Biosynthesis of Folates by Lactic Acid Bacteria and Propionibacteria in Fermented Milk

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Abstract

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Folate producing ability of several strains of *Bifidobacterium longum*, *Bifidobacterium bifidum*, *Streptococcus thermophilus* and *Propionibacterium freudenreichii* subsp. *shermanii* was evaluated. As substrate, UHT milk with 1.5% fat content treated with additional laboratory sterilisation was used. Fermentation was conducted at 37°C and 30°C in the case of *Propionibacterium*. 5-Methyltetrahydrofolate (5-MTHF) concentrations were determined using HPLC method. All strains of *Streptococcus thermophilus* tested showed 5-MTHF production. More than six-fold increase was found in the 5-MTHF content in comparison with control (increase = 3.69 μg 5-MTHF/100 g) after 12 h fermentation. *Bifidobacterium longum* strains were recognised as mild folate producers with max. 73% increase in the 5-MTHF content (increase = 0.48 μg 5-MTHF/100 g) after 12 h fermentation. The *Propionibacterium freudenreichii* subsp. *shermanii* strains tested did not basically influence the 5-MTHF levels during milk fermentation. In all cases, maximum 5-MTHF concentration was reached between 6 and 12 hours of fermentation. Large differences in the 5-MTHF production were found among individual strains within species. By a careful testing of the folate production ability of microbial strains used in the production of fermented milk, an enhancement of the natural folate content can be achieved.

Keywords: folate; fermentation; milk

Folates represent an essential nutrition component involved in many metabolic pathways, mainly in carbon transfer reactions such as purine and pyrimidine biosynthesis and amino acid interconversion. The daily recommended intake for an adult varies between 200 and 400 μg. Before and during pregnancy, the double dose is recommended for women since folate deficiency has been associated with the incidence of neural tube defects during the embryo development (Shaw *et al.* 1995; Daly *et al.* 1995). A low folate intake has been associated with the risk of cardiovascular diseases. Low plasma folate concentrations correlate with elevated levels of homocystein which has been recognised as a risk factor in the coronary heart disease (Brattstrom 1996; Morrison *et al.* 1995). Furthermore, there seems to exist a relation between a low folate status and the incidence of certain forms of cancer (Ames 1999).

The term folate is used as the generic descriptor for all derivatives of pteroic acid that demonstrate vitamin activity in humans. Pteroylmonoglutamic acid (trivial name folic acid) is not a natural physiological form of the vitamin. The pteridine ring of natural folates is reduced to 7,8-dihydrofolate or 5,6,7,8-tetrahydrofolate. These reduced forms can be substituted with one-carbon adduct attached to nitrogen positions 5 or 10 or both. All folate

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compounds exist predominantly as polyglutamates containing from five to seven glutamate residues in γ-peptide linkage (Ball 1994).

The methods applied for food folate analysis include microbiological assay, HPLC, ligand binding, and radioimmunoassay. Folate analysis is complicated by the variety of natural vitamin forms, variable γ-glutamyl polymer lengths, and folate instability (Eitenmiller & Landen 1999). Microbiological assay serves as the traditional method of folate analysis. Although the method is quite unspecific and crude, most data on the folate levels compiled in food tables are still based on this method. Several HPLC methods have been developed for the determination of folate in foods with the aim to separate and detect the individual forms of folates. So far, no HPLC method has been approved as suitable for food analysis in general. Extraction, enzymatic pretreatment, and sample purification must be optimised for various types of foods (Forssén *et al.* 2000).

Milk is a well known source of folates. The food composition tables and review papers based on microbiological assay report folate values in cow's milk in the range of 5–7 μg/100 g. Most HPLC studies indicate 5-methyltetrahydrofolate (5-MTHF) as the major form of folates in milk (Forssén *et al.* 2000). Fermented milk products are reported to contain even higher amounts of folate. The values reported vary widely. The folate contents in yoghurts commercially available in the Netherlands varied between less than 2 and more than 10 μg per 100 g (Smid *et al.* 2001). In 22 low fat commercial yoghurts from US market, the folate contents of 6.5 ± 1.7 μg/100 g were found by microbiological assay after conjugase treatment (Johnston *et al.* 2002). This high level is the result of the production of additional folates by bacteria. However, many bacteria synthesise this cofactor by themselves from simple precursors but some auxotrophic bacteria, including many lactic acid bacteria, have a strict growth requirement for folic acid (Hugenholtz *et al.* 2002). The production and consumption of folates by the microorganisms applied will be probably the most important factor determining the folate level in fermented milk products.

