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Applicability of a *Lactobacillus amylovorus* strain as co-culture for natural folate bio-enrichment of fermented milk

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ABSTRACT

The ability of 55 strains from different *Lactobacillus* species to produce folate was investigated. In order to evaluate folic acid productivity, lactobacilli were cultivated in the folate-free culture medium (FACM). Most of the tested strains needed folate for growth. The production and the extent of vitamin accumulation were distinctive features of individual strains. *Lactobacillus amylovorus* CRL887 was selected for further studies because of its ability to produce significantly higher concentrations of vitamin (81.2 ± 5.4 μg/L). The safety of this newly identified folate producing strain was evaluated through healthy experimental mice. No bacterial translocation was detected in liver and spleen after consumption of CRL887 during 7 days and no undesirable side effects were observed in the animals that received this strain. This strain in co-culture with previously selected folate producing starter cultures (*Lactobacillus bulgaricus* CRL871, and *Streptococcus thermophilus* CRL803 and CRL415) yielded a yogurt containing high folate concentrations (263.1 ± 2.4 μg/L); a single portion of which would provide 15% of the recommended dietary allowance. This is the first report where a *Lactobacillus amylovorus* strain was successfully used as co-culture for natural folate bio-enrichment of fermented milk.

1. Introduction

Folates (vitamin B9), the natural form of folic acid, are closely related to coenzymes under physiological conditions and act primarily as one-carbon unit transporter for the biosynthesis of DNA and RNA (Shane, 2001), and in the synthesis of many other vitamins (Hanson and Roje, 2001). In addition, folates possess antioxidant properties protecting animal genomes from the attack of free radicals (Duthie et al., 2002). Any deficiency in folate levels can lead to characteristic health disorders such as neural tube defects (Safi et al., 2012) or raise cardiovascular and colon cancer risks (Boushey et al., 1995; Morrison et al., 1996). Vitamin B9 deficiency may arise as a result of an increase in the nutritional requirements, an insufficient intake (Papastoyiannidis et al., 2006) or sometimes due to malabsorption caused by certain gastrointestinal diseases and cancer treatments (Sreeramulu et al., 1996). In contrast to plants and primitive organisms, which at least partially can synthesize this essential compound, human homeostasis depends on an exogenous supply of the vitamin in order to prevent nutritional deficiency (Gangadharan et al., 2010; LeBlanc et al., 2010). Since 1998, countries like USA, Canada and Argentina (2002), have adopted flour fortification programs with extra vitamins to offset nutritional deficiencies (LeBlanc et al., 2010). Even though global public health efforts have focused on folate fortification and supplementation in order to prevent neural tube defects in early pregnancy, many countries have not adopted mandatory folic acid food fortification programs because this chemical form of the vitamin may mask early clinical manifestations of vitamin B12 deficiency (Bailey and Ayling, 2009; Morris and Tangney, 2007), alteration in the activity of the hepatic dihydrofolate reductase enzyme (Bailey and Ayling, 2009) or promote cancer (Baggott et al., 2012; Ulrich and Potter, 2006).

Since natural folates, such as 5-methyltetrahydrofolate, that are normally found in foods and produced by microorganisms do not mask B12 deficiency (Scott, 1999), this folate form would be a more efficient and secure alternative than supplementation with folic acid (Lamers et al., 2006). Furthermore, it was shown that synthetic folic acid is absorbed and transported to the liver where it is reduced and a portion is methylated (Wright et al., 2003). In contrast, natural folates (such as 5-MTHF) are reduced and methylated before being absorbed, making them more bioavailable than folic acid (Asrar and O'Connor, 2005).

The use of vitamin-producing microorganisms is thus a more natural and economically viable alternative that can be used in the production of foods with elevated concentrations of folates that are less likely to cause undesirable side-effects.

Several studies have indicated that certain strains of lactic acid bacteria (LAB) have been shown to be able to synthesize natural folates (Aryana, 2003; Lin and Young, 2000). Furthermore, the use of LAB in fermentation processing constitutes an effective biotechnological strategy to increase folate levels in milk (Laiño et al., 2013; Wouters et al., 2002). However, the ability of microbial cultures to produce folate varies

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considerably, because it is a strain-dependent trait (LeBlanc et al., 2013). Most authors claim that *Streptococcus (S.) thermophilus* normally produce folates (Iyer et al., 2010), whereas *Lactobacillus delbrueckii* subsp. *bulgaricus* (*L. bulgaricus*) is considered a folate consumer (Kneifel et al., 1991; LeBlanc et al., 2011; Rao et al., 1984). For this reason some research groups used metabolic engineering for development of overproducing strains (Hugenholtz et al., 2002; Sybesma et al., 2003). Previous studies from our laboratory have demonstrated for the first time that some native strains of *L. bulgaricus* were able to synthesize folates (Laiño et al., 2012); and recently, by a judicious selection of folic acid-producing lactic acid bacteria, a combination of *L. bulgaricus* and *S. thermophilus* was proposed and resulted in an two-fold increase in folate levels compared to the commercial yogurts (Laiño et al., 2013).

