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Research Letter

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Screening of lactic acid bacteria producing folate and their potential use as adjunct cultures for cheese bio-enrichment

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One sentence summary: Five LAB strains were able to provide natural folate bio-enriched cheese; the folate content increased with ripening up to by 54% after 30 d and up to 113% after 60 d.

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ABSTRACT

Lactic acid bacteria (LAB) can be used to increase the folate in foods by *in situ* fortification. Seventy LAB were screened for their ability to produce folate during growth in de Man, Rogosa and Sharpe/M17 broth. *Lactobacillus casei, Lactobacillus plantarum, Lactobacillus paracasei* subsp. *paracasei*, *Lactobacillus rhamnosus, Lactobacillus delbrueckii* subsp. *bulgaricus*, *Streptococcus thermophilus*, *Lactococcus lactis* subsp. *lactis*, *Enterococcus faecium* and *Enterococcus lactis* were able to synthetize folates in the medium, even if to a different extent. The 47 folate-producing strains were further analyzed by microbiological assay, for total, extra and intracellular folate. *Enterococcus faecium* VC223 and *E. lactis* BT161 were able to produce in cultural medium 123,625.74 ± 8.00 ng/ml and 384.22 ± 5.00 ng/ml of folate, respectively. Five strains were further examined for their ability to synthesize folate in cheese. The folate content increased with ripening up to by 54% after 30 d when *L. casei* VC199 was used and up to 108% and 113% after 60 d, with *L. paracasei* SE160 and *E. lactis* BT161 respectively exceeding 100 ng/100g. Results encourage the use of specific LAB to obtain natural folate bio-enriched dairy products improving folate intake.

Keywords: lactic acid bacteria; folate; cheese; vitamin; fortification; functional food

INTRODUCTION

Tetrahydrofolate molecule and its numerous derivatives are generally known as folate, water-soluble vitamins belonging to the B-group (Capozzi *et al.* 2012; LeBlanc *et al.* 2014). Folic acid is the chemically synthesized form of folate and is commonly used for food fortification. Folic acid (pteroylglutamic acid) is monoglutamate form, while all-natural vitamin forms are polyglutamates (Hoffpauer and Bonnette 1998, Rydon 2016).

Folates are important because they are involved, as cofactors of metabolic enzymes, in vital pathways such as deoxyribonucleic acid replication, cell division (Duthie *et al.* 2002), repair and methylation, biosynthesis of nucleotides and amino acid metabolism (Cossins and Chen 1997; Hanson, Gage and Shachar-Hill 2000; LeBlanc *et al.* 2010; Greppi *et al.* 2017). Furthermore, they play an important role in the prevention of serious health problems, such as defects in the formation of the neural tube, megaloblastic anemias, Alzheimer's and coronary diseases and colorectal, breast and pancreas cancer (Giovannucci *et al.* 1998; Van Guelpen *et al.* 2006; Morris and Tangney 2007; Lin *et al.* 2013; Da Silva *et al.* 2016).

Humans are incapable of synthesizing this group of vitamins, and they consequently have to be obtained exogenously (LeBlanc *et al.* 2011). The major sources of folates are green leafy vegetables, legumes, nuts, fruits, liver, egg yolk, certain cheeses and fermented dairy products (Eitenmiller and Landen 1999; Arcot and Shrestha 2005; Greppi *et al.* 2017). Even when eating

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foods that are naturally rich in folate, daily level of intake is often not adequate to meet the nutrient requirements. Consequently, deficiencies are very frequent in many parts of the world, not only in developing countries, but also in some industrialized countries (Konings *et al.* 2001). For this reason, many countries have adopted laws to enforce the fortification of certain foods. However, a number of studies have shown that high intakes of folic acid, the chemically synthesized form of folate, can cause adverse health effects such as the masking of the early hematological manifestations of vitamin B12 deficiency, alteration in the activity of the hepatic dihydrofolate reductase enzyme (Bailey and Ayling 2009; Da Silva *et al.* 2016; Rosenberg and Selhub 2018) or promotion of cancer (Melnyk *et al.* 1999; Ulrich and Potter 2006; Baggott, Oster and Tamura 2012).

A natural enrichment of folates, by contrast, such as those found in foods or produced by certain microorganisms (Lin and Young 2000; Kariluoto *et al.* 2006; Santos *et al.* 2008), does not cause adverse health effects in individuals (Wright, Dainty and Finglas 2007). In this context, many studies have shown that the folate is frequently increased in certain fermented milk products such as yogurt, buttermilk and cheese due to the production by lactic acid bacteria (LAB) (LeBlanc *et al.* 2011). For this reason, several researchers have focused on the biosynthesis of folate using LAB strains (Sybesma *et al.* 2003) that could be an economically feasible alternative to chemical fortification and production of high-vitamin-containing products. In literature, large differences in folate biosynthesis by different LAB strains have been reported. In particular, many strains of *Lactococcus lactis*, *Streptococcus thermophilus*, *Leuconostoc* spp. and *Bifidobacterium* species were able to produce high amount of folate while, among the *Lactobacillus* species, only *Lactobacillus plantarum* could produce folate, whereas other species were shown to consume it (Sybesma *et al.* 2003; Pompei *et al.* 2007; Nor *et al.* 2010).