In general, *Lactobacillus* strains do not produce folates with the exception of *Lactobacillus plantarum* (Crittenden *et al.* 2003). *Streptococcus thermophilus* was reported to produce folates (Rao *et al.* 1984), however, great differences have been observed in the production ability of individual strains. By application of different *Streptococcus thermophilus* strains used in yoghurt productions, the folate contents ranging from 2 up to 15 μg/100 g were found (Smid *et al.* 2001). Some other lactic acid bacteria – *Lactococcus lactis*, *Leuconostoc lactis* (Sybesma *et al.* 2003), *Bifidobacterium longum* (Lin & Young 2000) were reported to produce folates. Both *Streptococcus thermophilus* and *Bifidobacterium* species were recognised as folate producers by Crittenden *et al.* (2003). Some strains of *Propionibacteria*, well known vitamin B_{12} producer, can produce large amounts of folates (Hugenholtz *et al.* 2002).

The aim of this work was to evaluate the ability of the selected microbial species and strains to produce folates in order to explore the possibility of increasing the folate level in fermented milk through natural synthesis by the bacterial cultures.

MATERIAL AND METHODS

Organisms and fermentation conditions. Three strains of *Bifidobacterium longum* (No. 95, 134, 241), two strains of *Bifidobacterium bifidum* (No. 93, 94), three strains of *Streptococcus thermophilus* (No. 131, 144, 280) and three strains of *Propionibacterium freudenreichii* subsp. s*hermanii* (No. 160, 163, 805) from the Collection of dairy micro-organisms Lactoflora Milcom were used for substrate inoculation. All strains were stored in freeze-dried or deep frozen forms and, except for *Propionibacterium* strains, they were propagated in sterilised milk. *Propionibacterium* strains were grown in broth with whey, yeast extract and calcium lactate. After microscopic examination, cultures or their mixtures were used for inoculation. For inoculation, 2%, 1%, and 5% of inoculum was applied with *Bifidobacterium*, *Streptococcus thermophilus*, and *Propionibacterium*, respectively.

UHT milk with 1.5% fat content treated by additional laboratory sterilisation (114°C/20 min) represented the substrate. This substrate with minimal residual microflora and with a relatively low content of natural folates allowed a better recognition of changes caused by fermentation. Substrate without inoculation was used as a control sample. Fermentation was conducted at 37°C and 30°C in the case of *Propionibacterium*, without agitation. Samples were taken after 6, 12, and 18 hours of fermentation, subjected to extraction and hydrolysis, stored at –18°C until purification and quantification. Folate concentrations were calculated from two parallel determinations. The production ability of the strains tested was evalu-

ated in relation to 5-MTHF content in the substrate without inoculation found at time 0.

5-MTHF determination. 5-MTHF concentrations were determined using HPLC method based on the methods published by Vahteristo *et al.* (1996) and Finglas *et al.* (1999). 20 g sample was homogenised in 20 ml of 0.075M phosphate buffer, pH 6, with 0.052 mol/l ascorbic acid and 0.1% 2-mercaptoethanol in ultrasonic bath after flushing with nitrogen, the samples were kept for 10 min in boiling water, rapidly cooled, and the volume was then made up to 50 ml. Centrifugation at 26 000 g, 2°C, for 15 min followed (Ultracentrifuge KRi Jouan). pH of the supernatant was adjusted with acetic acid solution to 4.9. Amount of 1 ml of hog kidney conjugase, prepared according to Gregory *et al.* (1984), was added to 5 ml of the adjusted extract, the mixture was flushed with nitrogen and kept in thermostat at 37°C for 2 h under regular agitation. Enzymes were inactivated by placing the sample into a boiling water bath for 5 min. The extracts were rapidly cooled and centrifuged at 46 000 g for 10 min at 2°C. SPE on AccuBond SAX columns 500 mg (Agilent) was used to purify the sample extracts with Visiprep™ vacuum manifold (Supelco). 1 ml of deconjugate was applied to the column. The retained sample was washed with 6 ml water and eluted from the column with 4 ml of 0.1M sodium acetate containing 10% sodium chloride and 1% ascorbic acid. For HPLC analysis, the liquid chromatograph HP 1100 equipped with the fluorescence detector G 1321 A (Agilent) and Lichrospher 100 RP 18 column (5 µm, 250 × 4 mm) with a guard column (Merck) was applied. The mobile phase consisted of 8% acetonitril in 0.03M potasium phosphate buffer, pH 2.3. After 20 min of isocratic elution, the acetonitril proportion was changed to 17% within 5 min. The run time was 20 min, the interval between injections was 35 min. The flow of 1 ml/min and the sample loop of 100 µl were used. The excitation and emission wavelengths were set at 280 and 360 nm, resp. quantification was based on an external standard method. The standard of 5-MTHF was obtained from Schircks Laboratories. It was dissolved in freshly prepared 0.05M sodium borate solution, pH 9.3, with 0.4% 2-mercaptoethanol, further diluted in 0.01M phosphate buffer pH 7 with 0.4% 2-mercaptoethanol, and the concentration was measured by UV spectrophotometry (Finglas *et al.* 1999). A sample of 5-MTHF standard (5-MTHF content around 0.7 µg) was included in every group of the samples analysed and underwent the whole sample treatment. The method was validated by analyses of CRM – milk powder 421 (certified value 25 µg 5-MTHF/100 g dry matter) obtained from Fluka. The results were examined by the Grubb's test at the $P = 0.05$ level of significance. The mean value obtained in 9 independent determinations was 26.37 µg/100 g dry matter with the relative standard deviation equal 7.61%. The mean standard recovery for 5-MTHF added to dried milk was 85.3 ± 4.4%.

