms and culture media

The strain *S. thermophilus* CRL803 was obtained from the culture collection of Centro de Referencia para Lactobacilos (CERELA, San Miguel de Tucumán, Argentina). This strain was previously selected for its folate-producing ability during a screening of 92 LAB strains belonging to the species *Lactobacillus (L.) delbrueckii* subsp. *bulgaricus* and *S. thermophilus* (Laiño et al., 2012).

The microorganism was activated in LAPTg broth, containing (w/v) 1.5% peptone, 1% tryptone, 1% yeast extract, 1% glucose and 0.1% Tween 80 (pH 6.5 ± 0.2), for 16 h at 42 °C.

Fermentations were carried out in modified LAPTg growth medium (mLAPTg) where yeast extract was omitted because of its high folate content.

Solid agar medium was prepared by the addition of 1.5% agar (Oxoid Ltd, Basingstoke Hampshire, UK) to the liquid medium.

2.2. Fermentation experiments

pH-controlled fermentations were performed in a 2.0 L BioFlo C32 fermentor (New Brunswick Scientific Co., Edison, N.J.) containing 1.5 L of mLAPTg culture medium. Agitation of 100 rpm was applied, temperature was maintained at 37 °C or 42 °C, and pH was automatically controlled at 5.0 or 6.0 with sterile 3N NaOH. A 16 h culture was washed twice with sterile saline solution (0.85% NaCl), suspended in 10 mL saline solution, and added to the fermentation vessel to reach an optical density of 0.1 at 580 nm (OD_{580}) [representing 10^5 colony-forming units (CFU)/mL]. Fermentation proceeded for 24 h; and samples were aseptically withdrawn from fermentation vessel at 0, 2, 4, 6, 8, 10, 12, and 24 h of incubation to evaluate: cell growth (OD_{580}); cell viability (CFU/mL; using the plate dilution method in LAPTg agar plates incubated at 37 °C for 48 h) and folate production.

Fermentations with free pH (without pH control) were carried out in the same conditions where the initial pH of the medium was 6.5 ± 0.2 and pH of sample at each time point was measured.

2.3. Flow cytometry viability analysis

Analysis was performed on a BD FACSCan flow cytometer (Becton–Dickinson, Co., Sparks, Maryland, USA). A combination of forward (FSC) and side scatter (SSC) was used to discriminate bacteria from background. Dyes (PI, propidium iodide, and TO, tiazol orange, 1 μL each one) were added to an aliquot (100 μL) of sample, and mixed by vortexing 30 s. Data (200,000 events) was collected with CELLQuest software (version 3.1; Becton Dickinson), and further analysed with FlowJo v7 software (Tree Star, USA). Previous to flow cytometric analysis, 10 μL of dyed samples were taken, and observed at 40 × with a fluorescent microscope Axio Scope A.1 (Zeiss, Oberkochen, Germany). Pictures were acquired with Axio Vision Software Rel. 4.8.2 (Zeiss, Oberkochen, Germany).

2.4. Folate determination

Samples (1 mL) of LAB-containing mLAPTg, aseptically withdrawn at different incubation times, were mixed with 1 mL of protection buffer (0.1 mol/L 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 (Laiño, Zelaya, Juárez del Valle, Savoy de Giori, & LeBlanc, 2015), was performed in all samples.

Folate determination was performed using a modified microbiological assay using *L. casei* subsp. *rhamnosus* NCIMB 10463 as indicator strain as was previously described (Laiño et al., 2012). In every case, non-inoculated samples analysed simultaneously, were used as controls.

2.5. Analysis of transcriptional levels of fol genes by quantitative real-time PCR (RT-qPCR)

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). 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. thermophilus* strains (LMD9, CNRZ1066 and LMG18311) 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.

Total RNA from growing cultures of *S. thermophilus* CRL803 was extracted at different times (2, 4, 6, 8, 10 and 12 h) obtained during fermentations at 42 °C, using a NucleoSpin® RNA kit (Macherey-

Table 1
Genes and corresponding primer sequences used for real-time-PCR analysis.