The aim of this study was to identify a folate producing strain isolated from regional Argentinean artisanal dairy products in order to use it as a co-culture for the natural folate bio-enrichment of fermented milk.

2. Materials and methods

2.1. Microorganisms and growth conditions

All LAB strains used in this study belonged to the culture collection of the Centro de Referencia para Lactobacilos (CERELA-CONICET, Tucumán, Argentina) and are listed in Table 1. Lactobacilli were grown in routine without agitation in De Man-Rogosa-Sharpe (MRS) broth culture media (De Man et al., 1960) at 37 °C for 16 h, whereas

Table 1
Folate concentration (μg/L) of different species of *Lactobacillus* in FACM at 37 °C after seven passages. Values are expressed as means (standard deviation, SD). ND (Not detected).

Microorganisms	Strain code (CRL)	Growth in FACM	Folates (μg/L)		
			Extracellular (SD)	Intracellular (SD)	Total (SD)
L. amylovorus	887	+	68.3 (3.4)	12.9 (1.3)	81.2 (5.4)
L. acidophilus	43	+	5.1 (0.8)	2.3 (0.6)	7.4 (0.8)
L. acidophilus	44	−	ND	ND	ND
L. acidophilus	45	−	ND	ND	ND
L. acidophilus	258	−	ND	ND	ND
L. acidophilus	730	−	ND	ND	ND
L. acidophilus	924	−	ND	ND	ND
L. acidophilus	1014	−	ND	ND	ND
L. acidophilus	1064	+	21.9 (2.3)	15.3 (1.4)	37.2 (3.1)
L. casei	431	+	1.5 (0.3)	0.0	1.5 (0.4)
L. casei subsp. *casei*	238	−	ND	ND	ND
L. casei subsp. *casei*	239	−	ND	ND	ND
L. casei subsp. *casei*	1100	−	ND	ND	ND
L. fermentum	141	−	ND	ND	ND
L. fermentum	220	−	ND	ND	ND
L. fermentum	251	+	0.0	0.2 (0.08)	0.2 (0.05)
L. fermentum	345	−	ND	ND	ND
L. fermentum	573	−	ND	ND	ND
L. fermentum	574	−	ND	ND	ND
L. fermentum	646	−	ND	ND	ND
L. fermentum	661	−	ND	ND	ND
L. fermentum	722	−	ND	ND	ND
L. fermentum	944	−	ND	ND	ND
L. fermentum	955	−	ND	ND	ND
L. fermentum	973	+	4.3 (0.6)	2.6 (0.4)	6.9 (0.9)
L. paracasei subsp. *paracasei*	59	−	ND	ND	ND
L. paracasei subsp. *paracasei*	66	−	ND	ND	ND
L. paracasei subsp. *paracasei*	72	−	ND	ND	ND
L. paracasei subsp. *paracasei*	75	+	3.4 (0.9)	26.6 (2.7)	30.1 (3.2)
L. paracasei subsp. *paracasei*	76	+	7.5 (0.9)	15.6 (2.5)	23.1 (2.8)
L. paracasei subsp. *paracasei*	84	−	ND	ND	ND
L. paracasei subsp. *paracasei*	206	−	ND	ND	ND
L. paracasei subsp. *paracasei*	207	−	ND	ND	ND
L. paracasei subsp. *paracasei*	208	+	21.6 (2.8)	17.1 (2.1)	38.7 (4.2)
L. paracasei subsp. *paracasei*	678	+	8.2 (1.2)	1.1 (0.3)	9.2 (1.3)
L. paracasei subsp. *paracasei*	997	−	ND	ND	ND
L. paracasei subsp. *paracasei*	1004	−	ND	ND	ND
L. plantarum	41	+	3.8 (0.8)	15.4 (1.2)	19.2 (2.3)
L. plantarum	51	+	1.8 (0.4)	9.6 (1.1)	11.4 (2.3)
L. plantarum	103	+	16.7 (3.4)	40.5 (4.2)	57.2 (5.2)
L. plantarum	140	+	1.8 (0.4)	6.4 (0.9)	8.2 (1.2)
L. plantarum	219	+	1.5 (0.3)	12.2 (1.7)	13.7 (1.9)
L. plantarum	363	+	4.1 (0.8)	15.8 (1.3)	19.9 (1.8)
L. plantarum	428	+	22.9 (2.7)	18.5 (1.6)	41.4 (3.6)
L. plantarum	651	−	ND	ND	ND
L. plantarum	681	+	14.4 (1.2)	7.7 (1.3)	22.1 (2.4)
L. plantarum	691	−	ND	ND	ND
L. plantarum	725	+	3.8 (0.9)	10.1 (1.2)	13.8 (1.7)
L. plantarum	759	+	14.7 (1.3)	12.1 (1.1)	26.8 (3.2)
L. plantarum	769	+	3.5 (0.8)	13.6 (1.8)	17.2 (1.9)
L. plantarum	778	+	2.9 (0.5)	8.1 (1.7)	11.1 (1.2)
L. plantarum	785	+	2.8 (0.3)	20.6 (2.8)	23.4 (2.1)
L. plantarum	794	+	0.3 (0.05)	1.1 (0.06)	1.4 (0.3)
L. plantarum	936	+	16.3 (1.6)	10.5 (0.9)	26.8 (3.2)
L. plantarum	1081	−	ND	ND	ND

