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Effect of fructooligosaccharides and galactooligosaccharides on the folate production of some folate-producing bacteria in media cultures or milk

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ABSTRACT

The present work investigated the ability of *Bifidobacterium catenulatum*, *Bifidobacterium adolescentis*, *Lactobacillus plantarum*, *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* to produce folate in milk and complex media. Moreover, the effect of two prebiotics, fructooligosaccharides and galactooligosaccharides, on folate biosynthesis was also evaluated. Levels of the predominant folate forms, i.e., tetrahydrofolate and 5-methyltetrahydrofolate, were determined using high performance liquid chromatography after 0, 6, 10 and 24 h incubation. *B. catenulatum* (28.82 ± 2.02 μg 100 mL^{-1}) and *S. thermophilus* (19.03 ± 1.95 μg 100 mL^{-1}) produced the highest level of folate in complex media and milk, respectively. In most cases, the bacteria tested reached the maximum folate levels within 6 h and 10 h of incubation. The inclusion of prebiotics in the culture medium did not stimulate the synthesis of folate by any of the five bacteria studied, although it increased the rate of bacterial growth.

1. Introduction

Folate, or vitamin B_9, is an essential micronutrient of normal cellular functions, growth and development. This vitamin is required in many metabolic reactions, such as methyl group biogenesis and synthesis of nucleic acids, vitamins and some amino acids (Ames, 1999; Sarma & Duttagupta, 1995), and is an essential component of the diet. Folates are widespread in nature, mainly in green leafy vegetables, liver, cereals and dairy products such as yoghurt, mainly due to the fermentation carried out by lactic acid bacteria (LAB). However, lack of this soluble vitamin in the diet is one of the most common nutritional deficiencies in the world; mammals must absorb folate from their diet because they do not have the capability to synthesise it.

Traditionally, folate deficiency in humans has been associated with macrocytic or megaloblastic anaemia. However, nowadays, it is known that marginal folate deficiency or changes in its metabolism are also associated with other health problems, such as cancer and cardiovascular diseases, as well as neural tube defects in newborns (Boushey, Beresford, Omenn, & Moultulsky, 1996; Jennings, 1995; Shaw, Schaffer, Velie, Morland, & Harris, 1995; Wang et al., 2007). The daily recommended intake (DRI) of folate in the European Union (EU) is set at 200 μg for adults and 600 μg for women in the periconceptional period (Otten, Pitzi-Hellwig, & Meyers, 2006).

Folate intake can be increased by a number of strategies, in particular the consumption of foods naturally rich in folate such as green leafy vegetables, fruits and berries, beans, whole grain products and liver, the use of folic acid supplements, and the consumption of synthetic folic acid or fortification of natural food with folate. This last strategy has proved very useful in reducing health problems associated with folate mal-intake (Wegkamp, 2008), showing a reduction of the prevalence of neutral tube defects by 46% (De Wals et al., 2007). However, it was recently shown (Smith, 2007) that a high-level intake of chemically synthesised folic acid might have some adverse health effects, masking the diagnosis of a vitamin B_{12} deficiency and possibly leading to irreversible neurological damage (Morris, Jacques, Rosenberg, & Selhub, 2007).

Another important factor is the high relation between intake of synthetic folic and the increase of risk of colorectal cancer risk (Kennedy et al., 2011). In addition, a recent report has suggested that folic acid supplementation during pregnancy is associated with an increased risk of respiratory infections in newborns (Håberg, London, Stigum, Nafstad, & Nystad, 2009). For this reason, there is a growing interest in the biofortification of foodstuff with natural folates. In this regard, the use of LAB for the improvement of the nutritional value of fermented food products and/or contributing health benefits to these products has been proposed.

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LAB are able to synthesise folate; specifically, this group of bacteria show increased growth rate in the presence of prebiotics (Bouhnik et al., 2004). Prebiotics can be classified according their chemical constituents and degree of polymerisation, resulting in a large variety of compounds. However, the majority of studies have been carried out with FOS and GOS (Macfarlane, Steed, & Macfarlane, 2008). These two prebiotics have shown beneficial effects on health, decreasing the risk of cardiovascular disease (Rault-Nania et al., 2006) and cancer (Rafter et al., 2007), increasing bone mineralisation (Abrahms et al., 2005) and protecting against gastrointestinal diseases (Brunser et al., 2006; Cummings, Christie, & Cole, 2001). The last effect is mainly due to the growth of bifidobacteria and lactobacilli, protecting the gut environment against incursion by invasive microorganisms, thereby playing an important role in the maintenance of gut homeostasis. However, no studies have determined whether these two prebiotics affect the bacterial synthesis of folate.

Thus, considering that prebiotics stimulate growth of bifidogenic bacteria, which produce produce water-soluble vitamins, it was of interest to determine the production of folate could be enhanced when these bacteria are grown in the presence of fructooligosaccharides (FOS) and galactooligosaccharides (GOS). The aim of the present work was thus to investigate the effect of these two prebiotics (FOS and GOS) on folate biosynthesis by several folate-producing bacteria in media cultures and milk.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Bifidobacterium catenulatum DSMZ 16992, *Bifidobacterium adolescentis* DSMZ 20083, *Lactobacillus plantarum* DSMZ 2601, *Lactobacillus delbrueckii* ssp. *bulgaricus* DSMZ 20081 and *Streptococcus thermophilus* DSMZ 20617 were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany (DSMZ) GmbH (German collection of microorganisms and cell cultures, http://www.dsmz.de/microorganisms). *L. plantarum* and *L. bulgaricus* strains were grown in Man Rogosa Sharpe (MRS) broth (Oxoid, Hampshire, England). *B. catenulatum* and *B. adolescentis* strains were grown in MRS broth supplemented with L-cysteine (0.05 g 100 mL^{-1}; Sigma, St. Louis, MO, USA). *S. thermophilus* was grown in both MRS broth and in trypticase soy–yeast extract (TSYE) broth (BD, Franklin Lakes, NJ, USA). All strains were also grown in 12% (w/v) non-fat dry milk (NDM; Difco Laboratories, Detroit, MI, USA) autoclaved at 121 °C for 15 min. All strains were anaerobically incubated at 37 °C for 24 h and serially transferred at least three times prior to assays. The inoculum (10^5 cfu mL^{-1}) was the same for all bacteria strains in every medium studied.