The aim of the present work was to evaluate the ability of 70 LAB strains belonging to 9 different species to produce or consume folate when grown in medium containing folates and their precursors using for the first screening Enzyme Linked Immuno Sorbent Assay (ELISA). All the producing strains (n = 47) were further studied with the microbiological assay (MA) and the differentiation between total, intra- and extracellular folate was carried out to better investigate the conditions for the synthesis of folate. The best five vitamin-producing strains were then tested in experimental cheese-making for the development of folate bio-enriched cheeses. We decided to use goat milk because of the increased interest of the consumers for goat milk products due to their health and nutritional value (Verruck, Dantas and Prudencio 2019).

MATERIALS AND METHODS

Bacterial strains and their maintenance

The 70 LAB strains used in this study were obtained from the CNR-ISPA Milano (National Research Council, Institute of Sciences of Food Production, Milan) collection. The strains belonged to different species: *Lactobacillus casei* (n = 7); *Lactobacillus delbrueckii* subsp. *bulgaricus* (n = 6); *Lactobacillus paracasei* subsp. *paracasei* (n = 2); *L. plantarum* (n = 15); *Lactobacillus rhamnosus* (n = 7); *Lc. lactis* subsp. *lactis* (n = 15); *Enterococcus faecium* (n = 5); *Enterococcus lactis* (n = 5); and *St. thermophilus* (n = 8). The enterococci strains did not harbor any of the virulence genes reported by European Food Safety Authority (EFSA 2012; Morandi, Silvetti and Brasca 2013, 2015).

The lactobacilli were grown in de Man, Rogosa, and Sharpe (MRS) broth (Biolife, Milano, Italy) at 30°C overnight with exception of *L. delbrueckii* subsp. *bulgaricus* that was cultured at 43°C. Enterococci, lactococci and streptococci were cultured in M17 broth (Biolife) at 37°C, overnight. All the strains were incubated in aerobic conditions and were sub-cultured twice in their respective broths for activation.

Screening for LAB with efficient folate-producing ability

For identification of folate-producing and folate-consuming LAB, each strain was inoculated at 1% (v/v) in 10 ml of the cultural broth and incubated under its own optimal conditions, for 24 h, in triplicate. Then, samples were vortexed intensely for 10 min and heated for 3 min in boiling water afterwards, then cooled to 20–25°C. They were centrifuged for 10 min at 3000 x g. The upper layer was aspirated and kept at −20°C until quantification was performed as described below. A sample of each batch of MRS and M17 broth underwent the same conditions, to quantify folate initially present in the medium.

Folate quantification by ELISA

Quantification of total folate was performed using ELISA (Abnova Corporation, Taipei, Taiwan). Folic acid conjugate was coated to a microplate with 96 wells. Each sample extract was used in ELISA plate in triplicate (three wells for each sample extract). The procedure used was that recommended by the manufacturer's instructions. The microplate was mixed before the absorbance was measured at 450 nm within 30 min with a microtiter plate reader (TECAN, Infinite F200 PRO, Männedorf, Switzerland). Controls were kept without inoculum. A standard curve for folic acid was obtained by using known concentrations from 0 to 400 ng/ml (ready to use) supplied with the kit. The folic acid concentration (ng/ml) corresponding to the extinction of each sample was read from the calibration curve.

Determination of total, intra- and extracellular folate by microbiological assay

The 47 folate-producing strains as evidenced by ELISA assay (12 cocci and 35 rods) were further tested by using a MA. The differentiation between total, intra- and extracellular folate was performed. The strains were inoculated (1%, v/v) in their own cultural broth and at their own temperature (see the first paragraph), and incubated for 24 h.

Sample preparation

The 47 folate-producing strains as evidenced by ELISA assay (12 cocci and 35 rods) were further tested by using a microbiological assay. The strains were inoculated (1%, v/v) in their own cultural broth and at their own temperature (see the first paragraph), and incubated for 24h. Cells and supernatants were recovered from 5 mL of a full-grown cell culture (OD600nm 1.0) and were used to measure intra- and extracellular folate concentration respectively. Briefly, a centrifugation step (12,000 x g, 10 min, 20°C) was performed to separate the supernatant from the cells. The supernatant was diluted 1:1 in 0.1 M phosphate buffer (pH 7.2) (Sigma Chemical Co., St. Louis, MO, USA) and 0.1% w/v sodium ascorbate (Sigma). The cells were washed with 0.1 M phosphate buffer (pH 7.2) and 0.1% w/v sodium ascorbate, and resuspended in 5 ml of the same buffer then they were lysed with lysozyme