streptococci were grown for 16 h at 42 °C without agitation in LAPT broth culture media containing (w/v) 1.5% peptone, 1% tryptone, 1% yeast extract, 1% glucose and 0.1% Tween 80 (Czeizel et al., 1994).

2.2. Selection of folate-producing strains

Lactobacilli strains were screened for the ability to grow in the folate-free synthetic medium. Previously, the activated cultures were washed three times with saline solution (0.85% w/v NaCl) and resuspended in this solution at the original culture volume. This cell suspension was used to inoculate at 4% (v/v) folate-free culture medium (Folic Acid Casei Medium (FACM), Difco, Becton, Dickinson, and Co., Sparks, Maryland) and incubated without agitation at 37 °C for 18 h. After growth, this washing–resuspension procedure was repeated, and the resulting LAB solution was used to inoculate at 2% (v/v) fresh FACM. Cultures showing good growth, as observed by increased turbidity, were propagated seven times in the same medium before the measurement of folate concentrations in the supernatants or cell extracts. The strains that were unable to grow in FACM were not used for further studies.

2.3. Samples preparation and folate determination

After the last incubation, two samples were taken to determine the concentration of extracellular and intracellular folates. A sample (500 μl) of LAB-containing FACM was taken. The 500 μl of protecting buffer (0.1 mol/L phosphate buffer, pH 6.8) containing 1.5% (w/v) ascorbic acid was added and mixed to prevent vitamin oxidation and degradation. Then the procedure was followed by immediate centrifugation for 5 min at 10,000*g*. The supernatant was collected (extracellular folate sample) and the pellet was resuspended in 500 μl of protecting buffer (intracellular folate sample). Both samples were then boiled (100 °C) for 5 min, centrifuged for 6 min at 10,000*g*, and resulting supernatant stored at −70 °C until analysis.

In the case of fermented milk, samples (500 μl) were mixed with 500 μl of protection buffer. The resulting mixture (1 ml) was boiled (100 °C) for 5 min to precipitate proteins and release folate from binding proteins present in milk and was then centrifuged (10,000*g* for 6 min). The supernatant was collected and stored at −70 °C for total folates determination.

Folate determination was performed using a modified microbiological assay using *Lactobacillus casei* subsp. *rhamnosus* NCIMB 10463 as indicator strain as was previously described (Laiño et al., 2013). In every case, non-inoculated samples analyzed simultaneously, were used as controls.

2.4. Folate production kinetics in folate-free culture medium

The strain selected as the best folate producer was inoculated in folate-free culture medium (Folic Acid Casei Medium (FACM), Difco, Becton, Dickinson, and Co., Sparks, Maryland, USA) at an initial optical density at 580 nm (OD_{580}) of 0.1, incubated at 37 °C for 24 h, and samples (2 ml) were aseptically withdrawn at 0, 2, 4, 6, 8, 10, and 24 h from the fermentation vessel and immediately cooled on ice to determine folate concentrations, growth parameters (pH, OD_{580nm}), and cell viability as determined by colony forming units (CFU)/ml).

2.5. Taxonomic identification of selected strain as the best folate producer

2.5.1. Phenotypic and biochemical tests

The selected strain (*Lactobacillus* spp.) was identified by phenotypic and biochemical tests. These included Gram reaction; oxidase-reaction; reduction of 1% (w/v) nitrate in MRS broth; bacterial growth, recorded at 15, 37 and 45 °C; gas production from glucose; and hydrolysis of arginine (in MRS broth without glucose and meat extract but supplemented with 0.3% arginine (Sigma Chemical Co., St. Louis, MO)); ammonia (NH_3) production was tested by addition of Nessler's reagent. Isomers of lactic acid were evaluated by an enzymatic method (Gawhen and Bergmeyer, 1974; Gutmann and Walhlefeld, 1974). The sugar fermentation pattern was determined by using the API 50 CH as specified by the manufacturer (API-BioMérieux, Marcy l'Etolie, France).