2.2. Bacterial growth and folate synthesis

This study evaluated the effect of two prebiotics on folate biosynthesis. The first prebiotic was oligofructose (Raftilose P95®, minimum content 95%) (Beneo-Orafti, Tienen, Belgium) as FOS; the second prebiotic was 4′-galactosyl-lactose (Oligomate 55P®, minimum content 55%; Yakult Pharmaceutical Industry Co., Ltd., Tokyo, Japan) as GOS. Prebiotics were added to milk and complex media to a final concentration of 1.0 g 100 mL^{-1}.

Culture media and milk, with or without prebiotics, were collected after 0, 6, 10 and 24 h of bacterial growth and folate levels were analysed by high performance liquid chromatography (HPLC). At the same time, bacterial growth was evaluated by plating in agar medium. *L. plantarum* and *L. bulgaricus* were enumerated on MRS agar plates (Oxoid), *B. adolescentis* and *B. catenulatum* were enumerated on MRS agar plates containing L-cysteine (0.25 g 100 mL^{-1}) and *S. thermophilus* was assayed on MRS and TSYE agar plates (Scharlau, Barcelona, Spain). All plates were incubated anaerobically at 37 °C for 72 h.

2.3. Folate standards

Individual folate standards (6R,S)-5,6,7,8-tetrahydrofolic acid calcium salt (H_4-folate), (6R,S)-5-methyl-5,6,7,8-tetrahydrofolic acid sodium salt (5–CH_3–H_4-folate) and (6R,S)-5-formyl-5,6,7,8-tetrahydrofolic acid sodium salt (5–HCO–H_4-folate) were obtained from Dr. Schirck's Laboratories (Jona, Switzerland). Standard solutions were prepared according to the method described by Van den Berg, Finglas, and Bates (1994).

2.4. Extraction and deconjugation of folates from samples

Folates from bacterial cultures were extracted following the procedure described by Pfeiffer, Rogers, and Gregory (1997) and Konings (1999). The eluted sample was weighed, and the purified extracts were kept under refrigeration for no longer than 2 h before they were placed in the autosampler and injected onto the HPLC. The extraction, deconjugation and purification procedures were carried out under subdued light to prevent photodegradation of folates.

2.5. High performance liquid chromatography analysis of folates

Folates were determined using a Merck-Hitachi 7000 (Merck, Darmstadt, Germany) HPLC equipped with a fluorescence detector (LaChrom, Merck-Hitachi, model 7485). A LiChrosphere® 100 RP-18 (5 μm) column (Merck), protected with a guard column (LiChroCART® 4-4, Merck), was used to separate the folate compounds following the method described previously (Iniesta, Perez-Conesa, Garcia-Alonso, Ros, & Periago, 2009). The precision of the HPLC analysis including sample extraction, deconjugation and purification showed recoveries of spiked folates on the samples studied at a level of 50 ng mL^{-1} that ranged from 75 to 100% for H_4-folate, from 70 to 99% for 5–CH_3–H_4-folate and from 80 to 100% for 5–HCO–H_4-folate. The coefficient of inter- and intra-assay variation for folate analysis was below 10%. The limits of quantification were 2.34 ng mL^{-1} for H_4-folate, 2.67 ng mL^{-1} for 5–CH_3–H_4-folate and 34.20 ng mL^{-1} for 5–HCO–H_4-folate. The folate content was expressed as micrograms per 100 mL of fresh medium in all samples.

2.6. Statistical analysis

Results were expressed as mean value ± standard deviation from four replicates (every strain was incubated at four different times) based on fresh weight (FW). Analysis of variance (ANOVA) was used to test the variation in the content of folates amongst the different LAB for a given sampling time, as well as the variation in the content of folates due to the effect of the prebiotics. Tukey's pairwise comparison was used to determine significant differences between means. Differences were considered to be significant at $p < 0.05$. Statistical analysis of the data was performed using the SPSS 15.0 software package for Windows (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Growth of lactic acid bacteria

Bacterial growth of all strains was studied in complex medium and in non-fat milk, with and without FOS (1.0 g 100 mL^{-1}) and GOS (1.0 g 100 mL^{-1}) (data not shown). In most strains, the growth

curves showed a similar pattern; the cultures reached stationary phase after approximately 10 h of incubation, giving a higher number of colony forming units in complex medium than in milk, with the exception of *S. thermophilus*. When a prebiotic was added to the medium, bacterial counts were higher than in medium without this additive. Overall, for *B. adolescentis*, *B. catenulatum* and *S. thermophilus*, growth in MRS or milk, the addition of FOS induced a higher growth rate than GOS. In contrast, lactobacilli showed a higher growth rate in the presence of GOS than in the presence of FOS, as seen for growth of *S. thermophilus* in TSYE broth.