(Bioseutica, Lugano, Switzerland) was added to a final concentration of 100 mg/kg and incubated for 5 min at 37°C. All samples (cells and supernatant) were boiled at 100°C for 5 min in order to obtain folate release from cells and from folate-binding proteins. Since the MA is less sensitive to longer chain polyglutamyl folate (Iyer and Tomar 2013), the enzymatic deconjugation of the samples to complete hydrolysis of polyglumates to simpler monomeric form was performed by incubating samples with pig pancreatin (Sigma) for 2h at 37°C in the dark. Thereafter, samples were heated at 95°C for 30 min in a water bath and were cooled to 30°C. One ml of the sample extracts was transferred in sterile tubes and centrifuged for 5 min at 8,000 x g, then analysed by MA assay (Sybesma *et al.* 2003; Nor *et al.* 2010).

Folate quantification by microbiological assay

The quantification of extracted folate (total, as well as extra- or intracellular) was carried out by a MA based on the growth of the indicator strain *L. rhamnosus* on 96-well microtiter plates (VitaFAST® Folic Acid from R-Biopharm AG, Darmstadt, Germany). The wells were filled by adding 150 μl of folic acid assay medium (contained in the kit) and 150 μl of an unknown or reference sample in sterile water. For reference samples, folic acid was dissolved in the sterile water at a concentration ranging from 0 to 1.28 μg/100 g (ml). Control wells were not inoculated to check for sterility of the procedure.

Each sample and standard were analyzed in triplicate. The plates were then incubated in the dark at 37°C for 44–48 h and the growth was measured at 620 nm by using a microplate reader (TECAN, Infinite F200 PRO) as the bacteria grow until the vitamin is consumed. The intensity of metabolism or growth in relation to the extracted folic acid was measured as absorbance and expressed as optical density, OD). The folate concentration of the samples was determined by comparing the OD obtained for treated samples with that obtained for the standard curve prepared.

Cheese manufacture

The five most active LAB strains (*L. casei* VC199, *L. paracasei* subsp. *paracasei* SE160, *L. plantarum* VS513, *E. faecium* VC223 and *E. lactis* BT161) were used to produce goat cheeses naturally enriched with folate. Experimental and control cheese-making trials were conducted by 'Il Boscasso cheese factory' (Ruino, Pavia, Italy) as previously described by Albano *et al.* (2018). Briefly, 10 l of goat raw milk (3.5% fat w/w) pre-warmed to 34 °C were inoculated with 10^7 CFU/ml of each test strain along with a commercial freeze-dried thermophilic lactic culture (TC 00, Santamaria s.r.l., Burago di Molgora, Italy). Cheeses manufactured only with the industrial starter culture was used as control. Coagulation took place at 35°C and was obtained within approx. 25 min by the addition of calf rennet; curd was then cut into maize size grains and gently stirred for additional 10 min, then placed in round moulds (height 6 cm, diameter 12 cm) for 20 hours before dry salting and ripened up to 60 days. Three groups of cheese-makings were carried out, each with its own control; two cheese-making trials were performed for each test strain.

Folate content in cheese

Folate content in cheese was determined as previously reported by Johnston et al. (2002). Briefly, a total of 500 mg of cheese was accurately weighted into a vial to which 10 mg of pig pancreatin (Sigma), 20 mL of 0.1 M phosphate buffer (pH 7.2) and 0.1% sodium ascorbate were also added. Samples were incubated for 2 h at 37°C, in the dark, then heated at 95°C for 30 min in a water bath and cooled to 30°C. The mixture were centrifuged for 5 min at 8000 x g and the supernatant sterilized through 0.22 μm filter. Folate level in cheese was then determined by MA, as described before. All the assays were performed in triplicate.

Statistical analysis

Data analysis was carried out using the Minitab version 14.0 software. The significance of differences was analyzed statistically by analysis of variance. A difference of $P < 0.05$ was considered significant. All analyses were conducted in triplicate.

RESULTS AND DISCUSSION

Screening for LAB with efficient folate-producing ability

Folate is an essential vitamin, which is involved in many of the most important metabolic pathways. The recommended daily intake for an adult is 200–400 μg of folate (400–600 μg for pregnant women) and food fortification programs have been adopted in many countries to increase folate intake (Institute of Medicine 1998; FAO/WHO 2002; Saubade *et al.* 2017). Numerous researchers have reported that LAB have the ability to synthesize folate during fermentation (Saubade *et al.* 2017) and suggested the use of selected starter cultures to explain food folate bio-enrichment. In the present study, an initial screening on the ability of the 70 LAB strains to synthetize or utilize folate in culture medium was done with the ELISA test that is based on the specific interaction of a polyclonal antibody with its antigen, folate. Immunoassays are generally highly sensitive and have high and specific affinity interaction that occurs even in complex matrices. In addition, the ELISA test performed on a microtitration plate format is fast proving to be well suited to routine food analysis (Finglas and Morgan 1994; Arcot, Shrestha and Gusanov 2002).