2.5.2. Polymerase chain reaction

Total cellular DNA was isolated and oligonucleotide primers (PLB16, 5′-AGAGTTTGATCCTGGCTCAG-3′; and MLB16, 5′-GGCTGCTGGCACGT AGTTAG-3′) were used to amplify the variable (V1) region of the 16S ribosomal RNA gene, as described previously (Hébert et al., 2000). Polymerase chain reaction amplicons were sequenced at the CERELA-CONICET (Tucumán, Argentina) using an ABI 3130Hitachi Genetic Analyzer (Applied Biosystems–Life Technologies, Buenos Aires, Argentina), and highly similar sequences were searched using the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

2.6. Detection of bacterial interactions (well-diffusion agar assay)

Interactions among lactic acid strains were investigated. Ten milliliters of MRS (lactobacilli) or LAPTg agar (streptococci) were melted, kept at 45 °C, and vigorously mixed with 70 μl of an overnight culture of lactobacilli (*L. bulgaricus* CRL871 or *Lactobacillus amylovorus* CRL887) or streptococci (*S. thermophilus* CRL803 and CRL415), and poured into Petri dishes. Wells of 5 mm in diameter were made in the agar layer, and 30 μl of the cell-free supernatant of each strain was placed into each well. After 24 h of incubation at 37 °C, the plates were examined for zones of inhibition which would be an indication that these produce antibacterial substances.

2.7. Safety evaluation of *L. amylovorus* CRL 887 using an animal model

L. amylovorus CRL887 was grown in MRS broth during 16 h at 37 °C. The culture was centrifuged (6000*g*, 5 min), and the pellet was washed twice with 0.1% (w/v) peptone solution and suspended in reconstituted skim milk before being administered to mice.

Twenty BALB/c mice of 6 weeks of age, weighing 25–30 g, were obtained from the closed inbred colony, maintained (12-h light cycle, 22 ± 2 °C) at CERELA-CONICET (Tucumán, Argentina) and were fed *ad libitum* with water and balanced rodent diet (Cooperación, Argentina; containing 32% protein, 5% fat, 2% fiber, and 60% nitrogen-free extract). The mice were separated into two groups: the experimental group that received the *L. amylovorus* strain and the control group that did not receive bacterial supplementation.

L. amylovorus CRL887 was given to the mice at a concentration of 1×10^9 CFU LAB/day/animal in the drinking water during 7 days. The control group consisted of mice that received 10% non-fat milk under the same conditions as the test group. All mice were fed with conventional balanced diet *ad libitum*.

Animal growth (live weight), food, and water intake were determined on a bi-daily basis. All animal protocols were pre-approved by the Animal Protection Committee of CERELA (CRL-BIOT-LT-20142A) and followed the latest recommendations of the Federation of European Laboratory Animal Science Associations and the Asociación Argentina para la Ciencia y Tecnología de Animales de Laboratorio. All experiments comply with the current laws of Argentina.

2.7.1. Organ samples collection

At the end of the feeding period (7 days), mice were anesthetized with an intraperitoneal injection of a solution containing ketamine (Holliday, Buenos Aires, Argentina) and xylacin (Rompum, Bayer S.A., Buenos Aires, Argentina) to obtain a final concentration of 100 μg and 5 μg/kg body weight, respectively. Animals were bled by cardiac puncture. The spleen and liver were aseptically removed and kept on ice until used.

2.7.2. Microbial translocation

Microbial translocation to extra-gut organs was determined following previously described protocols (LeBlanc et al., 2004). Briefly, the spleen and liver were homogenized in sterile peptone solution, and serial dilutions were plated in triplicate in the following media: MRS for enumeration of lactobacilli, McConkey for analysis of enterobacteria, brain–heart infusion (BHI) for enumeration of anaerobic and aerobic bacteria.

Plates were incubated for 48 h at 37 °C under aerobic and anaerobic conditions, and number of colony forming units (CFU) were counted and expressed as CFU/g of organs.

2.8. Milk fermentation with L. amylovorus CRL887 as co-culture

2.8.1. Milk preparation

The milk was obtained by reconstituting 10% (w/v) Svelty Calcio Plus skimmed milk powder (Nestle, Argentina) in water (45 °C), pasteurized at 87 °C for 30 min and then was cooled down in a water bath until the temperature of 42 °C was reached.

2.8.2. Starter cultures, yogurt fermentation and storage

The cultures employed were *L. amylovorus* CRL887 and the yogurt starter mixture culture containing *S. thermophilus* CRL803 and CRL415, and *L. bulgaricus* CRL871 (cocci:bacilli rate = 2:1) previously selected because it was the mixture that most increased the folate concentrations in yogurt (Laiño et al., 2013). The microorganisms were individually inoculated (2% v/v) in 200 ml reconstituted non-fat powdered milk and incubated 16 h. After that, each individual culture was used to inoculated 200 ml reconstituted non-fat powdered milk (total inoculum of 2% v/v, 0.5% v/v for each strain, coco:bacilli rate 2:2) and incubated in a water bath at 42 °C. At this dilution, the initial viable cell count of each individual strain was approximately 5.7 ± 0.3 CFU/ml. Samples were withdrawn at 0, 4, 6, 8 and 24 h of fermentation.