RESULTS AND DISCUSSION

5-MTHF production by *Bifidobacterium* strains

Five strains of *Bifidobacterium* were tested using sterilised UHT milk as substrate. Samples were

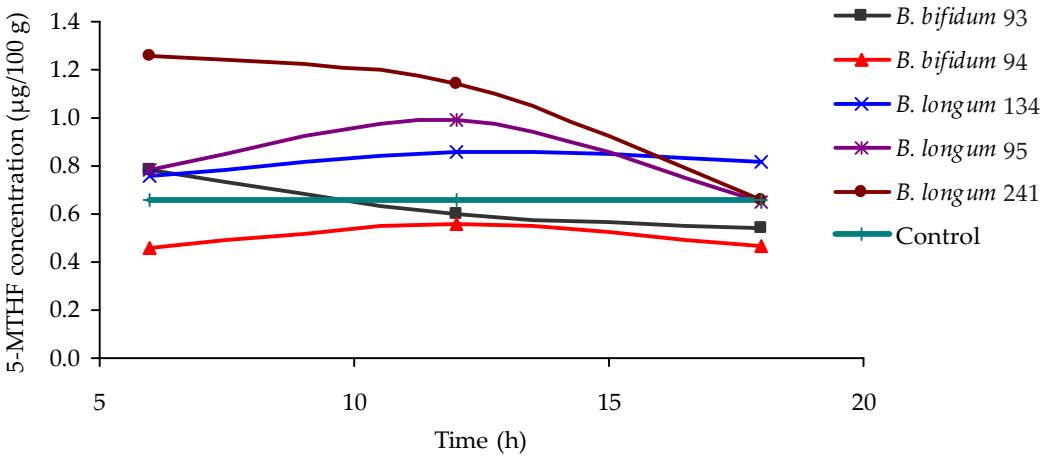

Figure 1. Time course of 5-MTHF production by *Bifidobacterium* strains

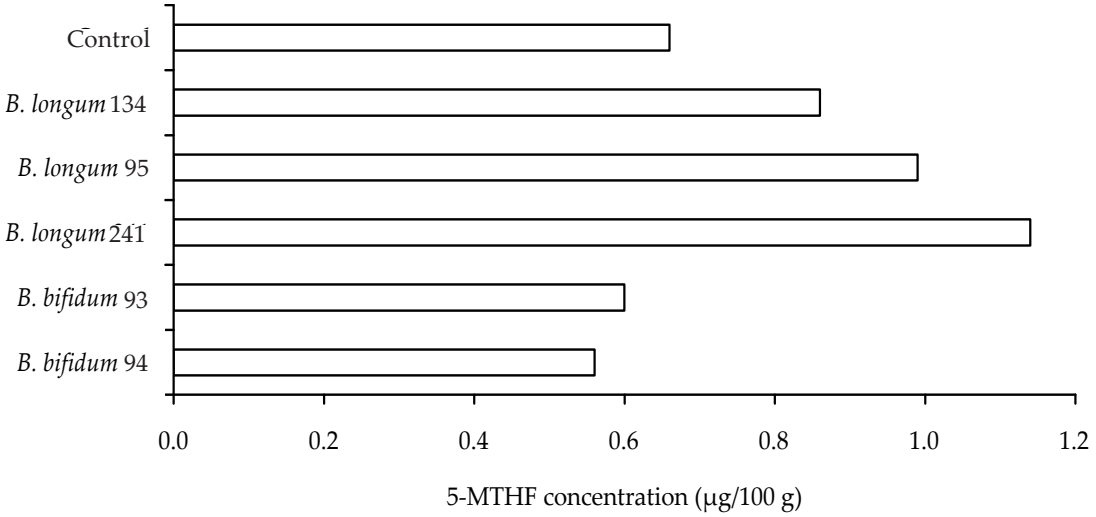

Figure 2. 5-MTHF production by *Bifidobacterium* strains (12 h fermentation)

taken after 6, 12 and 18 h of fermentation at 37°C (Figures 1 and 2).

The results show that the individual strains and species of *Bifidobacterium* differ in 5-MTHF production. Maximum value of 5-MTHF content with 2% inoculation and fermentation at 37°C was found between 6 and 12 h of fermentation except for the strain *Bifidobacterium bifidum* 93. After 12 h of fermentation, a decline of 5-MTHF content was observed. All tested strains of *Bifidobacterium longum* demonstrated 5-MTHF production. On the contrary, both *Bifidobacterium bifidum* strains revealed 5-MTHF levels below the initial contents in the substrate. The initial content of 5-MTHF in the substrate – 0.66 μg/100 g – was used as a reference value. After 18 h of fermentation, no significant change in 5-MTHF concentration occurred in the substrate. The strain *Bifidobacterium longum* 241 was recognised as the most effective producer with the absolute 5-MTHF increase of 0.48 μg/100 g, i.e. two times more in comparison with the weakest *Bifidobacterium longum* strain numbered 134. Lin and Young (2000) and Crittenden *et al.* (2003) recognised *Bifidobacterium longum* as a folate producer as well. The values demonstrated by Crittenden *et al.* (2003) were around 1 μg/100 g of total folate. The selected strains from Lactoflora collection, however, did not produce significant amounts of 5-MTHF in comparison with the results of Lin and Young (2000) who found two strains of *Bifidobacterium longum* even superior to *Streptococcus thermophilus* strains in its production.