3.2. Folate accumulation in bacterial cultures grown in prebiotic-free medium

Folate levels produced by the five bacteria strains tested in both non-fat autoclaved milk and complex media after 0, 6, 10 and 24 h of incubation at 37 °C are shown in Fig. 1. In 8 of 11 cases, both in milk and in complex media, the highest level of folate production was observed within 6 h and 10 h; specifically, *B. catenulatum* in MRS and *S. thermophilus* in milk produced the highest levels of folate. These values were significantly ($p < 0.05$) higher than those observed for the remaining strains. In contrast, *L. plantarum* produced the lowest levels of folate at 10 h of incubation amongst the five bacteria strains tested. All strains of bacteria tested in this study accumulated more 5–CH_3–H_4-folate, from 2.21 μg 100 mL^{-1} for *L. plantarum* to 21.57 μg 100 mL^{-1} for *B. catenulatum* after growing for 10 h in MRS and from 2.53 μg 100 mL^{-1} for *L. plantarum* to 11.01 μg 100 mL^{-1} for *L. delbrueckii* when they were grown in milk for 10 h. Meanwhile, in the case of H_4-folate, the results obtained were from 2.26 μg 100 mL^{-1} for *B. adolescentis* to 6.72 μg 100 mL^{-1} for *B. catenulatum* after growing for 10 h in MRS and from 0.83 μg 100 mL^{-1} for *B. adolescentis* to 1.94 μg 100 mL^{-1} for *B. catenulatum* when they were grown in milk during 10 h. Moreover, higher levels of folate were observed in complex media than in non-fat autoclaved milk at 10 h of incubation with the exception of *S. thermophilus* grown in TSYE (Fig. 2), where both values are similar.

3.3. Folate accumulation in bacterial cultures grown in FOS-containing medium

The effect of FOS on folate production by different LAB and bifidobacteria was studied at different times (Figs. 2 and 3). In general (7 of 11 cases), for bacteria grown in milk and complex media, the highest level of accumulation of folate was observed within 6 h and 10 h.

Cultures of *B. catenulatum* at 24 h (23.5 μg 100 mL^{-1}) and *L. plantarum* at 10 h (11.21 μg 100 mL^{-1}) showed the highest levels of folate accumulation in MRS + FOS and milk + FOS, respectively. When *L. plantarum* was grown in MRS, or *S. thermophilus* in TSYE, both in the presence of FOS, folate levels were higher than when they were grown in the prebiotic-free media. In contrast, the remaining strains showed significantly ($p < 0.05$) lower folate levels when grown in the prebiotic-supplemented medium. Again, the five bacteria strains tested produced more 5–CH_3–H_4-folate than H_4-folate when FOS was added to the medium. The results obtained for 5–CH_3–H_4-folate ranged from 3.37 μg 100 mL^{-1} for *L. delbrueckii* to 16.63 μg 100 mL^{-1} for *B. catenulatum* after 10 h of growth in MRS + FOS and from 0.12 μg 100 mL^{-1} for *L. delbrueckii* to 9.30 μg 100 mL^{-1} for *B. catenulatum* when they were cultured for

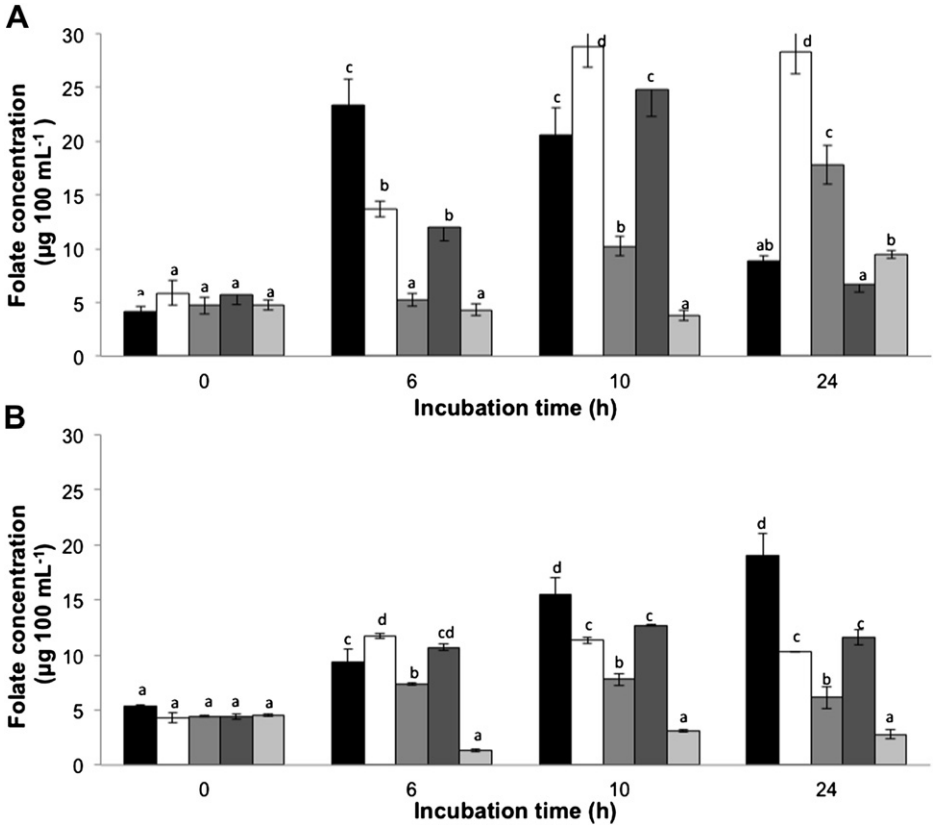

Fig. 1. Folate production in cultures of *S. thermophilus* (■), *B. catenulatum* (□), *B. adolescentis* (▩), *L. delbrueckii* (■), *L. plantarum* (□) in (A) MRS and (B) milk after 0, 6, 10 and 24 h of anaerobic incubation at 37 °C. Different lowercase letters within each incubation time (0, 6, 10 and 24 h) indicate significant differences ($p < 0.05$) in mean folate content among the five bacterial species tested. The error bars represent standard deviation.