The results about the *in vitro* folate-producing capacity evaluated by ELISA test of the 70 LAB strains after 24 h of growth in MRS or M17 are shown in Fig. 1.

The incubation time was set to 24 h because the final aim of the study was the selection of strains capable of producing folate in food as a result of the fermentation process. The results were corrected for initial folate present in the medium, with negative values indicating folates consumption. All the 9 species (*L. casei*, *L. plantarum*, *L. paracasei* subsp. *paracasei*, *L. rhamnosus*, *L. delbrueckii* subsp. *bulgaricus*, *St. thermophilus, Lc. lactis* subsp. *lactis*, *E. faecium* and *E. lactis*) were able to synthetize folates, and most of the LAB strains (42 out of 70) were able to produce folate in the medium, even if to a different extent (Fig. 1).

In *L. casei* group (n = 7), all the strains were able to produce folate, the total folate production ranging between 3.33 and 7.29 ng/ml. A higher production was evidenced among *L. plantarum* strains (n = 15), that were found to produce folate in cultures between 5.64 and 34.41 ng/ml, with an average of 23.20 ng/ml. In particular, one strain produced 5.64 ng/ml, 11 strains produced between 17.10 and 29.46 ng/ml and three strains produced more than 30 ng/ml (VS513, SE148 and SE140). Both the two *L. paracasei* subsp. *paracasei* strains produced folate with an average of 1.50 ng/ml while in *L. rhamnosus* group (n = 7), the total folate production ranged between −4.62 and 8.87 ng/ml. Two strains consumed folate from the medium, two strains were not able to produce folate in MRS medium (their maximum

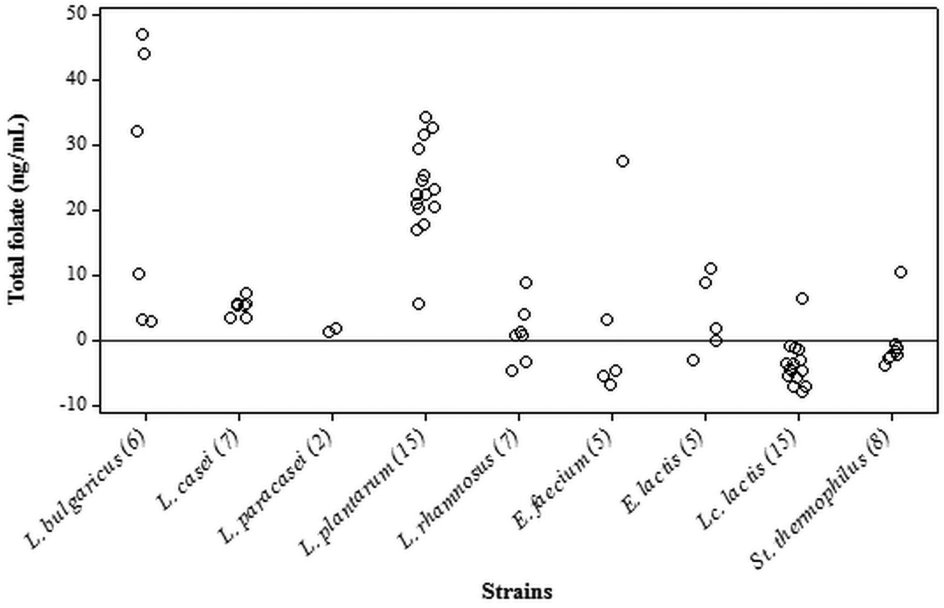

Figure 1. Production or consumption of folate by 70 LAB strains incubated 24 h in folate-containing medium (MRS or M17 broth). Results are corrected for initial folate present in the medium, with negative values indicating folates consumption. For each species, the number of strains tested is indicated in parenthesis and each dot represents one strain. Data are mean values derived from three independent experiments analyzed by ELISA assay.

folate production was 0.60 ng/ml) and three strains produced between 1.28 and 8.87 ng/ml.

All the *L. delbrueckii* subsp. *bulgaricus* strains (n = 6) produced folate widely differing among the strains: four strains produced between 2.86 and 32.23 ng/ml and two strains produce more than 40 ng/ml (A753 and A751). Seven out of eight *St. thermophilus* strains consumed folate from the M17 medium while one strain produced 10.46 ng/ml. Among *Lc. lactis* subsp. *lactis* strains (n = 15), only one strain was able to produce detectable amount of folate (1.21 ng/ml), while all the others were folate-consuming strains. In *E. faecium* group (n = 5), three strains consumed folate and two produced between 3.05 and 27.64 ng/ml. Among *E. lactis* (n = 5), one strain consumed folate (BT159), one neither consumed nor produced (BT220) and three strains produced folate up to 11.08 ng/ml (Fig. 1).