5-MTHF production by *Streptococcus thermophilus* strains

Three strains of *Streptococcus thermophilus* were tested with sterilised UHT milk as substrate. The samples were taken after 6, 12 and 18 h of fermentation at 37°C (Figures 3 and 4).

All strains of *Streptococcus thermophilus* tested proved to produce 5-MTHF. Substantial differences in the range of 0.54–3.69 μg/100 g were found between individual strains. The most productive strain *Streptococcus thermophilus* 144 revealed an increase of 3.69 μg/100 g in relation to the control. The initial content of 5-MTHF in the control sample was 0.60 μg/100 g. Maximum values were found at 12 h of fermentation. Two additional repeated independent experiments using this strain resulted in increases of 3.60 and 3.07 μg/100 g after 12 h fermentation, thus showing a good repeatability in the production and fermentation conditions. The results were in a good agreement with Lin and Young (2000) who found with 2 strains of *Streptococcus thermophilus* after 6 h fermentation maximal production of 2.39 and of 3.63 μg/100 g by HPLC. Microbiological assay was used in the study of Crittenden *et al.* (2003); the production of folates in reconstituted skim dry milk by 7 strains of *Streptococcus thermophilus* was around 4 μg/100 g. In modified M17 broth 3, the tested strains of *Streptococcus thermophilus* showed the production of 2.3–4.0 μg/100 ml of extracelular folates determined after deconjugation by micro-