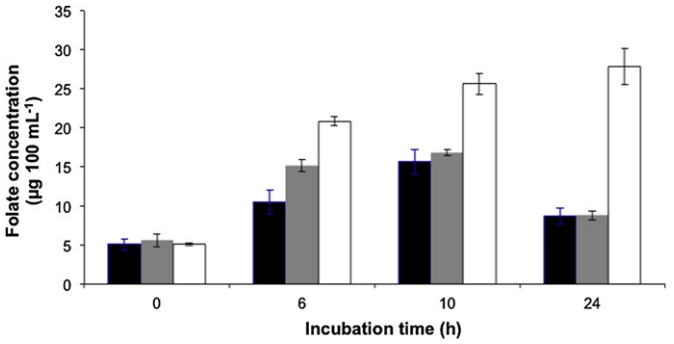

Fig. 2. Total folate content produced by *S. thermophilus* in TSYE (■), TSYE + FOS (■) and TSYE + GOS (□) at 0, 6, 10 and 24 h of anaerobic incubation at 37 °C.

10 h in FOS-supplemented milk. In the case of H_4-folate, the observed concentrations of this vitamin were from 1.69 µg 100 mL^{-1} for *L. delbrueckii* to 4.76 µg 100 mL^{-1} for *L. plantarum* after growing for 10 h in MRS + FOS and from 0.02 µg 100 mL^{-1} for *L. delbrueckii* to 2.49 µg 100 mL^{-1} for *L. plantarum* when they were grown in milk + FOS during 10 h. Moreover, bacteria showed higher folate levels in complex media than in non-fat autoclaved milk after 24 h of incubation. In fact, in all cases, the final folate concentration in milk was only slightly higher or lower than the initial concentration of the micronutrient, whereas in complex media the folate concentration increased over time (except for both lactobacilli, where a maximum content was obtained at about 10 h).

3.4. Folate accumulation in bacterial cultures grown in GOS-containing medium

To determine whether the folate production by the five bacteria strains assessed might be affected by the presence of the second prebiotic studied, the folate concentration was determined after 0, 6, 10 and 24 h in cultures grown in milk and complex media supplemented with GOS (10 g 100 mL^{-1}) (Figs. 2 and 4). *S. thermophilus* showed the highest levels of folate accumulation, both in MRS (12.28 µg 100 mL^{-1} at 24 h) and milk (12.26 µg 100 mL^{-1} at 10 h), and this difference was statistically significant ($p < 0.05$) compared with the remaining strains. In contrast, *L. delbrueckii* (1.37 µg 100 mL^{-1} at 10 h) and *L. plantarum* (0.35 µg 100 mL^{-1} at 24 h) showed the lowest folate levels in MRS and milk, respectively, with statistically significant differences compared with the other bacterial strains tested.

With the exception of *S. thermophilus* grown in TSYE (Fig. 2), all five strains tested showed lower folate levels in GOS-supplemented media than in prebiotic-free media. Furthermore, in 6 of 11 cases, the folate levels were lower in the presence of GOS than in the presence of FOS. It should be noted that *S. thermophilus*, *B. adolescentis* and *L. delbrueckii* produced higher concentrations of folate when they were grown in GOS-supplemented milk than in FOS-supplemented milk. In the case of complex media, only *B. adolescentis* culture in MRS and *S. thermophilus* in TSYE induced a higher folate production in the presence of GOS than in the presence of FOS.

All the bacterial strains tested, except *B. catenulatum* and *L. delbrueckii*, showed higher folate levels in complex media + GOS