In order to evaluate the shelf-life, when the pH reached 5.2 ± 0.2 (after 4 h of incubation) the fermented milks were removed from the incubator, and transferred to a cold room at 6–8 °C and stored for 28 days. After 1, 7, 14, 21 and 28 days of storage, samples were taken to determine folate concentrations, pH and cell viability (CFU/g). Folate production of each individual strain was also evaluated in milk in the same manner.

Viable cells (CFU/g) of *S. thermophilus* were enumerated by plating different dilutions on M17 agar (Biokar Diagnostics, Beauvais, France) which were then incubated during 48 h at 42 °C. Lactobacilli did not grow on M17 medium because it contains sodium β-glicerophosphate that inhibits the growth of both *L. amylovorus* CRL887 and *L. bulgaricus* CRL871 (data not shown). *L. amylovorus* CRL887 was enumerated on modified MRS agar that contained starch 1% (w/w) instead of glucose. In this medium only *L. amylovorus* CRL887 was able to form colonies, growth of streptococci or *L. bulgaricus* CRL871 were not observed on this media after incubation during 48 h at 37 °C (data not shown). *L. bulgaricus* CRL871 were enumerated on LBS agar plates (Difco, Becton, Dickinson, and Co., Sparks, Maryland) that were incubated at 48 h at 37 °C. In this medium, streptococci did not grow; however, both lactobacilli did (data not shown). Since *L. bulgaricus* CRL871 and *L. amylovorus* CRL887 produced distinct morphological features of the colonies (shapes and sizes), it was possible to enumerate each strain separately (data not shown). The counts in LBS medium for *L. amylovorus* CRL887 were similar to those obtained in MRS-starch medium and previous assays showed that no growth incompatibilities existed between *L. amylovorus* CRL887 and *L. bulgaricus* CRL871 (data not shown).

2.9. Statistical analysis

The results were obtained from three independent experiments and each data point was measured in triplicate (n = 9). All values were expressed as means ± standard deviations (SD). Statistical analyses were performed with the software package SigmaPlot for Windows Version 12.0 (Systat Software Inc., Chicago, IL, USA) using ANOVA GLM followed by a Tukey's post hoc test, and differences were considered statistically significant at $p < 0.05$.

3. Results and discussion

With the aim of improving starter cultures that would increase natural folates contents in fermented foods, 55 wild-type *Lactobacillus* strains, isolated from a wide range of artisanal dairy products in Northwestern Argentina, were screened for the ability to grow in a folate-free synthetic medium. In the absence of folate, 30 strains died within three passages and 25 grew abundantly in FACM even after seven subcultures (Table 1).

The folate concentration in cell-free supernatants was measured by microbiological assay of 24-h cultures. Folate was found in the supernatant of strains belonging to *Lactobacillus fermentum* (2), *Lactobacillus acidophilus* (2), *Lactobacillus* spp. (1), *Lactobacillus plantarum* (15), *Lactobacillus paracasei* (4), and *L. casei* (1). Most of *L. plantarum* strains (83%) were folate producers unlike *L. fermentum* where only 16% of tested strains showed this behavior. These results indicate that folate production is not a common characteristic of the species but seemed to strain specific traits as were reported previously (Laiño et al., 2012; Pompei et al., 2007).

Significant strain-to-strain differences in folate concentrations, ranging between 0.2 and 81.2 ng/ml, were observed. To clarify whether the strains accumulated folate or excreted it into the medium, the intracellular folate concentration was determined.

Intracellular folate accumulation was also strongly heterogeneous, as it ranged between 15% and 71% of total vitamin production (obtained by dividing the intracellular concentration by the total folate concentration). Similar observations were reported for bifidobacteria (Pompei et al., 2007), where intracellular folate concentrations were also strain dependent. From the strains tested in this work, *Lactobacillus* spp. CRL887 produced the highest extracellular folate levels (68.3 ± 3.4 μg/L) equivalent to 85% of their total folate production, thus this strain was selected for further studies.

As a first attempt to identify *Lactobacillus* spp. CRL887, the physiological characterization of this strain was evaluated using the API 50 CH system. The strain grew on and fermented amygdalin, cellobiose, galactose, maltose, mannose, sucrose, and trehalose (data not shown). Acid was not produced from salicin, mannitol, melibiose, raffinose, rhamnose, and ribose. The strain did not produce gas or acetoin from glucose. It was thermophilic and grew at 37 and 45 °C, but not at 15 °C. The strain was unable to produce ammonia from arginine and formed DL-lactic acid isomer. On the basis of their biochemical and physiological characteristics, the strain was classified as *L. amylovorus* CRL887. These data were confirmed by 16S rRNA gene sequences analysis. The amplified 16S rRNA genes of the strain CRL887 showed a sequence similarity of 99% with the 16S rRNA DNA sequences of *L. amylovorus* JCM7722 and *L. amylovorus* GRL1112.