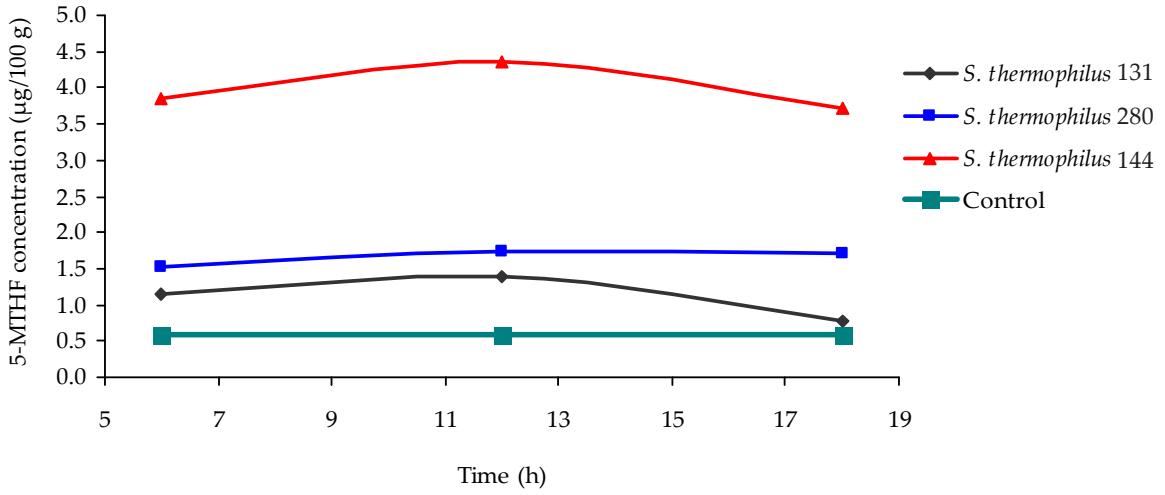

Figure 3. Time course of 5-MTHF production by *Steptococcus thermophilus* strains

biological assay (Sybesma *et al.* 2003). However, this recent work reported a substantial increase in the folate content after cell disruption and identified the dominant forms as 5-formyl and 5,10-methenyltetrahydrofolate. More research seems to be necessary to specify the folate spectrum produced in milk substrate.

5-MTHF production by *Propionibacterium freudenreichii* subsp. *shermanii* strains

Three strains of *Propionibacterium freudenreichii* subsp. *shermanii* were tested with sterilised UHT milk as substrate. The samples were taken after 6, 12, and 18 h of fermentation at 30°C. With regard to the sensorial properties, the butter starter was used simultaneously in the fermentation (Figure 5). The results show only slight differences between the individual strains. The level of inoculation did not basically influence the 5-MTHF production. Absolute increase was low reaching max. 0.12 µg/100 g in relation to the sample containing milk and the butter starter. The strains tested were not able, under the conditions applied, to produce significant amounts of 5-MTHF, comparable with the results of Hugenholtz *et al.* (2002), who found some *Propionibacteria* strains as productive as *Streptococcus thermophilus*.

It is possible to summarise that, among the species tested *Streptococcus thermophilus* strains proved to be the dominant folate producers in milk. All strains of *Streptococcus thermophilus* tested showed

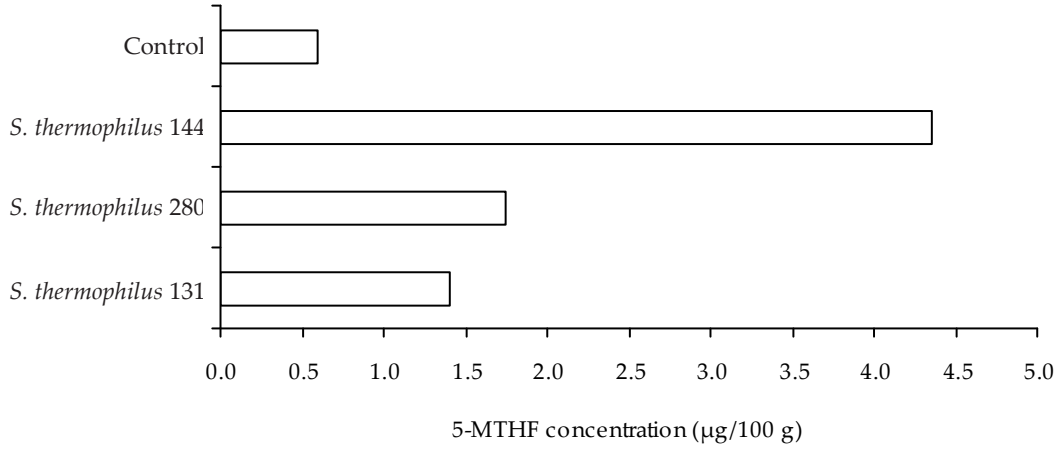

Figure 4. 5-MTHF production by *Steptococcus thermophilus* strains (12 h fermentation)