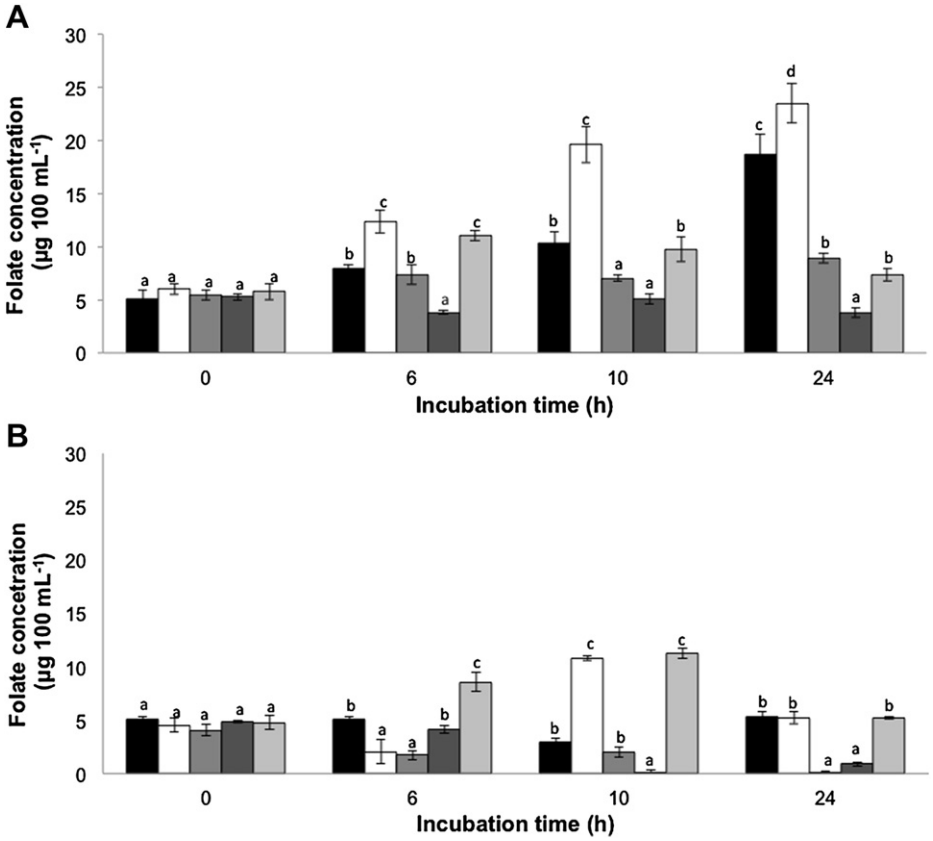

Fig. 3. Folate production in cultures of *S. thermophilus* (■), *B. catenulatum* (□), *B. adolescentis* (■), *L. delbrueckii* (■), *L. plantarum* (■) in (A) MRS + FOS and (B) Milk + FOS after 0, 6, 10 and 24 h of anaerobic incubation at 37 °C. Different lowercase letters content within each incubation time (0, 6, 10 and 24 h) indicate significant differences ($p < 0.05$) in mean folate content among the five bacterial species tested. Error bars represent standard deviation.

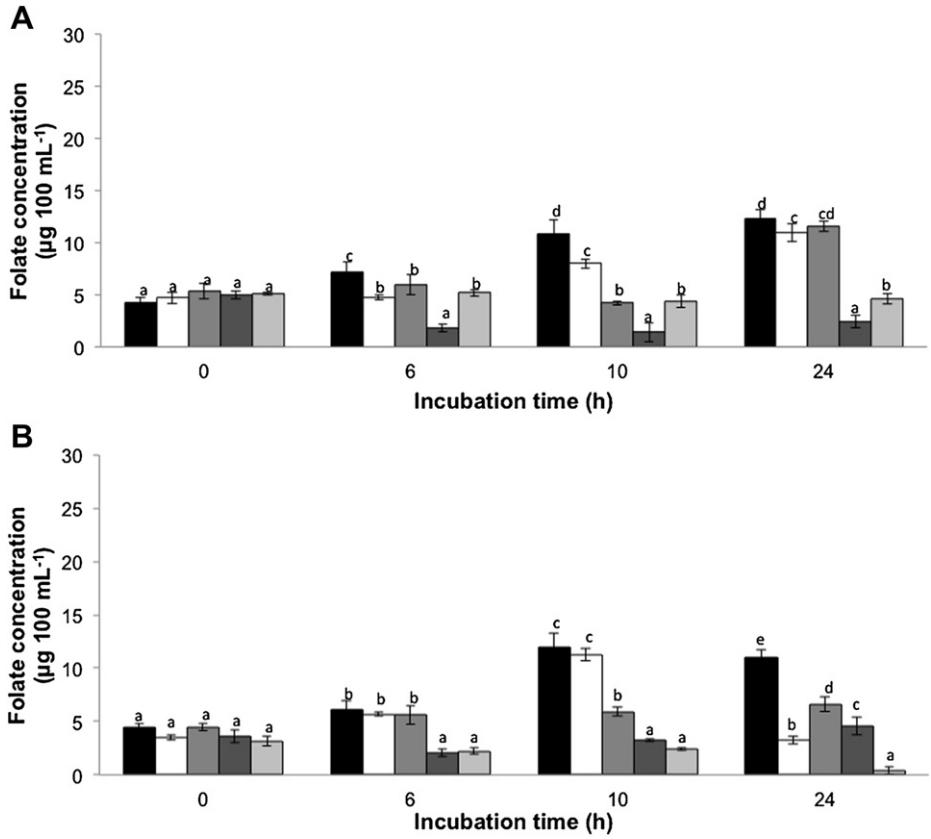

Fig. 4. Folate production in cultures of *S. thermophilus* (■), *B. catenulatum* (□), *B. adolescentis* (▩), *L. delbrueckii* (■), *L. plantarum* (▩) in (A) MRS + GOS and (B) milk + GOS after 0, 6, 10 and 24 h of anaerobic incubation at 37 °C. Different lowercase letters in within each incubation time (0, 6, 10 and 24 h) indicate significant differences ($p < 0.05$) in mean folate content among the five bacterial species tested. Error bars represent standard deviation.

than in non-fat autoclaved milk + GOS (from +1.22 µg 100 mL^{-1} for *S. thermophilus* to +4.86 µg 100 mL^{-1} for *B. adolescentis*). Again, all bacteria strains accumulated more 5–CH_3–H_4-folate than H_4-folate (the other predominant form) when the GOS-supplemented medium was used.