The growth kinetics of *L. amylovorus* CRL887 in folate-free medium is shown in Fig. 1a. The strain began the log phase of growth after 4 h incubation reaching the stationary growth phase at 8 h. Viable counts reached values of 4.7 ± 0.2 10^8 CFU/ml after 8 h of incubation; and these values remained constant after 24 h.

Vitamin production was evident after 4 h of bacterial growth; reaching concentrations three times higher after 10 h incubation compared to that found at 4 h of incubation (80 ± 2 and 28 ± 3 μg/L, respectively) as observed in Fig. 1b. As incubation time increased, the microorganism showed extensive excretion of folate into the external medium (Fig. 1b). The results showed that *L. amylovorus* CRL887 was not only capable of growing in the absence of folates, but also possessed the ability to produce this vitamin which was growth associated and occurred mostly during the exponential growth phase. The ability to produce folate by a strain of *L. amylovorus* has, to our knowledge,

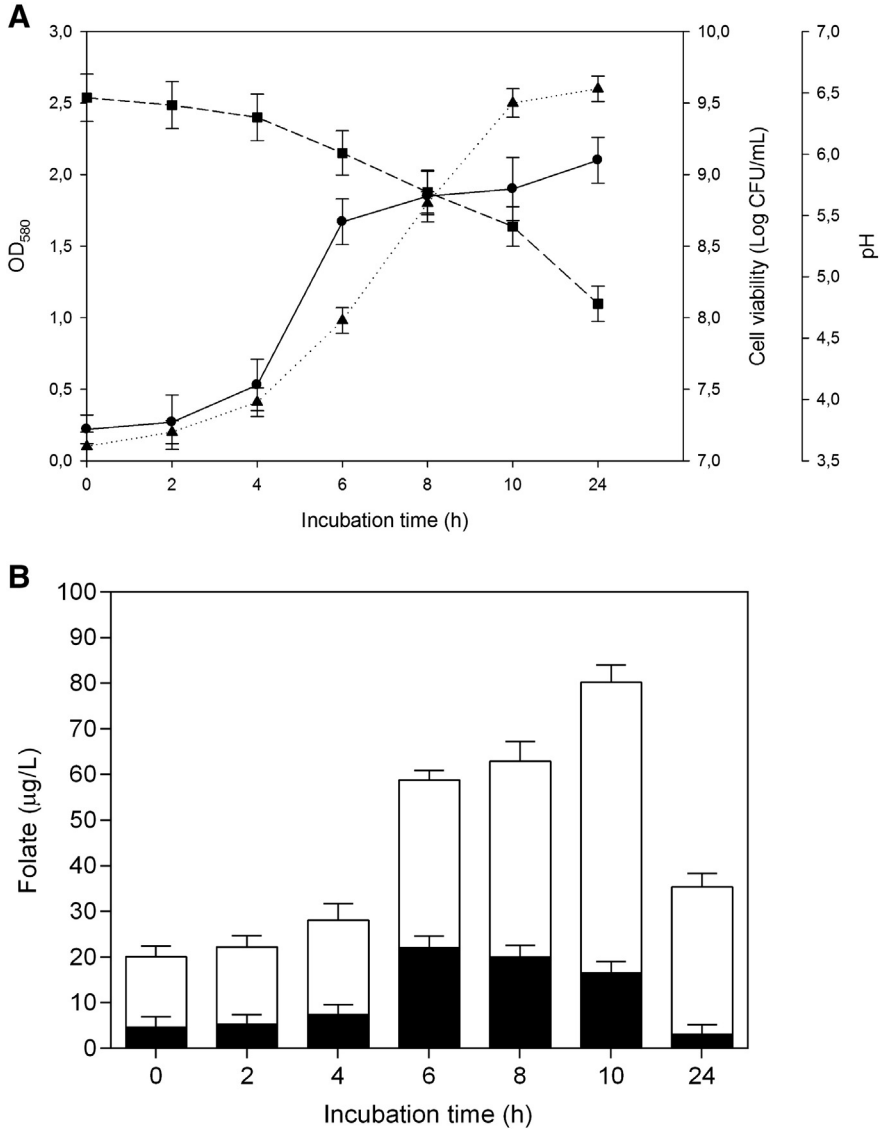

Fig. 1. A) Growth kinetics of *L. amylovorus* CRL887 in FACM at 37 °C. Round symbol represents viable cell enumeration (log CFU/ml), square symbol with discontinuous line represents pH and triangular symbol with dotted line represents OD at 580 nm (OD_{580}). B) Folate production by *L. amylovorus* CRL887 in FACM at 37 °C. Full bars represent intracellular folates and empty bars represent extracellular folates expressed in µg/L. Time is expressed as hour (h). Values are expressed as the means ± SD.