Gene	Primers	Sequence (5′–3′)
folE	For	TGGGGATAAGGTGACAGGAC
	Rev	CCTCGAGGATTAAGGGCTTC
folQ	For	CAGGGCTTCACAAAGTGACA
	Rev	TAGCACCAGCTAAACGCTCA
folK	For	GCTTGCTTGAGACGGAAGTT
	Rev	CCTGACAAGCACTCAGCAAG
folP	For	CGGAATTTGTAACCGAGGAA
	Rev	CTGGCAGTGGCTGTCTTGTA
folC1	For	ATCATTGAAGCAGGGATTGG
	Rev	GCTATCGCCCAAAGTTTCAG
folC2	For	GGTCTTGGGCAATACTCAT
	Rev	ATTGCTATTTGGGCGGAAG
folA	For	ATTGCTATTTGGGCGGAAG
	Rev	CATGCCATCAAAGGTCACAC
gyrB	For	CTCCAAAGAAGGCTTGCATC
	Rev	CCACGACCATCATCAACAAC
recA	For	TGTAGGACTTCAAGCGCGTA
	Rev	TCCACCTGGGGTAGTCTCTG

Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer's recommendations. Gene expression was compared from samples taken under constant pH 6 cultivation condition (cells grown in mLAPTg during batch fermentations at constant pH 6.0) respect to the reference condition (cells grown in mLAPTg during batch fermentations at free-pH).

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

RT-qPCR reaction was performed using SensiFastTM 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 RT-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.

The fold change of each transcript in each sample relative to the reference condition was measured in triplicates, normalized to internal control gene *recA* and calculated according to the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001). 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 twofold lower or higher than reference condition.

2.6. 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 $p < 0.05$ was considered significant.

3. Results

Growth and folate production by *S. thermophilus* CRL803 were evaluated in mLAPT medium at different incubation temperatures (37 or 42 °C) during batch fermentation at constant pH 6.0 and 5.0 and uncontrolled pH.

Temperature affected folate production: its concentrations were approximately 40% higher at 42 °C than at 37 °C in both controlled (pH 5.0 and 6.0) and uncontrolled pH fermentation conditions (data not shown). The control of pH also enhanced folate production between 2- and 3-fold compared with free pH batch fermentations with folate levels being considerably higher when *S. thermophilus* CRL803 was grown at pH 6.0 (240 ± 14 μg/L) than at pH 5.0 (148 ± 9 μg/L) after 6 h-fermentation at 42 °C (Fig. 1). Intra- and extra-cellular folate concentrations also reached their maximal amounts (89 ± 5 and 151 ± 9 μg/L, respectively) at the same time point at pH 6.0 (data not shown).

The strain grew well in mLAPT medium in all batch fermentation conditions assayed, as reflected by its viable cell counts (Fig. 2) where maximal cell counts (7.5 ± 0.2–8.8 ± 0.2 log CFU/mL) were obtained at 4 h of incubation after which the number of cells decreased slightly. The OD_{580nm} values were highest at 6 h of incubation at pH 6.0 than at pH 5.0 and at free-pH conditions (twofold and fourfold higher, respectively) and also significantly higher at pH 5.0 than in free-pH conditions where a twofold

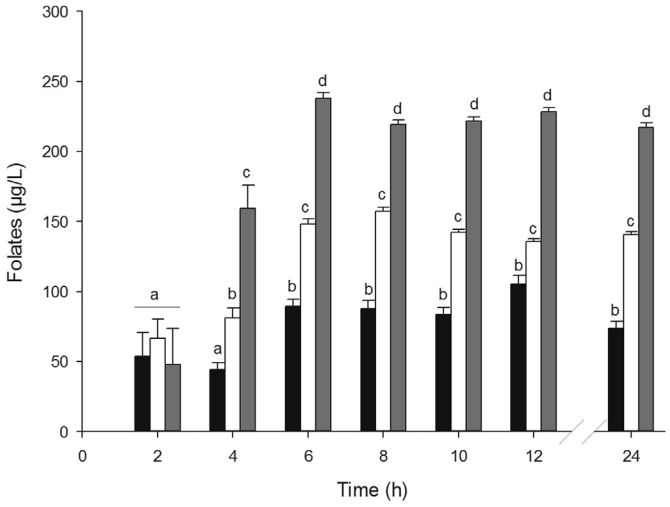

Fig. 1. Folate production by *S. thermophilus* CRL803 in mLAPTg medium at 42 °C during batch fermentation at a constant pH of 6.0 and 5.0 and free pH. Results are expressed as means ± standard deviation (SD). Black bars represent free-pH, empty bars represent pH 5.0, and gray bars represent pH 6.0.