4. Discussion

Five strains of folate-producin bacteria were screened to test the production of this vitamin after 0, 6, 10 and 24 h of incubation at 37 °C. In this research, as in most studies, *S. thermophilus* showed the highest levels of folate production in milk, yielding higher concentrations compared with other LAB or bifidobacteria (Crittenden, Martinez, & Playne, 2003; Iyer, Tomar, Uma, & Singh, 2010; Sybesma, Starrenburg, Tijsseling, Hoenfnagel, & Hugenholtz, 2003a). Sybesma et al. (2003a) suggested that *L. plantarum* is the only lactobacillus species able to produce folate. However, this research has shown that folate production also occurs with *L. delbrueckii*, mainly when it is grown in the absence of prebiotics. It is known that para-aminobenzoic acid (pABA) limits folate production (Santos, Wegkamp, de Vos, Smid, & Hugenholtz, 2008; Wegkamp, van Oorschot, de Vos, & Smid, 2007), emphasising that most lactobacilli are unable to synthesise this acid. Consequently, within the genus *Lactobacillus*, folate production is possible only when pABA is present in the medium (Wegkamp et al., 2007). Although pABA was not added to either the milk or MRS medium in this study, the amount of this compound naturally present in the two media was sufficient for both lactobacilli tested to produce folate in milk and MRS. In most cases, both in milk and in complex media, the highest level of folate accumulated within 6 h and 10 h, and folate levels decreased as fermentation continued, which is consistent with previous observations (Holasova, Fiedlerova, Roubal, & Pechacova, 2004; Lin & Young, 2000). Furthermore, Rao, Reddy, Pulusani, and Cornwell (1984) demonstrated that cultures of lactic acid bacteria not only synthesise but also utilise folate in their metabolism, a fact that might explain the results observed in this study after 10 h of incubation. Gregory, Sartain, and Day (1984) and Vahteristo, Lehikoinen, Olliliainen, and Varo (1997) reported that 5–CH_3–H_4 is the predominant form of folate in foods, including fruits, vegetables and dairy products. All strains of LAB and bifidobacteria tested in this study also accumulated more 5–CH_3–H_4 than H_4-folate. This trend also concurs with the results observed by Lin and Young (2000).

The five bacteria selected were further analysed for folate production following growth in the presence of two different prebiotics, FOS and GOS. The use of prebiotics in the culture medium did not stimulate the synthesis of folate in the five bacteria strains studied, although it increased the rate of bacterial growth. In line with this result, numerous studies have highlighted the bifidogenic activity of both GOS and FOS (Kunz & Rudloff, 1993; Mitsuoka, Hidaka, & Eida, 1987; Pérez-Conesa, López, & Ros, 2007). In vitro studies have shown that FOS encourages the growth of intestinal bacteria, in particular the bifidogenic population, which includes both bifidobacteria and lactobacilli (Velazquez, Davies, Marett, Slavin, & Feirtag, 2000). In a study with longer incubation times and different strains of bifidobacteria, the growth of *Bifidobacterium breve* and *Bifidobacterium longum* peaked after 20–25 h of incubation in the presence of β-(2,6)-FOS, remaining constant until the fifth day (Marx, Winkler, & Hartmeier, 2000). A similar trend was seen in this study, but *B. adolescentis*, *B. catenulatum* and

S. thermophilus showed a more sustained growth in the presence of FOS than in the presence of GOS. However, *S. thermophilus* showed a higher growth in the presence of GOS in TSYE broth than in the presence of FOS. Lactobacilli also showed a higher growth rate in the presence of GOS, which was not surprising because the bifidogenic effect of GOS has been extensively studied (Bouhnik et al., 1997; Ito et al., 1990; Tanaka et al., 1983).

In the case of *B. adolescentis* in milk + FOS, *B. catenulatum* in MRS + GOS , *L. plantarum* in milk and MRS (both supplemented with GOS), and *S. thermophilus* in MRS + FOS, the most likely reason for reduced bacterial production of folate in the presence of a prebiotic is the higher growth rate shown in such conditions. In fact, Sybesma et al. (2003a) reported that the folate production was further stimulated when growth was inhibited by the presence of growth-inhibiting concentrations of several antibiotics and in the presence of high salt concentrations. The reason for increased folate production when growth is inhibited is not yet fully understood. It could be that, under conditions of low growth rate, one of the folate precursors (GTP) accumulates due to the decreased synthesis of DNA and RNA. Interestingly, in an earlier study, it was reported that overproduction of GTP-cyclohydrolase I, the first enzyme involved in folate biosynthesis, leads to increased production of folate by *Lactococcus lactis* (Sybesma et al., 2003b).

Another possible explanation for the lower folate production in the presence of a prebiotic is the high amount of acetic acid and lactic acid that bacteria produce when grown on media supplemented with oligosaccharides (Chick, Shin, & Ustunol, 2001). There may be a negative relationship between the acidic pH of the medium and the microbial production of folate. Furthermore, the acidic pH may also lead to the destruction of some labile folate forms when they are excreted into the surrounding medium (Paine-Wilson & Chen, 1979). In line with this deduction, Sybesma et al. (2003a) observed increased folate production by LAB when the pH of the culture medium remained constant. In agreement with this finding, many studies have found that enzymes involved in the folate synthesis of various microorganisms possess most activity at high pH values (between 7.3 and 9.5) of the culture medium (Ballantine, Volpe, & Delves, 1994; Rao, 2000; Yoo, Han, Ko, & Bang, 1998). In this study, a notable decrease in the pH of the culture medium supplemented with prebiotic was also observed as the incubation time increased (data not shown).

5. Conclusion

The species of bacteria commonly used in fermented milk products can positively influence the folate content that milk or dairy products may provide to consumers. However, the addition of prebiotics (FOS or GOS) does not meet the purpose of increasing bacterial folate production, which is reached only in specific conditions depending on the bacteria strain used. More specifically, FOS increased folate production only in *L. plantarum*, whereas FOS reduced it in the other bacteria studied. In the case of the other prebiotic tested, only *S. thermophilus* showed increased folate production when this bacterium was grown in GOS-supplemented TSYE medium.

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References

Abrahms, S. A., Griffin, I. J., Hawthorne, K. M., Liang, L., Gunn, S. K., Darlington, G., et al. (2005). A combination of prebiotic short- and long-chain-type fructans enhances calcium absorption and bone mineralization in young adolescents. *American Journal of Clinical Nutrition, 82*, 471–476.