never been described before; in fact, this species was reported to require folate for their growth (Fujisawa et al., 1992; Nakamura, 1981; Rogosa et al., 1961). The folate producing ability of this strain was then evaluated in milk. When CRL887 was used to ferment milk as an individual strain, it was observed that it did modify folate concentrations in milk which remained constant even after 24 h of incubation (data not shown). This could be due to the fact that this strain showed reduced growth (reaching values of only $7.2 \pm 0.2 \times 10^6$ CFU/ml after 24 h incubation with an initial inoculum of $4.6 \pm 0.3 \times 10^5$ CFU/ml). These results show that *L. amylovorus* CRL887 is not only able to produce folates in the absence of folates, as shown in the folate-free medium (Fig. 1b), but also in its presence since milk contains 50 ± 9 µg/L of folates suggesting that the strain produces the same amount of the vitamin that it requires for its own growth. This is the first report of a *L. amylovorus* strain that produces folates, and as of now, published reports have shown that this species lacks many of the genes of the folate biosynthetic pathway (Kant et al., 2011). With these promising results, the search for the genes involved in folate biosynthesis by *L. amylovorus* CRL887 is essential to understand to see how this strain acquired this interesting property.

Although there are few reports of the use of *L. amylovorus* in commercial food production, there is increasing evidence that strains of this species possess many beneficial properties such as the ability to produce phytase (Sreeramulu et al., 1996), to assimilate cholesterol (Grill et al., 2000) produce antifungal agents (Ryan et al., 2011) and it has been demonstrated that some *L. amylovorus* strains produce bacteriocins active against some strains of the *Lactobacillus* lineage such as *L. delbrueckii* subsp. *bulgaricus* and *Lactobacillus helveticus* (Gawhen and Bergmeyer, 1974). Recently, Laiño et al (2013) selected strains of *S. thermophilus* (CRL803 and CRL415) and *L. bulgaricus* CRL871 capable of producing folates. Since the use of *L. amylovorus* as a co-culture in yogurt fermentation seems promising, the interactions among the strains were evaluated. No growth inhibition between the strains tested in our study (*L. amylovorus, L. bulgaricus* and *S. thermophilus)* was observed (data not shown) suggesting that they are compatible for co-cultures.

Also, the safety of this newly identified folate producing strain was evaluated. Healthy adult BALB/c mice were fed during 7 days with a relatively high dose of *L. amylovorus* CRL887 to determine if the strain causes translocation to the liver or spleen. No bacterial translocation was detected in liver and spleen after consumption of CRL887 during 7 days and no undesirable side effects were observed (such as weight loss, changes in hair thickness, behavioral modifications, etc.) in the animals that received this strain (data not shown). Folate concentrations in serum were also measured and no significant differences were observed in mice that received *L. amylovorus* CRL887 compared to those that did not receive bacterial supplementation (data not shown). This result is not surprising since the animals used in this study receive a balanced diet that contains more that the daily recommended concentration of folates, thus increasing their folate intake was not expected to increase folate concentrations since excess folate, as is the case for all water soluble vitamins, would be excreted in urine. The evaluation of folate concentrations using folate-depleted animals is currently being studied.

Hence, taking into account its ability to produce folates, its compatibility with other strains and its safety, *L. amylovorus* was evaluated as a co-culture along with previously selected yogurt starter cultures in order to evaluate the potential to increase folate concentrations in this fermented milk product.

L. amylovorus CRL887 as co-culture in yogurt fermentation produced high folate levels (263 ± 12 μg/L) after 4 h of incubation, with a slight but significant decrease in these values up to 24 h at 42 °C (Fig. 2), but remaining over 200 μg/L. These elevated folate values reached with the addition of *L. amylovorus* CRL887 as a co-culture are significantly higher (a 46% increase was observed) than those reported for the same combination of folate producing strains (180 ± 3 μg/L) without *L. amylovorus* CRL887 or with other combinations of yogurt starter cultures (Laiño et al., 2013) and those reported by other combination of species (Holasova et al., 2004, 2005; Wouters et al., 2002). Also, it is important to remark that the addition of *L. amylovorus* CRL887 with the yogurt starter cultures not only increased folate concentrations, but also decreased the incubation time necessary to reach the maximal concentrations since the peak of production was after 4 h of incubation compared to 6 h of incubation necessary to reach maximal folate concentrations with the starter cultures alone (Laiño et al., 2013). At the beginning and at the end of yogurt elaboration (4 h of fermentation), a differential viable cell count was performed to determine the extent of growth of starter cultures and *L. amylovorus* CRL887. Viable cell counts showed that both starter cultures and *L. amylovorus* CRL887 grew after fermentation, being *S. thermophilus* ($2{,}9 \pm 0.4 \times 10^8$ CFU/g of yogurt) the most predominant after the incubation period. In the case of *Lactobacillus* species, even when *L. amylovorus* CRL887 ($6.5 \pm 0.2 \times 10^7$ CFU/g of yogurt) grew 1 log unit lower than *L. bulgaricus* CRL871 ($3.4 \pm 0.3 \times 10^7$ CFU/g of yogurt) (Fig. 3), the growth was enough to increase folate levels up to 263 ± 12 μg/L (Fig. 2). This is a very promising result since a reduction in incubation time would lead to decreased costs associated with the production of bio-enriched yogurts.