Overall, by ELISA test, *L. delbrueckii* subsp. *bulgaricus* and *L. plantarum* species appeared to provide the highest folate content, but the production was found to differ substantially depending on the strain.

The use of ELISA-based kits for folate determination is limited due to a varying affinity for the different forms of folates (Finglas and Morgan 1994; Arcot and Shrestha 2005). Generally, ELISA test has a much lower response to folate derivatives other than folic acid, thereby underestimating the natural folate content of foods. This statement was confirmed in the present study, since folate content in the culture medium after LAB growth determined by ELISA was significantly lower in comparison with microbiological analysis ($P < 0.001$).

Folate determination with microbiological assay

Based on the results obtained by the ELISA test, 47 out of the 70 LAB strains were chosen for further analysis by MA. In particular, we focused our attention on the 35 folate-producing *Lactobacillus* strains, and the three highest folate-producing strains of *St. thermophilus, Lc. lactis, E. faecium* and *E. lactis* species. MA serves as the traditional and most versatile method of folate analysis and is the only food folate method given official status by American Association of Analytical Chemists (Iyer and Tomar 2009). So, these strains were analyzed by MA for extracellular (in cell-free supernatants) and intracellular (after cell lysis) folate production and these two forms were used to determine total folate production.

The presence of folate in the bacterial cell is of great importance when ones want to use bacteria for in situ bio-fortification of food. If the food product is cooked, it will allow the bacterial cell lysis, thus liberating all folate produced (both intra- and extracellular). On the other hand, if the food is consumed raw, intracellular folate may stay entrapped inside the bacterial cell, and thus may not be available for absorption. Therefore, if bacteria are consumed alive, without cooking of the food, extracellular production will be preferred, since bacteria would excrete their metabolites in the human gastrointestinal tract (Strozzi and Mogna 2008). In humans, folate produced by the microbiota in the small intestine is assimilated by the host and the extracellular folates are mostly monoglutamates, whose absorption seems to be higher than that of polyglutamates (LeBlanc *et al.* 2007; Ohrvik and Witthoft 2011; Greppi *et al.* 2017). For these reasons, we wanted to investigate not only the total folate production in a folate containing medium, but whether the production was mainly intra- or extracellular.

Folate quantification was performed after 24 h of incubation in MRS or M17 broth and the results are shown in Tables 1 and 2.

With exception of *L. plantarum* VC194, MA confirmed that all the *Lactobacillus* strains were folate-producing strains. The results highlighted also that, in most strains (cocci or rods), folate production levels determined with MA were significantly higher than those of ELISA test (Fig. 1. Table 1-2). The difference is reasonably due to the fact that MA quantifies the total folate concentration including polyglutamyl folate while ELISA method corresponds only to folic acid.

Table 1. Extra- and intracellular folate content after incubation of 35 rod strains in MRS broth.

Species	Strain	Extracellular folate (ng/mL)	Intracellular folate (ng/mL)
Lb. casei	SV54	21.81 ± 2.0	18.42 ± 1.2
Lb. casei	VC199	35.74 ± 1.5	4.99 ± 0.3
Lb. casei	SV226	8.32 ± 1.0	7.06 ± 0.1
Lb. casei	VC235	6.12 ± 1.0	5.82 ± 0.8
Lb. casei	VC201	5.88 ± 1.0	9.90 ± 0.1
Lb. casei	BT199	8.50 ± 2.0	4.20 ± 0.1
Lb. casei	BT147	1.20 ± 1.1	3.30 ± 0.2
Lb. plantarum	VS513	72.99 ± 2.0	14.51 ± 0.8
Lb. plantarum	VS166	47.69 ± 1.0	36.11 ± 3.0
Lb. plantarum	VS377	40.96 ± 2.0	25.04 ± 2.0
Lb. plantarum	SE90	36.77 ± 5.0	19.23 ± 0.6
Lb. plantarum	SE112	27.67 ± 2.0	22.83 ± 2.0
Lb. plantarum	SE125	28.60 ± 2.0	7.40 ± 1.0
Lb. plantarum	VC114	22.90 ± 1.0	12.30 ± 0.2
Lb. plantarum	SE148	20.83 ± 2.0	11.27 ± 0.9
Lb. plantarum	SE196	5.63 ± 3.0	21.60 ± 0.5
Lb. plantarum	SE140	8.79 ± 3.0	18.20 ± 0.5
Lb. plantarum	VS516	5.30 ± 2.0	21.70 ± 0.9
Lb. plantarum	VC233	9.79 ± 4.0	4.21 ± 1.0
Lb. plantarum	BT205	4.14 ± 1.0	9.46 ± 0.7
Lb. plantarum	VC212	3.45 ± 1.0	2.65 ± 0.2
Lb. plantarum	VC194	− 7.90 ± 1.0	2.19 ± 1.5
Lb. paracasei subsp. *paracasei*	VC213	13.50 ± 0.2	6.47 ± 0.2
Lb. paracasei subsp. *paracasei*	SE160	16.40 ± 0.5	4.63 ± 0.1
Lb. delbrueckii subsp. *bulgaricus*	A751	9.10 ± 1.0	2.90 ± 0.5
Lb. delbrueckii subsp. *bulgaricus*	A753	11.20 ± 1.0	7.30 ± 0.6
Lb. delbrueckii subsp. *bulgaricus*	233	0.00 ± 0.1	6.00 ± 0.2
Lb. delbrueckii subsp. *bulgaricus*	123	1.00 ± 0.6	4.50 ± 0.2
Lb. delbrueckii subsp. *bulgaricus*	132	0.00 ± 0.5	2.50 ± 0.1
Lb. delbrueckii subsp. *bulgaricus*	133	0.10 ± 0.1	2.10 ± 0.5
Lb. rhamnosus	SV60	2.60 ± 0.6	10.00 ± 0.1
Lb. rhamnosus	VC221	10.00 ± 0.8	6.00 ± 0.5
Lb. rhamnosus	VC222	2.00 ± 0.2	4.00 ± 0.2
Lb. rhamnosus	VC228	0.00 ± 0.2	2.00 ± 0.4
Lb. rhamnosus	SE150	0.20 ± 0.1	2.00 ± 0.3