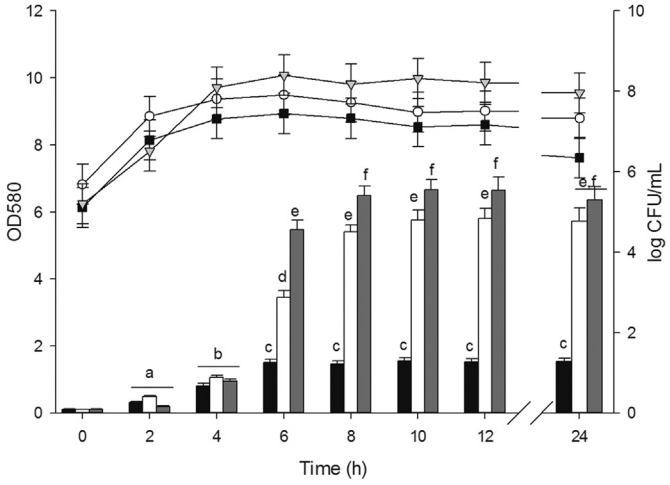

Fig. 2. Cell growth [cell viability (log CFU/mL), OD_{580}] of *S. thermophilus* CRL803 grown in mLAPTg medium at 42 °C with constant pH of 6.0 and 5.0 and free pH. Results are expressed as means ± SD. [a-f]Means with different letters differ significantly ($p < 0.05$). Black bars represent OD_{580} at free-pH, empty bars represent pH 5.0, and gray bars represent pH 6.0. Black squares represent log CFU/mL at free-pH, empty circles represent pH 5.0, and gray triangles represent pH 6.0.

increase was observed at the same time point (Fig. 2).

To analyze the effect of environmental pH on *S. thermophilus* CRL803 viability, flow cytometry was used in samples taken in mLAPT medium at 42 °C during batch fermentation with free pH and constant pH of 6.0 where the highest differences in OD_{580} were observed (Fig. 2). During the first 6 h of incubation, viable cell populations both at free-pH and controlled pH of 6.0 remained constant; after which viable cells counts remained unchanged at pH 6.0, while at free-pH, this sub-population decreased significantly (supplemental data). The loss of viability resulted largely due to an increase of relative frequency of damaged cells population from about 2.6 ± 0.3% to 23.3 ± 3.5%, between starting and 8 h-incubation, respectively.

From these vitamin production results, the relative expression of *fol* genes of *S. thermophilus* CRL803 as a function of environmental pH was evaluated. To this end, a quantitative real-time PCR

(RT-qPCR) methodology was applied to allow comparison of the transcript levels of all *fol* genes in this strain grown in different culture conditions. Relative expression levels for each gene were determined by qPCR using cells grown in mLAPT, from 2 to 12 h-incubation, in constant pH of 6.0 and free pH (condition used as reference) batch fermentations. For *folE, folQ* and *folK* genes, the relative expression level was highest when the culture was in the exponential growth phase (6 h) (Fig. 3) which also corresponded to the maximal production point (Fig. 1). Before and after this time point, no differences in the relative expression level were observed. At 6 h of incubation, a 2- to 3-fold increase in relative expression level of these genes was found at pH 6.0 compared with non-constant pH. No significant differences in relative expression level of *folC1* and *folA* gene were detectable under all assayed conditions and only a small increase was observed for *folC2* after 6 h incubation.

4. Discussion

The use of folate-producing LAB is a more natural and economically viable alternative to fortification with chemically synthesized vitamin and could be used as bio-factories for the *in situ* delivery of the natural forms of folates that are less likely to cause undesirable side-effects (Laiño et al., 2015). This property is strain-dependent trait and is affected by culture conditions (Crittenden et al., 2003; Lin & Young, 2000; Sybesma et al., 2003). Previously, it was shown that *S. thermophilus* CRL803 has the ability to synthetize folate *de novo* in a folate-free culture medium and in complex matrix such as milk (Laiño et al., 2012; Laiño, Juárez del Valle et al., 2013). Here, as an approach to increase folate production by this strain, fermentations automatically controlled at pH values of 6.0 and 5.0 (or uncontrolled pH) and different incubation temperatures were performed. In this study it was shown that at constant pH 6.0 and 42 °C-incubation allowed enhancing folate production by this strain at most incubation times. In contrast, it was reported that *S. thermophilus* strains produced high folates levels when these were incubated at 37 °C. However, the highest folate production by *S. thermophilus* CRL803 in this study was similar to that reported previously by *S. thermophilus* B119 (Sybesma et al., 2003).

In the current study, *S. thermophilus* CRL803 showed partial excretion of folates into the external medium. This excretion of folate was observed to be strongly dependent on the pH of the cultivation medium. Under controlled growth conditions at pH 5.0, the cytoplasmic retention of folate in this strain was relatively low (11%), resulting in increased excretion of folate into the medium.