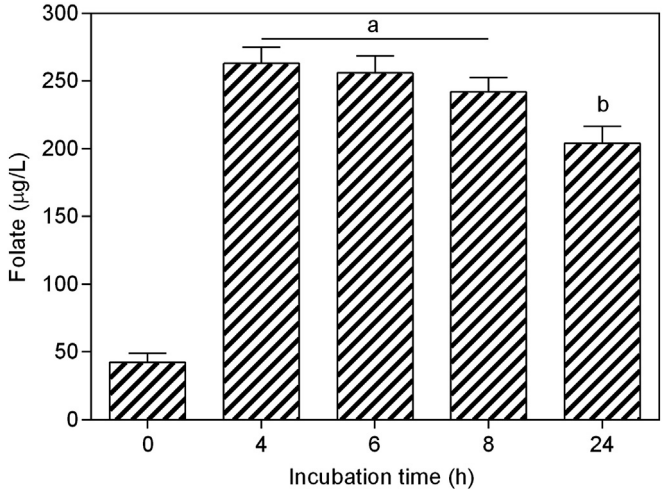

Fig. 2. Folate production by *L. amylovorus* CRL887 in co-culture with yogurt starters in milk at 42 °C for 24 h. Time is expressed as hour (h). Values are expressed as means ± standard deviation (SD). [a, b]Means with different letter differ significantly ($p < 0.05$).

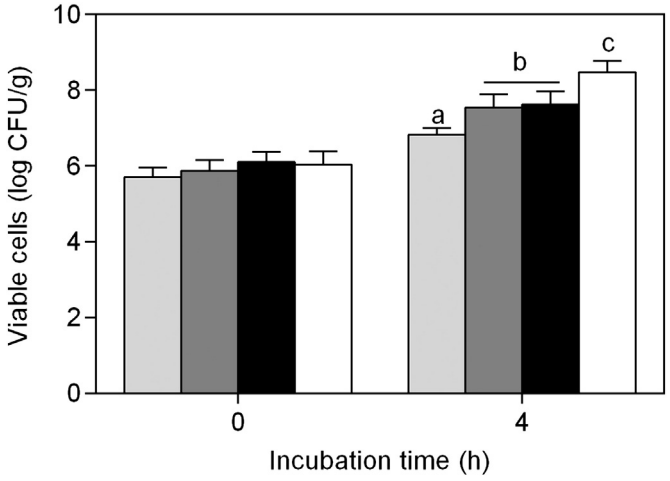

Fig. 3. Viable cell counts of *Lactobacillus* and *Streptococcus* in fermented milk. Time is expressed as hour (h). 0 h represents the beginning of fermentation (inoculum), and 4 h represents the end of fermentation. Values are expressed as means ± standard deviation (SD). [a–c]Means with different letter differ significantly ($p < 0.05$). Light gray bars represent *L. amylovorus* CRL887, dark gray bars represent *L. bulgaricus* CRL871, empty bars represent total *Lactobacillus* and full bars represent total *Streptococcus*.

The effect of storage of the fermented milk product at 4 °C was studied during 28 days. There were no significant variations in folate concentration during the 28 days of storage compared to the freshly prepared bio-enriched product (Supplemental data). Only a viable cell count decrease was detected on *L. bulgaricus* CRL871 during the last week of storage (Supplemental data), without significant variations in the rest of strains and pH during the 28 days of storage.

These results make the use of *L. amylovorus* CRL887 as co-culture feasible for the development of fermented milk naturally bio-enriched in folate. According to the World Health Organization, the recommended dietary allowance (RDA) for an average adult is 400 μg per day (FAO/WHO, 2002). This organization also established that a food can only be considered a good source of folate when it provides more than 10% of the RDA. Normally, milk, yogurt, cheese, and other foods are considered poor sources of folate since these contain between 20 and 50 μg/L (Ohio State University, 2005). In our study, the yogurt produced with selected yogurt cultures and co-cultivated with *L. amylovorus* CRL887, contained over 260 μg/L. Considering the daily consumption of a single 225 ml serving (Laiño et al., 2013), this fermented product would provide approximately 15% of the RDA, making it a very good source of folates.

4. Conclusions

Based on the results obtained in this study, it was shown for the first time, that a strain of *L. amylovorus* was able to grow and produce folates in a folate-free culture medium. *L. amylovorus* CRL887 was also shown to be effective as a co-culture together with conventional yogurt starter cultures and increased the folate concentrations of this fermented milk. Such products, with increased natural folate concentrations, could be

used as an alternative to current obligatory fortification protocols that use the chemical form (folic acid) of this vitamin.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijfoodmicro.2014.08.031.

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