Extracellular and intracellular folate concentrations were corrected for folate initially present in the medium.
Results are shown as mean and standard deviations of three independent experiments analyzed by MA.

Table 2. Extra- and intracellular folate content after incubation of 12 cocci strains in M17 broth.

Species	Strain	Extracellular folate (ng/mL)	Intracellular folate (ng/mL)
Lc. lactis subsp. *lactis*	FT26	18.1 ± 4.0	33.6 ± 1.0
Lc. lactis subsp. *lactis*	N16	112.3 ± 5.0	27.7 ± 1.0
Lc. lactis subsp. *lactis*	N26	123.8 ± 1.5	6.9 ± 3.0
St. thermophilus	BT232	21.0 ± 5.0	5.8 ± 2.0
St. thermophilus	SE95	99.2 ± 3.0	16.5 ± 4.0
St. thermophilus	VS436	65.9 ± 1.1	15.3 ± 3.0
E. faecium	VC185	61.2 ± 2.2	7.6 ± 2.0
E. faecium	VC187	72.7 ± 8.0	12.8 ± 2.0
E. faecium	VC223	116 642.9 ± 1.3	6982.8 ± 2.0
E. lactis	BT161	304.3 ± 6.0	79.9 ± 3.0
E. lactis	BT188	68.6 ± 1.2	18.4 ± 2.0
E. lactis	BT204	55.4 ± 2.3	5.1 ± 3.0

Extracellular and intracellular folate concentrations were corrected for folate initially present in the medium.
Results are shown as mean and standard deviations of three independent experiments analyzed by MA.

The ability to synthesize folate after 24 h of incubation and partition between excretion (extracellular) and accumulation (intracellular) varied considerably among and also within the species (Table 1), as previously reported by other authors (LeBlanc *et al.* 2007; Laiño, LeBlanc and Savoy de Giori 2012). Folate production for the tested *Lactobacillus* species (*L. casei* (4.50-40.74 ng/ml), *L. plantarum* (5.71-89.50 ng/ml), *L. paracasei* subsp. *paracasei* (19.97-21.03 ng/ml), *L. rhamnosus* (2.00-16.00 ng/ml), and *L. delbrueckii* subsp. *bulgaricus* (2.20-18.50 ng/ml), confirmed that *L. plantarum* is the best producer species, and enrichment levels were quite similar to those observed by other authors.

Among the 12 cocci the two highest folate-producing strains were *E. faecium* VC223 (123,625.74±8.00 ng/ml) and *E. lactis* BT161 (384.22±5.00 ng/ml), but also two out of three *Lc. lactis* subsp. *lactis* (N16 N26) and and *St. thermophilus* SE95 produced more than 100 ng/ml after 24h of incubation in M17 broth (Table 2).

In particular, *E. faecium* VC223 and *E. lactis* BT161 were able to produce significant amount of folate in M17 after 24h of growth not observed in previous studies. To the best of our knowledge, no other study reported a production of folate equal to that obtained by us with *E. faecium* VC223. The only data available on folate production by enterococci are those by Divya, Varsha and Nampoothiri (2012) that isolated an *E. faecium* strain producing 12.58±0.11 ng/ml after 7h of fermentation in folic acid assay medium, confirming previous data reported by Crittenden, Martinez and Playne (2003); no data are available about *E. lactis* ability to synthesize folate.