Ames, B. N. (1999). Micronutrient deficiencies cause DNA damage and cancer. *Food Science and Agricultural Chemistry, 1*, 1–15.

Ballantine, S. P., Volpe, F., & Delves, C. J. (1994). The hydroxymethyldihydropterin pyrophosphokinase domain of the multifunctional folic acid synthesis Fas protein of *Pneumocystis carinii* expressed as an independent enzyme in *Escherichia coli*: refolding and characterization of the recombinant enzyme. *Protein Expression and Purification, 5*, 371–378.

Bouhnik, Y., Flourie, B. L., D'Agay-Abensour, P., Pochart, G., Gramet, M., Durand, M. Y., et al. (1997). Administration of trans-galacto-oligosaccharides increases faecal bifidobacteria and modifies colonic fermentation metabolism in healthy humans. *Journal of Nutrition, 127*, 444–448.

Bouhnik, Y., Raskine, L., Simoneau, G., Vicaut, E., Neut, C., Flourié, B., et al. (2004). The capacity of nondigestible carbohydrates to stimulate fecal bifidobacteria in healthy humans: a double-blind, randomized, placebo-controlled, parallel-group, dose-response relation study. *American Journal of Clinical Nutrition, 80*, 1658–1664.

Boushey, C. J., Beresford, A. A., Omenn, G. S., & Moultulsky, A. G. (1996). A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. *Journal of the American Medical Association, 274*, 1049–1057.

Brunser, O., Gotteland, M., Cruchet, S., Figueroa, G., Garrido, D., & Steenhout, P. (2006). Effect of milk formula with prebiotics on the intestinal microbiota of infants after an antibiotic treatment. *Pediatric Research, 59*, 451–456.

Chick, H., Shin, H. S., & Ustunol, Z. (2001). Growth and acid production by lactic acid bacteria and bifidobacteria grown in skim milk containing honey. *Journal of Food Science, 66*, 478–481.

Crittenden, R. G., Martinez, N. R., & Playne, M. J. (2003). Synthesis and utilisation by yoghurt starter cultures and probiotic bacteria. *International Journal of Food Microbiology, 80*, 217–222.

Cummings, J. H., Christie, S., & Cole, T. J. (2001). A study of fructo-oligosaccharides in the prevention of traveller's diarrhoea. *Alimentary Pharmacology and Therapeutics, 15*, 1139–1145.

De Wals, P., Tairou, F., Van Allen, M. I., Uh, S. H., Lowry, R. B., Sibbald, B., et al. (2007). Reduction in neural-tube defects after folic acid fortification in Canada. *The New England Journal of Medicine, 357*, 135–142.

Gregory, J. F., Sartain, D. B., & Day, B. P. F. (1984). Fluorometric determination of folacin in biological materials using high performance liquid chromatography. *Journal of Nutrition, 114*, 341–353.

Håberg, S. E., London, S. J., Stigum, H., Nafstad, P., & Nystad, W. (2009). Folic acid supplements in pregnancy and early childhood respiratory health. *Archives of Disease in Childhood, 94*, 180–184.

Holasova, M., Fiedlerova, V., Roubal, P., & Pechacova, M. (2004). Biosynthesis of folates by lactic acid bacteria and propionibacteria in fermented milk. *Czech Journal of Food Sciences, 22*, 175–181.

Iniesta, M. D., Perez-Conesa, D., Garcia-Alonso, J., Ros, G., & Periago, M. J. (2009). Folate content in tomato (*Lycopersicon esculentum*). Influence of cultivar, ripeness, year of harvest, and pasteurization and storage temperatures. *Journal of Agricultural and Food Chemistry, 57*, 4739–4745.

Ito, M., Deguchi, Y., Miyamori, A., Matsumoto, K., Kikuchi, H., Kobayashi, Y., et al. (1990). Effects of administration of galactooligosaccharides on the human fecal microflora, stool weight and abdominal sensation. *Microbial Ecology in Health and Disease, 3*, 285–292.

Iyer, R., Tomar, S. K., Uma Maheswari, T., & Singh, R. (2010). *Streptococcus thermophilus* strains: multifactorial lactic acid bacteria. *International Dairy Journal, 20*, 133–141.

Jennings, E. (1995). Folic acid as a cancer-preventing agent. *Medical Hypotheses, 45*, 297–303.

Kennedy, D. A., Stern, S. J., Moretti, M., Matok, I., Sarkar, M., Nickel, C., et al. (2011). Folate intake and the risk of colorectal cancer: a systematic review and meta-analysis. *Cancer Epidemiology, 35*, 2–10.

Konings, E. J. M. (1999). A validated liquid chromatographic method for determining folates in vegetables, milk powder, liver and flour. *Journal AOAC International, 82*, 119–127.

Kunz, C., & Rudloff, S. (1993). Biological functions of oligosaccharides in human milk. *Acta Paediatrica, 82*, 903–912.

Lin, M. Y., & Young, C. M. (2000). Folate levels in cultures of lactic acid bacteria. *International Dairy Journal, 10*, 409–413.

Macfarlane, G. T., Steed, H., & Macfarlane, S. (2008). Bacterial metabolism and health-related effects of galacto-oligosaccharides and other prebiotics. *Journal of Applied Microbiology, 104*, 305–344.

Marx, S. P., Winkler, S., & Hartmeier, W. (2000). Metabolization of ß-(2,6)-linked fructose-oligosaccharides by different bifidobacteria. *FEMS Microbiology Letters, 182*, 163–169.

Mitsuoka, T., Hidaka, H., & Eida, T. (1987). Effect of fructooligosaccharides on intestinal microflora. *Die Nahrung, 31*, 427–436.