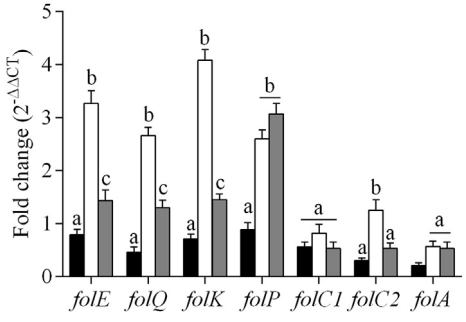

Fig. 3. Relative expression of *fol* genes in *S. thermophilus* CRL803 grown in mLAPT medium with constant pH of 6.0 and free pH incubated at 42 °C after 4 (black), 6 (white) and 8 h (grey) of incubation.
Results are expressed as means ± SD. [a-c]Means with different letters differ significantly for the same gene evaluated at the different time points ($p < 0.05$).

Sybesma et al. (2003) have reported that excretion of folate was stimulated at lower pH in *S. thermophilus* strains; in contrast, other authors observed that culture pH did not affect folate excretion by *S. thermophilus* BAA-250, *B. adolescentis* MB 239 and *Lactococcus lactis* strains (Burgess, Smid, & van Sinderen, 2009; Mousavi et al., 2013; Pompei et al., 2007).

Folate production by *S. thermophilus* CRL 803 both at constant pH and free pH was cell-growth-associated. Specific folate production was higher when the strain was grown at constant pH of 6.0 than at pH 5.0 or free pH, in agreement with literature data that report on the high pH optima (near 7.0) of all the enzymes involved in folate biosynthesis examined in several microorganisms such as *Lactobacillus leichmaniii* ATCC 7830 (Rao, 2000). The maximum folate production was observed at the beginning of the stationary phase (6 h incubation). These facts suggest that physiological state of cell is a factor affecting metabolite production. In lactic acid bacteria, the production of lactic acid and other acids can lead to an inhibition of their growth through different mechanisms. This could explain that cell growth determined by optical density was significantly higher at pH 6.0 with respect to uncontrolled conditions. Generally, elevated OD values are related in high cellular counts. It is well known that maintaining membrane integrity and intracellular environment is essential for normal cell functions. In our study, flow cytometric analysis was applied to profile the physiological status of the strain exposed at different environmental pH conditions. The use of multiple physiological probes allowed discriminating physiological heterogeneity within the bacteria population.

More damaged cells were found in cultures grown for 8 h-at free pH compared to those at controlled pH of 6.0. It has been reported that when lactic acid bacteria are exposed to various forms of sublethal stress, some cells fail to grow on nutrient agar but still exhibit metabolic activity (Rault, Bouix, & Béal, 2009). This might explain the high cell density (OD_{580}) found, after 6 h-incubation at constant pH 6.0 when cell counts (CFU/mL) remained low. In this condition, *S. thermophilus* CRL803 would be considered viable-but-non-culturable because the majority (93.7%) of the cells remained viable while cell counts remained unchanged. Even though the cells were not cultivable, they remained biologically active as shown by their capacity to continue producing folates, reaching the highest folate levels after 6 h incubation. These observations were supported by analysis of transcriptional levels of *fol* genes of *S. thermophilus* CRL803 by RT-qPCR. The expression of the *folE, folQ, folK* and *folP* genes of this strain was markedly increased when culture was grown, before 6 h-incubation, at constant pH 6 as compared to the reference condition. The direct correlation between the high levels of *fol* genes expression and the maximum folate production suggests that there is an up regulation of these genes at 6 h incubation. At pH 6.0, no significant increase of mRNA levels were detected before 6 h-incubation while the highest relative expression levels of *folE, folQ, folK* and *folP* genes, involved in *de novo* synthesis, were observed at this time. These results suggested that greater growth in optimal culture conditions would lead to higher synthesis of *fol* enzymes. No significant modifications were observed on mRNA levels of *folA* and *folC* genes respect to reference condition (free-pH).

Sybesma et al. (2003) demonstrated that it is only necessary to overexpress *folK* and *folE* to increase folate synthesis by *Lc. lactis*. These authors also reported that the overexpression of other genes coding for *p*ABA synthesis did not lead to increase folate concentration unless a simultaneous overexpression of *folK* and *folE* were performed. Subsequently, it was also reported that *S. thermophilus* possesses the complete pathway for folate biosynthesis from GTP or *p*-aminobenzoic acid (PABA) precursor, as well as *de novo* synthesis of PABA from chorismate (van de Guchte et al., 2006).

To the best of our knowledge, this is the first study on the expression of *fol* mRNA and their relation with folate synthesis, which is influenced by culture conditions, in *S. thermophilus*. It was shown that external pH significantly affects folate production that was associated to growth. Also, the physiological state in which the strains are found can also affect *fol* genes expression as demonstrated here by viable but non-culturable cells that express *fol* genes and produce folate. New studies using food models are needed to transfer this information to increase the potential use as a functional starter for the production of a healthier food with increased folate concentrations.

Disclosure statement

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.lwt.2017.07.004.

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