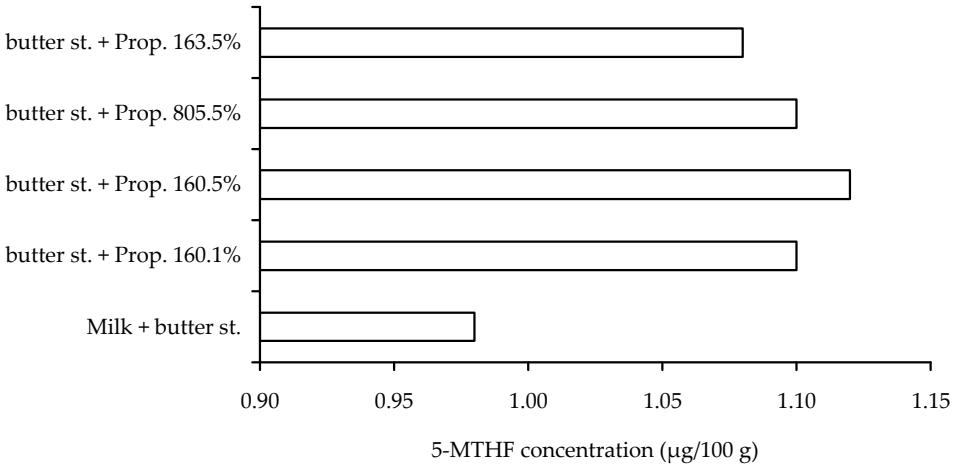

Figure 5. 5-MTHF production by *Propionibacterium* strains (butter st. = starter; Prop. = *Propionibacterium freudenreichii* subsp. *shermanii* strains)

5-MTHF production. More than six-fold increase was found in 5-MTHF content in comparison with the control (increase = 3.69 μg 5-MTHF/100 g) after 12 h fermentation. *Bifidobacterium longum* strains were recognised as moderate folate producers with max. 73% increase in 5-MTHF content (increase = 0.48 μg MTHF/100 g) after 12 h fermentation. *Propionibacterium freudenreichii* subsp. *shermanii* strains tested did not basically influence the 5-MTHF levels during milk fermentation. In all cases, maximum 5-MTHF concentration was reached between 6 and 12 h of fermentation. After 12 h of fermentation, a decline of 5-MTHF content was observed. Great differences in 5-MTHF productions were found in species among individual strains.

The results show that, by a careful testing of the folate production ability of microbial strains used in the production of fermented milk starters, formulations may be optimised and lead to natural enhancement of the folate content. *Streptococcus thermophilus* has the highest potential as concerns the natural folate enrichment in fermented milk products.

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Souhrn

Holasová M., Fiedlerová V., Roubal P., Pechačová M. (2004): **Biosyntéza folátů ve fermentovaném mléce bakteriemi mléčného kvašení a *Propionibacterium* sp.** Czech J. Food Sci., **22**: 175–181.

U vybraných kmenů *Bifidobacterium longum*, *Bifidobacterium bifidum*, *Streptococcus thermophilus* a *Propionibacterium freudenreichii* subsp. *shermanii* byla hodnocena schopnost produkovat foláty. Jako substrát bylo užito UHT mléko o obsahu tuku 1,5 %, ošetřené dodatečnou laboratorní sterilací. Fermentace byly prováděny při 37 °C, v případě *Propionibacterium* při 30 °C. Pro stanovení 5-methyltetrahydrofolátu (5-MTHF) byla užita metoda HPLC. Všechny kmeny *Streptococcus thermophilus* vykázaly produkci folátů. Po 12 h fermentace byl nalezen více než šestinásobný nárůst v obsahu 5-MTHF při srovnání s kontrolním mlékem (nárůst = 3,69 μg 5-MTHF/100 g). Produkce 5-MTHF kmeny *Bifidobacterium longum* byla nižší s max. zvýšením obsahu po 12 h fermentace o 73 % (nárůst 0,48 μg 5-MTHF/100 g). Testované kmeny *Propionibacterium freudenreichii* subsp. *shermanii* neměly za daných podmínek výraznější vliv na hladinu 5-MTHF. Ve všech případech bylo maximální koncentrace 5-MTHF dosaženo mezi 6. a 12. h fermentace. Značné rozdíly v produkci 5-MTHF byly nalezeny mezi jednotlivými kmeny. Testováním a výběrem produkčních kmenů pro výrobu fermentovaných mléčných výrobků lze dosáhnout zvýšení obsahu přirozených folátů.

Klíčová slova: foláty; fermentace; mléko

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