Morris, M. S., Jacques, P. F., Rosenberg, I. H., & Selhub, J. (2007). Folate and vitamin B-12 status in relation to anemia, macrocytosis, and cognitive impairment in older Americans in the age of folic acid fortification. *American Journal of Clinical Nutrition, 85*, 193–200.

Otten, J. J., Pitzi-Hellwig, J., & Meyers, L. D. (2006). *Dietary reference intakes - The essential guide to nutrient requirements*. pp 244–253. Washington, DC, USA: Institute of Medicine (IOM), National Academies Press.

Paine-Wilson, B., & Chen, T. S. (1979). Thermal destruction of folacin: effect of pH and buffer ions. *Journal of Food Science, 44*, 717–722.

Pérez-Conesa, D., López, G., & Ros, G. (2007). Prebiotic and symbiotic follow-up infant formulas on iron bioavailability in rats. *Food Science and Technology International, 13*, 69–77.

Pfeiffer, C. M., Rogers, L. M., & Gregory, J. F. (1997). Determination of folate in cereal-grain food products using trienzyme extraction and combined affinity and reversed-phase liquid chromatography. *Journal of Agricultural and Food Chemistry, 45*, 407–413.

Rafter, J., Bennett, M., Caderni, G., Clune, Y., Hughes, R., Karlsson, P., et al. (2007). Dietary synbiotics reduce cancer risk factors in polypectomized and colon cancer patients. *American Journal of Clinical Nutrition, 85*, 488–496.

Rao, K. N. (2000). Purification and characterization of dihydrofolate reductase from *Lactobacillus leichmannii*. *Indian Journal of Biochemistry and Biophysics, 37*, 121–129.

Rao, D. R., Reddy, A. V., Pulusani, S. R., & Cornwell, P. E. (1984). Biosynthesis and utilization of folic acid and vitamin B_{12} by lactic culture in skim milk. *Journal of Dairy Science, 76*, 1819–1828.

Rault-Nania, M. H., Gueux, E., Demougeot, C., Demigne, C., Rock, E., & Mazur, A. (2006). Inulin attenuates atherosclerosis in apolipoprotein E-deficient mice. *British Journal of Nutrition, 96*, 840–844.

Santos, F., Wegkamp, A., de Vos, W. M., Smid, E. J., & Hugenholtz, J. (2008). High-level folate production in fermented foods by the B-12 producer *Lactobacillus reuteri* JCM1112. *Applied and Environmental Microbiology, 74*, 3291–3294.

Sarma, J. D., & Duttagupta, C. (1995). Improved microbiological assay for folic acid based on microtiter plating with *Streptococcus faecalis*. *Food Biological Contaminants, 78*, 1173–1176.

Shaw, G., Schaffer, M. D., Velie, E. M., Morland, K., & Harris, J. A. (1995). Periconceptional vitamin use, dietary folate, and the occurrence of neural tube defects. *Epidemiology, 6*, 219–226.

Smith, D. (2007). Folic acid fortification: the good, the bad and the puzzle of vitamin B-12. *American Journal of Clinical Nutrition, 85*, 3–5.

Sybesma, W., Starrenburg, M., Kleerebezem, M., Mierau, I., de Vos, M., & Hugenholtz, J. (2003b). Increased production of folate by metabolic engineering of *Lactococcus lactis*. *Applied and Environmental Microbiology, 69*, 7101–7107.

Sybesma, W., Starrenburg, M., Tijsseling, L., Hoefnagel, M. H. N., & Hugenholtz, J. (2003a). Effects of cultivation conditions on folate production by lactic acid bacteria. *Applied and Environmental Microbiology, 69*, 4542–4548.

Tanaka, R., Takayama, H., Morotomi, M., Kuroshima, T., Ueyama, S., Matsumoto, K., et al. (1983). Effects of administration of GOS and *Bifidobacterium breve* 4006 on the human fecal flora. *Bifidobacteria Microflora, 2*, 17–24.

Vahteristo, L., Lehikoinen, K., Olliliainen, V., & Varo, P. (1997). Application of an HPLC assay for the determination of folate derivatives in some vegetables, fruits and berries consumed in Finland. *Food Chemistry, 59*, 589–597.

Van den Berg, H., Finglas, P. M., & Bates, C. (1994). FLAIR intercomparisons on serum and red cell folate. *International Journal for Vitamin and Nutrition Research, 64*, 288–293.

Velazquez, M., Davies, C., Marett, R., Slavin, J. L., & Feirtag, J. M. (2000). Effect of oligosaccharides and fibre substitutes on short-chain fatty acid production by human faecal microflora. *Anaerobe, 6*, 87–92.

Wang, X., Qin, X., Demirtas, H., Li, J., Mao, G., Huo, Y., et al. (2007). Efficacy of folic acid supplementation in stroke prevention: a meta-analysis. *Lancet, 369*, 1876–1882.

Wegkamp, A. (2008) *Modulation of folate production in lactic acid bacteria*. Ph.D. thesis dissertation, pp 176. Wageningen University, The Netherlands.

Wegkamp, A., van Oorschot, W., de Vos, W. M., & Smid, E. J. (2007). Characterization of the role of para-aminobenzoic acid biosynthesis in folate production by *Lactococcus lactis*. *Applied and Environmental Microbiology, 73*, 2673–2681.

Yoo, J. C., Han, J. M., Ko, O. H., & Bang, H. J. (1998). Purification and characterization of GTP cyclohydrolase I from Streptomyces tubercidicus, a producer of tubercidin. *Archives of Pharmacal Research, 21*, 692–697.