Different studies demonstrated that yogurt starter *St. thermophilus* and *L. bulgaricus* as well as *Lc. lactis* and *L. plantarum* have the ability to synthetize folate in cultural medium with or without folate (Padalino *et al.* 2012; Saubade *et al.* 2017). Moreover, although it has been reported that lactobacilli other than *L. plantarum* can not synthesize *de novo* this essential vitamin (Rossi, Amaretti and Raimondi 2011), in the present study, folate production was evidenced for most of the strains of the analyzed *Lactobacillus* species in accordance with other studies (LeBlanc *et al.* 2007; Laiño, LeBlanc and Savoy de Giori 2012; Greppi *et al.* 2017; Saubade *et al.* 2017).

Greppi *et al.* (2017) from a total of 151 LAB strains isolated from traditional cereal-based fermented food, including *L. fermentum*, *L. plantarum* and *L. paraplantarum,* evidenced only two strains (*L. fermentum* 6.2 and *L. plantarum* 8.2) producing over 110 ng/ml in MRS medium. These levels are also similar to those of LAB that were isolated from raw cereal materials (Salvucci, LeBlanc and Perez 2016) and fermented dairy foods (Laiño *et al.* 2014). A highest folic acid production ability was reported by Park *et al.* (2014) that found *L. plantarum* JA71 to contain 9.03 μg/ml of folic acid after 24 h of incubation in MRS broth.

We determined that of the 47 strains analyzed with MA, folates excreted into the external medium were preponderant in all the strains for *St. thermophilus* , *E. faecium* , *E. lactis* and *L. paracasei* subsp. *paracasei*. Extracellular and intracellular concentration ratio was strain-dependent for *Lc. lactis* subsp. *lactis* , *L. casei* and *L. plantarum* . In contrast, regarding *L. rhamnosus* and *L. delbrueckii* subsp. *bulgaricus* , intracellular folate represented the 80% and 76% of the total.

Among *L. casei* strains, five predominantly excreted folate (from 66.94% to 87.74% of the total folate), while two presented higher intracellular folate content (VC201 = 62.75% and BT147 = 73.34% of the total). A total of 10 out of 15 *L. plantarum* strains excreted folate with a mean percentage of 61.36%, four presented a preponderant cytoplasmic retention (between 74.83% and 60.68%) and one consumed folate (VC194 = −7.90 ng/ml). Two *L. paracasei* subsp. *paracasei* showed higher levels of extracellular folate (VC213 = 67.59% and SE160 = 78.00%), while in *L. delbrueckii* subsp. *bulgaricus* , two strains excreted the majority of the folate (A751 = 75.84% and A753 = 60.55%), and the remaining four accumulated most of the folate inside the cell. Among *L. rhamnosus* strains, only VC221 produced 62.50% of external folate, three presented values of intracellular folate between 90.91% and 66.67% and one produced only intracellular folate (VC228) (Table 1).

All 12 cocci excreted the majority of folate content (between 78.28% and 94.72% of the total content), with exception of the strain *Lc. lactis* subsp. *lactis* FT26 that presented a lower external folate content (Table 2).

The folate yield concentrations provided by *Lc. lactis* (varying between 51.00–140.05 ng/ml) and *St. thermophilus* (26.78–115.71 ng/ml) were comparable or higher than those obtained in similar studies (Lin and Young 2000; Sybesma *et al.* 2003; Padalino *et al.* 2012).

Mosso *et al.* (2018) determined folate concentration in cell-free supernatants (extracellular) and after cell lysis (intracellular) and determined folate production by 41 strains in cultural medium. Total folate production varied from 29–138 ng/mL. A total of 13 *L. sakei* excreted in average 67% of folates into the external medium. In contrast, in the other species, cytoplasmic retention of folate was preponderant, e.g. 78% in *L. fermentum* and 59% in *L. casei*. Nor *et al.* (2010) reported a comparable intra and extracellular folate for *Lc. lactis* NZ9000, *Lc. lactis* MG1363, *L. plantarum* I-UL4 and *L. johnsonii* DSM 20553.

As folate production strictly depends on culture condition, medium composition and food matrix, we investigated folate production of the five strains that produced the highest folate level and in particular extracellular folate, to prepare cheeses ripened to up to 60 d.

Folate production in cheese

The five strains showing the highest activity in the *in vitro* test were further examined for their folate-producing capacity in cheese: *L. casei* VC199, *L. paracasei* subsp. *paracasei* SE160, *L. plantarum* VS513, *E. faecium* VC223 and *E. lactis* BT161. Two experimental cheese-makings were conducted with each adjunct culture, and the cheeses were analyzed after 30 and 60 d to evaluate their activity throughout ripening. Although all five LAB had the capability to produce folate in cheese, folate content in the 30-d ripened cheeses (between 40.31 and 86.68 μg/100 g) was significantly higher in the experimental cheeses than in the control cheese ($P < 0.001$) when *L. paracasei* subsp. *paracasei* SE160, *L. casei* VC199 and *L. plantarum* VS513 were used as adjunct culture (Table 3). The greatest increase in folate level with respect to the control was observed for *L. casei* VC199 (54%), followed by *L. paracasei* subsp. *paracasei* SE160 (48%) and *L. plantarum* VS513 (42%). With further ripening up to 60 d, no significant difference was observed in the control cheeses (folate content was 50.94 μg/100 g in 30-d cheeses *vs* 54.14 μg/100 g in 60-d cheeses) while there were significant differences in the folate level in all the experimental cheeses inoculated with the selected strains. The highest bio-enrichment in cheeses was obtained after 60 d of ripening when lactobacilli were inoculated (folate content in cheese was 122.58 ± 6.03 μg/100 g and 104.52 ± 2.24 μg/100 g respectively for *L. paracasei* subsp. *paracasei* SE160 and *L. casei* VC199). The highest increase from 30 to 60 d of ripening was observed when *L. paracasei* subsp. *paracasei* SE160 (108.1% increase) and *E. lactis* BT161 (113.3%) were added.

Table 3. Folate content in cheeses produced with folate-producing LAB strains during ripening time. Control cheeses are included for each experimental cheese-making.

	30 days μg/100 g	60 days μg/100 g	*P*
Control_1	56.39 ± 3.19[a]	58.92 ± 3.84[a]	ns
L. paracasei subsp. *paracasei* SE160	83.28 ± 0.26[b]	122.58 ± 6.03[c]	*
L. casei VC199	86.68 ± 4.74[b]	104.52 ± 2.24[b]	*
Control_2	56.39 ± 3.19[a]	58.92 ± 3.84[a]	ns
L. plantarum VS513	80.20 ± 1.80[b]	102.25 ± 2.32[b]	**
Control_3	40.04 ± 4.64[a]	44.57 ± 1.60[a]	ns
E. lactis BT161	44.11 ± 2.59[a]	95.07 ± 2.65[c]	***
E. faecium VC223	40.31 ± 0.98[a]	50.18 ± 0.10[b]	**

Data expressed as mean ± standard deviations of triplicate determinations on two cheese-making trials.
*$P < 0.05$; **$P < 0.01$; ns: not significant; indicate significant difference in the same line.
Means with different superscripted lowercase letters in the same column are significantly different ($P < 0.05$).

Unlike expected, the lowest production was obtained with *E. faecium* VC223 (50.18 ± 0.10 μg/100 g).

Therefore, folate content increased with ripening in all the cheeses including the control cheeses, highlighting a natural production of vitamin B9. A significant increase was provided by the individual addition of the selected starter, even if to a different extent. These findings confirmed preliminary studies reporting that LAB increased folic acid content through their growth in milk (Lin and Young 2000; Padalino *et al.* 2012; Laiño *et al.* 2013; Da Silva *et al.* 2016; Saubade *et al.* 2017). A significant different behavior of the bacterial strains was highlighted comparing folate production in the culture medium and in cheese matrix. *Enterococcus faecium* VC223 that was able to produce 123,625.74 ± 8.00 ng/ml of folate in M17 after 24 h of growth was the least producer out of the five strains tested in experimental cheese-makings.

No published data are available about the use of different LAB species as folate producers in cheeses. Only Ayad (2009) increased folate concentration up to 11.1 μg 100 g^{-1} in cheeses made with *Lactococcus* cultures after 3 months. In a previous work, we demonstrated that the adjunct cultures had no negative affect on the sensory characteristics of the cheese, and the strains were still present in 60-d ripened cheese at level higher than 10^7 CFU/g and were able to survive *in vitro* digestion (Albano *et al.* 2018); thus the present findings demonstrate the potential capacity of the LAB strains carried by the cheese to colonize the gastrointestinal tract and to exert beneficial effects delivering folate to colonic-rectal cells.

CONCLUSION

LAB naturally occur during dairy fermentation, thus can be usefully exploited for *in situ* fortification of dairy products through fermentation. To our knowledge, this is the first study investigating the folate production capacity of different LAB species in cheese. It was shown that the addition of selected LAB strains during cheese-manufacturing can double the folate content in cheese after 30 d of ripening and that by prolonging the ripening of the cheese for up to 60 d, the content of folates further increases at level exceeding 100 μg 100 g^{-1}. Bio-enriched cheese consumption can contribute to achieve the recommended folate daily intake. The allocation of folate, which differs among and inside the LAB species, has to be taken into account in the selection of the strain for fermentation with regard to the food matrix. The five LAB strains considered in this study are candidates to develop novel bio-enriched fermented milk and dairy products to improve folate intake.

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